AN IMMUNOHISTOCHEMICAL AND MICROSATELLITE ANALYSIS OF NEPHROBLASTOMAS

by

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DECLARATION

This study represents original work by the author and has not been submitted in any form to another University. Where use was made of the work of others it has been duly acknowledged in the text.

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ABBREVIATIONS

A	Adenine
AI	Allelic imbalance
APC	Adenomatous polyposis coli
APE1	Apirine/apyrimidine endonuclease 1
AREG	Amphiregulin
ATP	Adenosine triphosphate
AXIN	Axins inhibitor protein
BER	Base excision repair
BWS	Beckwith-Wiedemann syndrome
C	Cytosine
CDK	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitor
CI	Chromosomal instability
Cip/Kip	CDK interacting protein or kinases inhibitory protein
CK1	Casein kinase 1
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
EGR1	Early growth response 1
FH	Favourable histology
G	Guanine
G1	Gap 1
G2	Gap 2
$GADD_{45}$	Growth arrest and DNA damage protein
GPC3	Glypican 3
GSK3	Glycogen synthase kinase 3
hMLH1	Human mut L homolgue 1
hMSH2	Human mut S homologue 2
HNPCC	Hereditary non-polyposis colorectal cancer
HPV	Human papillomavirus
IGF2	Insulin-like growth factor II
IGF2R	Insulin-like growth factor II receptor
ILNR	Intralobar nephrogenic rests

kb	Kilobase
LFS	Li-Fraumeni syndrome
LOH	Loss of heterozygosity
LOI	Loss of imprinting
Mb	Megabase
MDM2	Murine double minute 2 gene
MDR	Multidrug resistant
MEN1	Multiple endocrine neoplasia type 1
MMR	Mismatch repair
MRP1	Multidrug resistant related protein 1
MSI	Microsatellite instability
MSI-H	High frequency microsatellite instability
MSI-L	Low frequency microsatellite instability
MSS	Microsatellite stable
NR	Nephrogenic rests
NWTS	National Wilms tumour study
NWTSG	National Wilms tumour study group
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF-A	Platelet-derived growth factor A
PLNR	Perilobar nephrogenic rests
pRb	Retinoblastoma protein
RER	Replication error
S	Synthesis phase
Sdi1	Senescent cell derived inhibitor 1
SIOP	Société Internationale d' Oncologie Pédiatrique
T	Thymidine
TCF/LEF	T cell specific transcription factor and lymphoid enhancer
TGF-β	Transforming growth factor β
TGF-βRII	Transforming growth factor β receptor II
TSP1	Thrombospondin 1
VEGF	Vascular endothelial growth factor
WAGR	Wilms tumour, aniridia, genitourinary abnormalities, mental retardation
WT	Wilms tumour

ABSTRACT

The aims of this study were: (i) to determine the association between p53, bcl-2, pRb, p21, cyclin A and p-glycoprotein immunoexpression and prognosis, and (ii) to determine the frequency of loss of heterozygosity and microsatellite instability at 11p, 16q and mismatch repair gene loci and their association with prognosis, in nephroblastomas in South African children.

There were 138 cases (111 of whom received preoperative chemotherapy) in the immunohistochemical study and, 70 cases (48 with preoperative chemotherapy) in the microsatellite study. The following monoclonal antibodies were used after heat induced epitope retrieval; p53, bcl-2, pRb, p21, cyclin A and p-glycoprotein. Six polymorphic microsatellite markers were selected from the 11p region, 5 from the 16q region and 6 from the loci of known mismatch repair genes. Automated fluorescent DNA technology was used in the analysis. The results of the immunohistochemical and microsatellite studies were correlated with patient age, gender, preoperative chemotherapy, SIOP histological classification, SIOP histological risk group, clinicopathological stage, patient outcome and survival using χ^2, Fisher's exact test, Cox regression model and Kaplan-Meier estimates.

The majority of patients presented with advanced disease. Anaplastic tumours and high-risk histology were associated with high disease stage. Mortality was directly related to increasing stage and histological risk group. Multivariate analysis showed that clinicopathological stage was the only factor significantly associated with survival ($p<0.001$) (hr=5.6, 95%CI: 2.1-14.9).

High expression of p53 was more frequent in anaplastic tumours suggesting that *p53* mutations are common events in this tumour type ($p<0.001$). Despite the strong association with tumour histology, there was no association with stage. Although p53 expression was found to be a predictor of survival in the univariate analysis this was not retained in the multivariate analysis. Tumours treated with preoperative chemotherapy showed higher bcl-2 immunoreactivity ($p=0.027$ but lower levels of pRb ($p=0.040$) and cyclin A expression ($p<0.001$). All anaplastic tumours showed high expression of pRb compared to the other histological types ($p=0.003$). Expression of

pRb was significantly associated with survival in the univariate analysis but not in the multivariate analysis. High cyclin A expression was associated with high risk histology ($p<0.001$). Cyclin A expression was found to be a significant predictor of survival in both the univariate (hr=1.7; 95%CI 1.2-2.4; p=0.002) and multivariate analyses (hr=1.7; 95%CI 1.1-2.7; p=0.032). Although tumours with high risk histology were more likely to express high levels of p-glycoprotein, this did not reach significance.

LOH at 11p was seen in 64.7% of 68 informative cases. LOH at 11p13 was more frequent than LOH at 11p15. LOH for both 11p13 and 11p15 was found in 39.7% of all tumours. MSI at 11p was seen in 22.1% of informative cases. The majority showed MSI for one marker only. LOH 16q was seen in 66.7% of 66 informative cases. MSI at 16q was seen in 16.7% of cases. LOH for D16S496 and D16S520 appear to be related to tumour histology and risk group. The most frequent locus for LOH was 16q21-22, which is known to harbour important genes, such as, *E2F4* and E-cadherin. LOH for MMR markers was seen in 43.5% of 69 informative cases. MSI was seen in 11.6% of tumours. In the multivariate analysis there was no significant correlation between LOH at any of the loci studied and survival. There were no tumours with high frequency MSI. Low frequency MSI was of no clinicopathological significance.

The following conclusions are made: (i) *p53* mutations determined by high p53 expression is a frequent finding in anaplastic tumours, (ii) Bcl-2 may play a role in the chemoresistance of nephroblastomas, (iii) *Rb* gene alterations are not important in the development of nephroblastoma and anaplasia, (iv) Cyclin A expression is an independent predictor of survival, (v) p-glycoprotein may be responsible for the chemoresistance in a proportion of nephroblastomas, (vi) MSI is a rare occurrence in nephroblastoma and does not play a role in the development of nephroblastoma, (vii) LOH at 11p and 16q are frequent findings in nephroblastomas, (viii) LOH for the specific 16q markers (D16S496 and D16S520) may have an important prognostic role in nephroblastoma.

CHAPTER ONE

INTRODUCTION

Wilms tumour or nephroblastoma is an embryonal neoplasm of the kidney, widely accepted as arising from the primitive metanephric blastema. It is one of the common solid paediatric neoplasms seen at the academic hospital complex in Durban, South Africa. The number of patients with nephroblastomas admitted annually to King Edward VIII Hospital in Durban increased from 15 in 1984 to 25 in 1994.

In a 10-year retrospective study conducted at the above institution, 129 cases of nephroblastoma were analysed.[1] Clinicopathological stage and histological classification into favourable and unfavourable histology were found to be important predictors of patient survival. However, there was a subset of patients with low disease stage and favourable histology that had a poor outcome. This led to a search for other prognostic factors in this patient population.

Since the first description of nephroblastoma in the mid nineteenth century, there has been an on going search for pathological and clinical factors which influence prognosis. The National Wilms Tumour Study Group (NWTSG) in the United States of America and the Société Internationale d'Oncologie Pédiatrique (SIOP) studies in Europe have made major contributions to understanding the histogenesis, behaviour and histology of this sometimes enigmatic neoplasm. This resulted in the development of effective treatment protocols, and improved prognosis. Although there is still a small percentage of nephroblastomas, which ultimately will resist any therapy, considerable progress in the treatment of nephroblastoma has been achieved in the past few years. This was paralleled by important discoveries in the understanding of the complex genetics of this tumour and the close relationship with kidney and urogenital development.

Molecular biology studies of nephroblastomas have been a relatively recent development and although several putative Wilms tumour genes have been recognised, the role of these genes and their interaction with other genes and gene products is still poorly understood. Multiple Wilms tumour genes exist but there are two major genes implicated in nephroblastoma tumourigenesis, namely *WT1* and *WT2*. It has been suggested that a putative *WT3* gene is located on chromosome

16q. The microsatellite markers selected for this study are directed at the Wilms tumour gene loci on 11p and 16q.

The *WT1* gene at chromosomal band 11p13 has been cloned and is known to be important in the aetiology of at least some tumours by virtue of the identification of both germline and somatic mutations in patients with nephroblastomas. The most intensively studied Wilms tumour gene is the *WT1* gene, which acts as a tumour suppressor gene.[2,3] *WT1* probably plays a key role in initiating and maintaining mesenchymal and epithelial differentiation in the kidney, and is therefore essential for kidney development.[4] Loss of *WT1* function probably leads to aberrant differentiation resulting in unrestricted growth of metanephric blastemal cells in the embryonic kidney and nephroblastoma.[5]

A second locus, *WT2*, has been implicated in the development of nephroblastoma. This locus is also associated with the BWS[6-8] but understanding of the genetic mechanisms affecting this locus is dependent on understanding the mechanisms of the multiple genes in this region, including *H19/IGF2*, *CDKN1* (*p57*) and *KCNQ1*.[9,10]

The cell cycle is an extremely complex system and a family of protein kinases, called the cyclin dependent kinases (CDK) essentially governs cell cycle progression. Progression of the cell cycle is regulated by the accumulation and binding of cyclins, the displacement of inhibitors and specific phosphorylation of CDKs by activating kinases. Cyclins are cell cycle proteins that sequentially activate various CDKs along the cell cycle. p21 is a CDK inhibitor which mediates p53 induced G1 cell cycle block after DNA damage. Deregulation of the cell cycle is a characteristic feature of most malignant tumours. This disorganisation may occur at many levels in the cell cycle and often involves one or several of the proteins involved in the control and progression of the cell cycle (cell cycle proteins).

Activated CDKs phosphorylate various substances and pRb is one of the main targets. pRb is inactivated by CDK mediated phosphorylation in G1-S transition. Alterations of *Rb* have been observed in a wide range of neoplasms. pRb is considered to be inactivated when there is very weak or no nuclear staining.

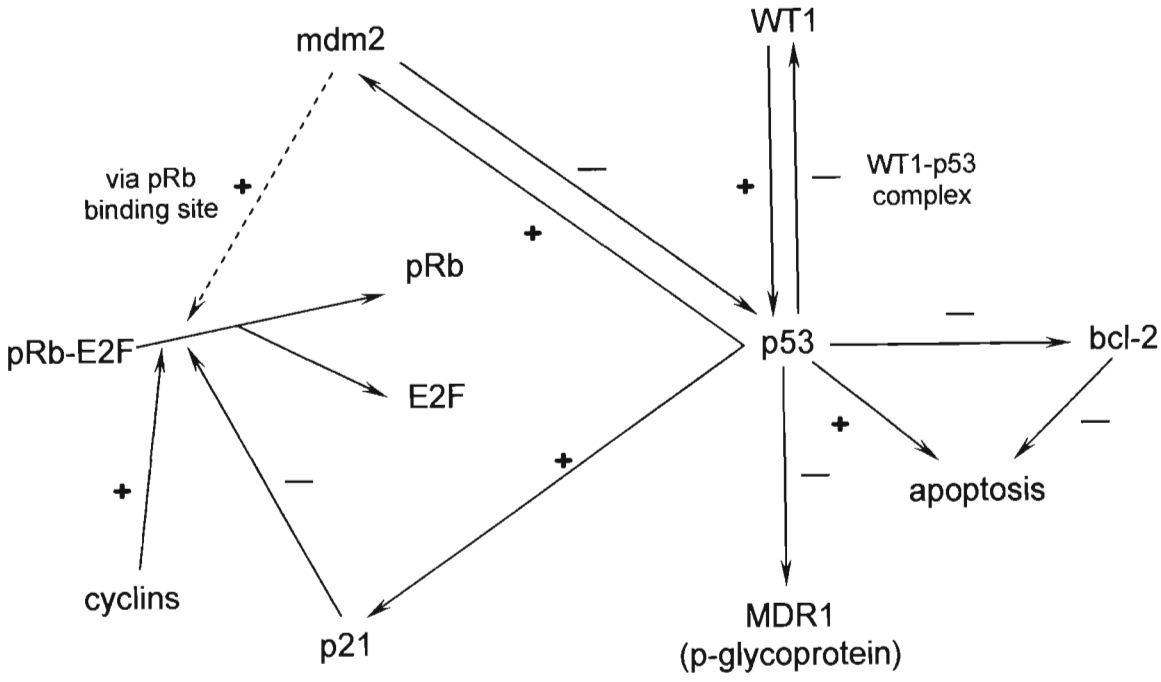


Figure 1: Inter-relations between markers

The *p53* gene can act as either a dominant oncogene or a recessive tumour suppressor gene. Alteration of the *p53* gene is the commonest genetic change associated with human malignancy. The p53 protein has multifunctional qualities. It has been shown to interact with a number of cellular proteins including WT1 protein (Figure 1).[11] An association between p53 and multidrug resistant gene (*MDR1*) has also been suggested since p53 may stimulate MDR1 expression (Figure 1). p53 acts as a checkpoint in the cell cycle at the G1-S transition by activating p21 CDK inhibitor (Figure 1). The chemotherapy response of p53 positive nephroblastomas has not been studied. However, in light of the proposed association between p53 and MDR1, investigation of this may be significant. p53 has also been associated with the induction of differentiation and the promotion of programmed cell death or apoptosis. An association between *p53* mutation and nephroblastoma with anaplasia, a subtype with unfavourable prognosis, has been reported. Immunohistochemical detection of p53 protein is related to gene mutations, which stabilise the protein, prevents its degradation and increases its half-life. The role of p53 alterations as a molecular marker of poor prognosis in nephroblastoma has been investigated.

The *bcl-2* gene is different from other known oncogenes in that its expression does not induce proliferation or transformation but allows cells to survive.[12] It induces cell

survival by blocking programmed cell death or apoptosis. Although bcl-2 was the first molecule to be implicated in regulation of apoptosis, it is now clear that p53 promotes apoptosis and has an action opposite to bcl-2 (Figure 1).

A study of both p53 and bcl-2 expression in relation to survival has been done on non-Hodgkin's lymphomas.[13] In lymphomas, the relationship between bcl-2 expression and survival was not found to be significant. The relationship between p53 expression and overall survival, on the other hand, was found to be significant. Combined expression of p53 and bcl-2 identified a group with poorer prognosis than those expressing p53 only. A similar study has not been done on nephroblastomas.

Microsatellites are short tandem repeat sequences of DNA that are scattered throughout the genome. They are stably inherited, unique to each individual and have a low inherent mutation rate. Typically, these tandem sequences consist of DNA repeats of 6 base pairs or fewer, and the total length of the stretch is fewer than 100 base pairs. These repeats are commonly located within the non-coding DNA sequences and their function is unknown. They are invaluable in genetic linkage studies because of their high degree of polymorphism. Currently, over 2000 polymorphic microsatellite loci have been identified in the human genome. In the space of a few years, microsatellite polymorphisms have assumed a major role in genomic analysis with applications as diverse as positional cloning, tumour biology, human evolution, medical diagnostics, and forensic identification. The major factors for this popularity are their informativeness, ubiquity in the genome, and PCR typability. A study which found microsatellite instability (MSI) in 3% of nephroblastomas suggested that defects in mismatch repair genes may contribute to the pathogenesis of nephroblastomas.[14] This initiated the present study which aimed to determine the frequency of MSI in nephroblastomas.

Since MSI is regarded as a marker of defective MMR system, it is used to identify underlying MMR defects. Nearly 86% of hereditary non-polyposis colorectal cancers (HNPCC) showed instability of microsatellite sequences compared to 16% of sporadic colorectal carcinomas.[15] This difference in frequency of MSI is due to the presence of germline mutations in mismatch repair genes in patients with HNPCC. The majority of HNPCC cases that have been genetically characterised are accounted for by

germline mutations in *hMSH2*, *hMLH1* and in attenuated cases, *hMSH6*.[16] Mutations in *hPMS2* and *hEXO1* are less frequent and are sometimes associated with 'atypical' HNPCC tumour phenotypes but are still awaiting additional validation regarding their role in the aetiology of colorectal cancers with MSI.[17] It is important to note, however, that only a small proportion (1–5%) of colorectal cancers is the result of HNPCC.[18,19] Although MSI has been studied in many extra-colonic neoplasms, to date there have been limited studies in nephroblastomas.

A defect in the mismatch repair system will lead to genetic alterations, and consequently abnormal proteins will be expressed. Therefore, there is a close interplay between the molecular and immunohistochemical components of this study. Studies looking at 11p, 16q and MMR genes in nephroblastomas using polymorphic microsatellite markers have not been conducted in nephroblastomas from South Africa. Therefore, this study will be the first to examine the expression of cell cycle proteins and abnormalities of the 11p, 16q and MMR loci.

The prognosis and management of cancer must be based not only on traditional clinical and histological factors, but also on a thorough understanding of the molecular mechanism involved. Investigation and analysis of the proteins involved in various cellular processes, such as, cell signalling, cell cycle arrest, differentiation and apoptotic cell death should help to elucidate the cause and progression of individual tumours.

CHAPTER TWO

LITERATURE REVIEW

2.1 Epidemiology and clinical features of nephroblastoma

Nephroblastoma is the most common primary malignant renal tumour of childhood and accounts for 80% of genito-urinary cancer in children under 15 years of age.[21] It affects approximately one child per 10 000 world-wide under the age of 15 years.[22] These tumours are uncommon in neonates and most cases occur in children between 1 and 3 years of age.

The incidence of nephroblastomas varies in different racial and ethnic groups. Blacks have a high incidence, whereas Whites appear to have an intermediate incidence and Orientals a low incidence.[23] In Britain the relative incidence rate in Asians is about half those in Whites, while West Indians have a rate more than twice the rate in Whites.[24]

Most patients, approximately 80%, seek medical attention because the mother or child-minder notices an abdominal mass. Other presenting features include abdominal pain (37%), fever (23%), haematuria (21%) and systemic hypertension.[23,25,26] In most series hypertension is seen infrequently, however in one series hypertension was found in 63% of patients.[27] Hypertension in nephroblastomas may be caused by renal artery compression or by renin production by tumour cells. Rarely patients may present with acute abdominal symptoms usually due to intratumoral haemorrhage.[28] In Third World communities patients tend to present with advanced disease and poor nutritional status, which has a negative influence on outcome.[29]

2.2 Biology of nephroblastoma

Wilms tumours are epidemiologically and histopathologically heterogeneous.[30,31] Cytogenetic and molecular studies conducted in numerous American and European centres show that the molecular pathogenesis of Wilms tumour is also complex involving more than one genetic locus and interactions between genetic and epigenetic factors.[8,32,33] Wilms tumour research has proven to be a very fertile ground for studying a wide range of fascinating biological problems: (i) the relationship between cancer and development, (ii) the control of normal genitourinary development, (iii) the primordial relationship of kidney and gonadal

development, (iv) the evolution of the genitourinary system, (v) the control of transcription during development and (vi) the processes of RNA editing and genomic imprinting.

An ever growing number of studies have now addressed the nature of these events: (i) the number of different loci involved in predisposition and tumour progression, (ii) the recessive or dominant mode of action of mutations in identified genes, (iii) their role in embryonic and adult development, (iv) the nature of epigenetic events underlying genomic imprinting, and (v) the role they play both in urogenital development and in tumourigenesis. Thus the genetics of Wilms tumour seems to contradict some of the well-established principles of tumourigenesis, including the two-hit hypothesis of Knudson, the recessive nature of mutations of tumour suppressor genes, and the expected presence of a tumour suppressor rather than a growth factor (oncogene) at the sites revealed by losses of alleles.[20,34,35]

2.2.1 Histogenesis of nephroblastoma

From a biological perspective, Wilms tumour is particularly fascinating, as it is believed to arise from pluripotent embryonic metanephric tissues persisting beyond foetal development,[36] and perhaps failing to respond to normal differentiation signals.[31] This provides a window for examining the mechanisms of early renal development and exploring the properties of embryonic kidney stem cells.

Nephroblastoma has been regarded as an example of altered development of the kidney.[34,35,37,38] An understanding of normal kidney development seems to be essential to define the molecular basis underlying its development. In humans, the kidney forms through the reciprocal epithelial-mesenchymal induction of two tissues, the ureteric bud and the metanephric mesenchyme. The metanephric mesenchyme induces the ureteric bud to branch and eventually form the collecting ducts. The mesenchyme then condenses around the ureteric buds and in response to inductive signals from the bud differentiates into epithelial cells, which go on to form the proximal and distal tubules and the glomerulus of the nephron. The uninduced blastemal cells undergo apoptosis.[39] The permanent kidneys begin to develop at day 35–37 in human embryos, and nephrogenesis is complete at approximately 36 weeks of gestation.

It is not surprising that nephroblastoma shows pathogenetic heterogeneity since it arises in a developmentally complex organ, the kidney. Nephrogenesis is an immensely complex process involving events occurring over many months, and it seems obvious that many different molecular events are involved. In this setting it seems reasonable that many types of molecular error might occur potentially leading to the formation of nephrogenic rests. Nephroblastomas appear to arise from these aberrant rests, which persist into early childhood. Normally such rests are transient structures that would disappear by 36 weeks of gestation.

The characterisation of *WT1* has provided insight into the mechanisms underlying normal kidney development and Wilms tumourigenesis.[5,20,38,40-43]

2.2.2 Clinical forms of nephroblastoma

Nephroblastoma arises in three distinct clinical scenarios: syndromic, familial and sporadic.

The syndromes in which nephroblastomas occur may be divided into those characterised by somatic overgrowth and those lacking overgrowth (Table 1).[44] Investigations of these clinically distinct entities have begun to clarify some aspects of the biology of nephroblastoma.

Table 1: Syndromes associated with nephroblastoma

With somatic overgrowth	**Without somatic overgrowth**
Beckwith-Wiedemann syndrome	Aniridia
Hemihypertrophy	WAGR syndrome
Perlman syndrome	Denys-Drash syndrome
Sotos syndrome	Familial nephroblastoma
Simpson-Golabi-Behmel syndrome	

Genetic studies indicated that nephroblastoma occurs with high frequency in four different syndromes: the WAGR (Wilms tumour, aniridia, genitourinary abnormalities, mental retardation) syndrome, the Denys-Drash syndrome, the Beckwith-Wiedemann syndrome, and the Perlman syndrome.

The WAGR syndrome (~1% of nephroblastoma cases) is almost always caused by a *de novo* deletion involving the 11p13 region. The two genes identified in the deleted region are *PAX6* and *WT1*. In rare cases the deletion may be inherited from one of the parents. The penetrance is on the order of 50%.[38]

In Denys–Drash syndrome (~0.5% of nephroblastomas), patients have pseudohermaphroditism and mesangial sclerosis, leading to inevitable early renal failure, coupled with a very high risk of nephroblastoma. It has become clear that *WT1* can also act as a dominant negative oncogene, implying that alterations in only one of the *WT1* alleles may contribute to abnormal cell growth. The existence of dominant negative *WT1* mutations is supported by analyses of the germline of children with the Denys-Drash syndrome.[45,46] In Denys-Drash syndrome, only one *WT1* allele harbours a mutation within the DNA binding domain suggesting that a dominant mutation can overcome the presence of the remaining wild-type allele.[47]

Beckwith-Wiedemann syndrome (~1% of nephroblastomas) results in a foetal overgrowth that predisposes to several paediatric malignancies, including nephroblastoma. While most BWS cases are sporadic, approximately 15% are familial or occur in association with chromosomal abnormalities.[20] The gene implicated in BWS has been mapped to 11p15.5.[6-8]

In Simpson–Golabi–Behmel syndrome[48] mutations in glypican-3 (*GPC-3*) have been found. This gene is expressed in both mesenchymal and ureteric bud derived tissue during embryonic renal development and is essential for normal renal branching morphogenesis.[49] The Simpson-Golabi-Behmel syndrome is characterised in part by pre- and post-natal somatic overgrowth and renal medullary cystic dysplasia.[48] Hence, *GPC-3* provides another link between Wilms tumourigenesis and renal development.

Perlman syndrome is characterised by renal dysplasia with nephroblastoma, foetal gigantism, foetal ascites, macrosomia, hyperplasia of the endocrine pancreas, typical facial appearance, and mental retardation in those who survive past early infancy.[50] The phenotype varies and some clinical features overlap those of the

Beckwith-Wiedemann syndrome. The syndrome is familial and is transmitted as an autosomal recessive trait. Cytogenetic studies have shown an extra band on the tip of the short arm of chromosome 11 (11p).

Congenital syndromes that predispose to nephroblastoma have been pivotal in unravelling the genetic factors implicated in nephroblastoma development. Patients with these syndromes often develop multiple tumours, either in both kidneys (bilateral disease) or in one kidney (multifocal disease). These syndromic tumours also tend to occur at an earlier age of onset than sporadic cases, which normally present with a single tumour at a later age.

Familial nephroblastoma, in which cancer susceptibility is transmitted from one generation to the next, represents only 1-2% of all cases[22,51] and falls far short of the incidence (30%) predicted by the two-hit hypothesis.[52] Linkage analyses in families susceptible to nephroblastoma excluded genetic loci on chromosome 11p (*WT1* and *WT2*) and 16q as possible familial predisposition genes.[53-55] In these rare familial cases, nephroblastoma is inherited as an autosomal dominant trait with incomplete penetrance[52,56] and linkage has been reported in these families to chromosomes 17q12-q21 (*FWT1*)[57] and 19q13.3-q13.4.[58] Comparative genomic hybridisation analysis has also implicated chromosomes 4q, 9p, 20p and 3q as regions that may harbour potential familial predisposition genes.[59] The critical rate-limiting steps in the development of familial nephroblastoma therefore seem to involve at least two separate genes.[57,58]

The majority of nephroblastomas occur in children with neither unusual physical features nor positive family history and are considered sporadic. In sporadic Wilms tumour, mutations in *WT1* are present in 10–15% of cases[60] and β-*catenin* activation mutations can be detected in 10% of tumours.[61] In addition, LOI of *IGF2* has been detected in up to a third of sporadic tumours[62,63] and might therefore have a role in these cases.

2.3 The genetics of nephroblastoma

2.3.1 Historical background

In 1964, Miller and collaborators were the first to suggest that nephroblastoma had a genetic basis based on their observation that nephroblastoma was associated with aniridia, genitourinary abnormalities, and hemihypertrophy.[64] Almost a decade later, Knudson and Strong proposed a two-hit genetic model of Wilms tumourigenesis.[52] In 1979, Uta Francke and her colleagues[65] found constitutional chromosome 11p13 deletions in patients with the WAGR syndrome, which suggested that perhaps this was one of the two genetic events of the two-hit model.[52] These findings provided the catalyst to search for the Wilms tumour suppressor gene.

Nephroblastoma was one of the three tumours that led Knudson and Strong to their two-hit hypothesis of carcinogenesis.[52] While retinoblastoma, one of the original three tumours, has met the criteria of their hypothesis fairly well, nephroblastoma has proved far more complicated. Based on the two-hit model, it has been suggested that nephrogenic rests might represent the first mutational event.[52,66] But that only a few intralobar nephrogenic rests (ILNRs) are found in the kidneys of children with constitutional chromosome disorders such as 11p13 deletion raises the possibility that the a "second hit" may be responsible for the development of nephrogenic rests. It has also been suggested that nephroblastoma induction in a nephrogenic rest might involve additional mutational events following the second hit. This suggestion is consistent with the observation that a number of other chromosomal defects, some of them repetitive, involving chromosomes 1, 8, 16, and many others, are present in nephroblastoma specimens.[67,68] Despite nephroblastoma being one of the most difficult genetic problems in molecular oncology, it has thus far been extremely informative.

The genetic basis of Wilms tumour is very complicated in comparison with the relatively simple situation found in another model paediatric tumour, retinoblastoma.[33,67] Firstly, unlike the situation for retinoblastoma, familial Wilms tumour is extremely rare. Secondly, Wilms tumour is found at high frequency in association with other congenital anomalies in at least four different syndromes. Thirdly, LOH of 11p loci is only seen in 30-50% of tumours depending on the

study,[8,35] compared with 70% in *Rb*.[69] In addition, LOH is not confined to region 11p13 but often extends into 11p15 and in some tumours LOH is restricted to the 11p15 region.[8] This would suggest that tumour suppressor genes are present in both 11p13 and 11p15, which may act alone, or in concert, giving rise tumourigenesis. Lastly, the rare cases of familial Wilms tumour are not linked to chromosome 11.

2.3.2 11p13 chromosomal locus

The first clue to the location of a putative gene involved in the development of nephroblastoma was the finding of constitutional deletions encompassing chromosome 11p13 in the WAGR syndrome[70] and 11p LOH in sporadic nephroblastomas. It has subsequently been determined that the WAGR deletion encompasses a number of contiguous genes, including the paired box gene *PAX6*, the calcium binding protein reticulocalbin and the zinc finger gene *WT1*.[32,44,71] Loss of one copy of *PAX6* is responsible for aniridia,[44] whereas mutations of one *WT1* allele may confer genitourinary defects,[44,72] in addition to constituting the first hit required for the development of nephroblastoma.[5,37]

Loss of heterozygosity (LOH) for 11p markers as a result of chromosome loss or deletion, loss of one chromosome and reduplication of the remaining homologue, or somatic recombination are reported in 40-50% of nephroblastomas.[35] Cowell *et al.*[73] suggested that allele loss on 11p is not always associated with *WT1* mutation as might have been expected from the general LOH dogma. This together with tumour LOH for markers telomeric of 11p13 and maintenance of heterozygosity for markers flanking chromosome 11p13 has suggested the presence of a second independent Wilms tumour suppressor locus on the short arm of chromosome 11.[33] Tumour LOH for chromosome 11p15 markers occurs in about 40% of nephroblastomas.[6] This second locus at chromosome 11p15 has already been designated *WT2* and may explain the association of nephroblastoma with BWS.

2.3.2.1 WT1 Structure

Although several loci have been implicated in the biology of nephroblastoma, the function and significance of only one tumour locus, the Wilms tumour suppressor

gene *WT1* at chromosome 11p13, has been studied in detail. Several groups simultaneously isolated the *WT1* gene in 1990, using positional cloning methods.[32,74,75] The *WT1* gene contains ten exons, covering approximately 50 kb[32,74-76] and encodes multiple 52–56 kDa protein isoforms generated via alternative mRNA splicing,[76] RNA editing[77] and non-AUG translational initiation.[78]

Three potential functional domains have been identified in the WT1 protein. The carboxyl (C) terminus of WT1 contains four zinc finger domains of the Krüppel Cys_2-His_2 class that mediate sequence specific DNA binding.[32,76] This motif is similar to those found in transcriptional regulators named early growth response 1 (EGR1), EGR2, Sp1, Krox-20, and Krüppel.[32,74,75] The WT1 amino (N) terminus is rich in proline and glutamine, a feature found in the transactivation domain of other transcription factors. Finally, a potential third domain of WT1 is encoded by the alternatively spliced exon 5, although the function of this protein domain remains unknown. WT1 shows an expression pattern restricted both temporally and spatially during development.

Together with the observed RNA editing,[77] there may be as many as 16 different WT1 isoforms. Of these, the most noteworthy are the variable insertion of exon 5 (alternative splice I), and the variable splice donor-site usage on exon 9 (alternative splice II). Exon 5 encodes a stretch of 17 amino acids just N-terminal of the four zinc fingers.[76] Alternative splice I is specific to mammals and, although its presence modifies *WT1* function in some cell-culture systems, its role *in vivo* remains unclear. The other alternative splicing event involving exon 9 results in the insertion of three amino acids (lysine-threonine-serine)(+KTS) between zinc fingers three and four.[76] This alternative splice II (+KTS) alters the DNA-binding properties of WT1,[79] and has been shown to affect the development of kidneys and gonads in mice and in humans affected by Frasier syndrome. Kidneys are abnormal and hypoplastic in the absence of the –KTS form of WT1 whereas the absence of the +KTS form causes defects in the function of glomerular podocytes.[80,81] The WT1 isoforms lacking KTS appear to function predominantly as transcription factors, whereas the isoforms containing KTS associate with splicing complexes in the nucleus and might be involved in RNA metabolism.[80,81]

2.3.2.2 *WT1* mutations

WT1 mutations represent an early genetic event in Wilms tumour development,[82,83] because mutations have been found in 'nephrogenic rests'.[84] Two to four percent of Wilms tumours are caused by an inherited mutation in the *WT1* gene.[85-87] However, the majority of nephroblastomas are later onset, unilateral, nonsyndromic, and likely to be sporadic. *WT1* mutations are seen in approximately 20% of sporadic nephroblastomas.[5] Mutations in *WT1* cause an arrest in nephrogenic development, presenting a persistent target of nephrogenic precursor cells for subsequent tumourigenic events. However, most sporadic Wilms tumours do express wild-type WT1, often at high levels, raising the possibility of other genetic lesions in downstream effectors. In the germline, heterozygous mutations in *WT1* are associated with renal and genitourinary abnormalities, implying a broader role for *WT1* in renal and gonadal development. In this situation, *WT1* appears to act as a classic loss-of-function tumour suppressor gene.[5]

Varanasi and collaborators[60] did not detect *WT1* mutations in the majority of sporadic cases analysed, despite having scanned the entire gene. They could not exclude the possibility of a mutational hot spot within *WT1* yielding a false negative result but they did not favour such an argument, because each amplified strand of the *WT1* gene was analysed individually.

Schumacher and colleagues[88] in the course of two studies analysed a total of 86 patients and found *WT1* mutations/deletions in 21 cases (24%). The mutation frequency was significantly different among the various histological subtypes: mutations were seen in 63% of tumours of stromal-predominant histology; 14% of tumours of triphasic histology; and 6% of tumours of blastemal-predominant histology. Of the 21 mutations, 17 were present in the germline, and 3 of these were deletions in patients with WAGR syndrome. Six of 10 stromal tumours with *WT1* mutations showed LOH of 11p13 markers, and one tumour had a second somatic mutation. These results confirm that the presence of *WT1* mutations correlates with the specific histological subtype of stromal-predominant or intralobar nephrogenic rest (ILNR)-like Wilms tumours. These findings suggest that the two-hit inactivation of *WT1* is operative in stroma-predominant Wilms tumours.[89] The

extensive rhabdomyomatous differentiation and the presence of *WT1* mutations may be used as a diagnostic tool to identify a tumour subtype that seems to respond poorly to chemotherapy. In contrast, blastemal-predominant tumours express wild-type WT1 and show early signs of epithelialisation.

Zhuang *et al.* studied the connection between the structure of Wilms tumour and expression of the *WT1* gene.[90] Their study showed that deletions of *WT1* gene are present in all three components of the Wilms tumour structure (blastema, epithelium and stroma).

2.3.2.3 WT1 expression

The *WT1* gene is expressed in a limited set of tissues during development, chief among those being the kidney, the gonads, the spleen and the mesothelium.[43,91] During early nephrogenesis, a low level of WT1 is first detected in the uninduced mesenchyme and it then increases dramatically during induction. *WT1* is expressed in the condensing blastemal cells, renal vesicles, and glomerular epithelium; all of these are regarded as renal precursor cells.[43] Expression then becomes restricted to the posterior part of newly formed epithelium and is limited to the podocytes of the adult kidney.

In contrast to its transient expression in the developing kidney, *WT1* is expressed continuously in mesothelial cells, Sertoli cells of the testis, and granulosa cells of the ovary.[92] All these tissues are of mesodermal origin and all, including the kidney, experience a mesenchymal to epithelial transition when *WT1* is being expressed. This hypothesis is supported by the findings that in nephroblastomas missing functional *WT1*, the mesenchymal cells can no longer differentiate into epithelia but can take on different fates, either stromal or myogenic.[88,89,93] In nephroblastomas, WT1 is seen in the malignant counterparts of those elements that express the protein during normal development (that is, blastema and glomeruloid structures) but is absent from stromal cells.[94]

2.3.2.4 WT1 function

Since the cloning of *WT1*, a wealth of experiments has been carried out to determine the function of *WT1* as a tumour suppressor and developmental

regulator. The expression pattern of the *WT1* gene in the kidney suggests that *WT1* may function at three different stages of development: the onset of nephrogenesis, the progression of nephrogenesis and the maintenance of normal podocyte function. Absence of *WT1* at the first stage results in failure of kidney development as has been observed in *WT1* null mice.[95] The blastemal cells fail to differentiate and undergo apoptosis. The *WT1* gene has been implicated in many processes like proliferation, differentiation and apoptosis. In agreement with these diverse functions is the growing list of putative target genes. Expression of the gene may allow a cell to respond appropriately to signals from its environment and the many isoforms may aid in fine-tuning this response.

Current evidence suggests that *WT1* plays a role in the development of nephroblastoma but *WT1* appears to be involved in only a subset of tumours since *WT1* gene mutations have so far been demonstrated in a limited number of cases. The action of *WT1* as a tumour-suppressor gene seems hard to reconcile with its essential pro-survival function in normal early renal differentiation. One possible explanation is that tumour cells with *WT1* mutations are able to escape apoptosis owing to the effects of other genetic events. In this situation, the strong association between mutations in *WT1* and β-*catenin* is particularly interesting. Some 10% of sporadic Wilms tumour specimens have inactivating mutations in *WT1*, and these same tumours often also contain mutations in β-*catenin* (*CTNNB1*).[61,96] This suggests that the wnt-signalling pathway, which is known to be vital in kidney development, may also be implicated in Wilms tumourigenesis. An alternative hypothesis is that the tumour-prone consequences of *WT1* inactivation only occur at a slightly later stage of renal development, when the failure of *WT1*-mediated differentiation might lead to immortalisation of renal precursors instead of apoptosis. This would produce a population of cells that are susceptible to additional transforming events and yet continue to be proliferative progenitors that can produce the blastemal, mesenchymal and epithelial cell types observed in nephroblastoma.

Recent studies have indicated that *WT1* can either activate or repress GC-rich promoter reporters, depending upon experimental conditions.[97] WT1 proteins can bind to a GC-rich EGR-like DNA sequence[79,82] and to a longer CT-rich sequence.[98]

A large number of genes have been proposed as targets of *WT1* transcriptional regulation, but only a few endogenous genes show altered expression when levels of WT1 are modulated. The activity of WT1 is modulated by protein–protein interactions, including self-association,[98] binding to the tumour suppressor p53[11] and to the transcriptional repressor PAR-4.[99] The association with p53 and PAR-4 may contribute to the ability of WT1 to repress the transcription of potential target genes.

Based on the prototype *WT1* responsive promoter, that of the immediate early gene *EGR1*, *WT1* has been thought to act by repressing expression of growth-inducing genes, including insulin-like growth factor II (*IGF2*), insulin-like growth factor receptor (*IGFR*), platelet-derived growth factor A (*PDGF-A*), epidermal growth factor receptor (*EGFR*), transforming growth factor β (*TGF-β*), *c-myc*, and others.[82] However, most genes with *WT1*-responsive promoters are not physiologically regulated by WT1.[100] In contrast, several target genes seem to be activated by *WT1 in vivo*, including *CDKN1A* (cyclin-dependent kinase inhibitor 1A, also known as *p21*),[101] *bcl-2*,[102,103] *AREG*,(amphiregulin) [104] and *CDH1* (E-cadherin).[105]

In agreement with its genetic inactivation in Wilms tumour, the role of *WT1* as a tumour suppressor is supported by the consequences of its ectopic expression in various cell lines, including Wilms tumour cell lines. For example, several experiments have documented suppression of colony formation and reduction of tumourigenesis in nude mice. In addition, G1 arrest and apoptosis have been associated with the direct activation of p21 and downregulation of EGFR, two known WT1 transcriptional targets,[100,101] indicating possible mechanisms for tumour suppression. The ability to suppress the expression of growth-associated genes may also contribute to the function of *WT1* as a tumour-suppressor gene.

2.3.3 11p15 chromosomal locus

There is at least one additional genetic alteration on 11p in WT. This putative chromosome 11p15 Wilms tumour suppressor gene has been associated with the BWS,[6-8] but understanding of the genetic mechanisms affecting this locus has awaited the identification of multiple genes in this region. 11p15 contains a cluster

of imprinted genes that have been shown to be involved in nephroblastoma tumourigenesis both by LOH and by epigenetic changes.[106,107]

There are various 11p15 expression abnormalities that could be relevant for Wilms tumour pathogenesis.[108] These abnormalities affect three imprinted genes at 11p15 (*BWR1C*, *CDKN1C*, and *IGF2*) and two genes involved in kidney development (*RET* and *GDNF*). These consist of: overexpression of *IGF2*, lack of expression of *BWR1C*, abnormal coexpression of *RET* and *GDNF*, and in some cases, very low expression of *CDKN1C*.

2.3.3.1 WT2

Other tumours that show LOH for 11p15 loci include embryonal tumours such as rhabdomyosarcoma, hepatoblastoma, adrenocortical carcinomas, and adult tumours such as breast cancer, non-small-cell lung carcinoma, pancreatic cancer associated with multiple endocrine neoplasia type 1 (MEN1), bladder carcinoma, hepatocellular carcinoma, and testicular cancer.[3] These observations suggest that the 11p15 gene may be important in the development or progression of many tumours, not only nephroblastoma.

The region harbouring *WT2* has been localised to the 11p15.5 region, from the insulin-like growth factor-II (IGF2) locus to the telomere, including the HRAS1 locus.[3] Mapping studies place both *H19* and *IGF2* in the minimal region defining *WT2*.[109] The *H19* gene satisfies several criteria for *WT2*: (i) it is silenced in all cases with LOH; (ii) its expression is extinguished by a gene-specific mechanism (DNA methylation) which is recognized as a mechanism for elimination of tumour suppressor gene activity; (iii) and this inactivation results in both loss of *H19* RNA and a reciprocal biallelic activation of *IGF2*.

2.3.3.2 Genomic imprinting and Beckwith-Wiedemann syndrome

According to Mendel's laws the activity of a gene should not depend on its parental origin.[110] In violation of these laws, there are a number of observations that point to differences in the activity of certain genes depending on whether they have been inherited from the mother or the father. The process by which alleles are marked in the two parental germlines for differential expression in the offspring is referred to as

genomic imprinting or, parental or gamete imprinting.[111,112] Genes whose expression is inhibited after passage through the mother's germline are referred to as 'maternally imprinted' and conversely genes whose expression is inhibited when transmitted by fathers are called 'paternally imprinted'. Imprinted genes are silenced possibly during gametogenesis. Although only a minority of imprinted genes have been identified, they play a crucial role in development and carcinogenesis.[113] Imprinting appears to be controlled by epigenetic modification, with DNA methylation suggested as an important epigenetic signal.[114] In imprinted genes there is differential methylation of one parental allele at specific sites, which correlates with expression or non-expression of that allele, depending on the mechanisms of transcriptional control in that particular gene.[115] The inhibition of gene expression at imprinted loci is often virtually complete, so that imprinted genes are monoallelically expressed in somatic tissues of the offspring. Genomic imprinting is reversible. A gene that has been imprinted by passage through one type of parent will be reactivated on passage through the opposite type of parent.[110]

The Beckwith-Wiedemann syndrome clearly involves various molecular alterations of the multiple imprinted genes within 11p15.5 region. In humans, imprinted genes are clustered into 2 distinct regulatory domains at 11p15.[106] Imprinting centres in these two domains have differentially methylated regions (DMRs). Domain 1, which has a telomeric location at 11p15, contains 2 genes: *IGF2* and *H19*. Domain 2, which has a centromeric location at 11p15, contains a spectrum of genes, 3 of which are implicated in Beckwith-Wiedemann syndrome: *KCNQ1* (potassium voltage-gated channel-subfamily Q or long QT syndrome gene), *KCNQ1OT1* (KCNQ1-overlapping transcript 1), and *CDKN1C* (cyclin dependent kinase inhibitor 1).[9,10]

The *H19* gene codes for a spliced, polyadenylated, biologically active nontranslated mRNA,[9,10] which is minimally expressed in undifferentiated cells, highly expressed in a large variety of foetal tissues at a stage when cells are differentiating and then declines in most differentiated tissues.[116,117] *H19* has been shown to inhibit embryo growth in transgenic mice.[118] Rainier *et al.*[63] found that *H19* is transcribed from the maternal allele (paternally imprinted), and *IGF2* from the paternal allele (maternally

imprinted). This was the first direct evidence for an imprinted gene in man. *IGF2 is* an important autocrine and paracrine growth factor in a wide variety of childhood and adult tumours. The physical linkage of the *H19* and *IGF2* genes in both mice and humans suggests that they constitute a functional imprinting domain but they are imprinted reciprocally.[119]

CDKN1C ($p57^{Kip2}$) is paternally imprinted and has been proposed as a candidate tumour suppressor gene and is a negative regulator of foetal growth.[120] *KCNQ1* is maternally expressed in most tissues. Mutations of this gene have been associated with cardiac arrhythmia syndromes. The 5' end of the *KCNQ1OT1* transcript overlaps with DMR2. The maternal allele of DMR2 is normally methylated and *KCNQ1OT1* is silenced while the paternal allele is expressed.

The following molecular alterations of these imprinted genes have been described in BWS[9,121-124]: (i) maternally inherited translocations and inversions in 1-2% of cases; (ii) uniparental disomy which affects both domains and is due to the loss of the maternal allele and duplication of the paternal allele. This occurs in 20% of cases; (iii) *CDKN1C* mutations are found in 5-10% of cases but are more frequent in familial BWS; (iv) hypermethylation of *H19* is seen in 10% of cases; (v) demethylation of a DMR at the 5' end of *KCNQ1OT1* is seen in 55% of cases and (vi) microdeletions have been described in a small number of cases.

Direct genetic evidence for this link between imprinting and cancer was identified when 70% of nephroblastomas were found to have biallelic *IGF2* expression.[62,63,125] Inactivation of *H19* due to hypermethylation of the normally unmethylated maternal allele was also present in a number of these cases.[125,126] Transcription of *H19* is significantly repressed in a majority of cases of nephroblastoma.[125,126] Loss of the maternal allele in Wilms tumour would cause loss of function of *H19* and $p57^{Kip2}$, and paternal duplication (uniparental isodisomy) could result in increased dosage of *IGF2*. Studies have found that nephroblastomas that lacked 11p15 LOH showed consistent biallelic expression of *IGF2*.[62,63] The term 'loss of imprinting' (LOI) has been coined to describe this alteration.[127] These results suggest that LOH at 11p15

and loss of imprinting both lead to the overexpression of a growth-promoting gene (*IGF2*) and the loss of expression of growth-inhibitory genes (*H19* and *CDKN1C*).

IGF2 is overexpressed in all nephroblastomas, including tumours from BWS patients.[128] A majority of nephroblastomas have a functional double dose of the active *IGF2* allele. *IGF2* also has a role in preventing apoptosis.[129] It is possible that enhanced levels of IGF2 within a precursor cell might interfere with the normal balance of apoptosis and differentiation and lead to clonal expansion of a cell population within the developing kidney.

2.3.4 16q chromosomal locus

The fact that more than one chromosomal region has undergone LOH in Wilms tumour[6-8] is not surprising since it is likely that multiple genetic events are required to give rise to a fully malignant phenotype. The presence of a third Wilms tumour gene has been suggested by the demonstration of LOH for markers at chromosome 16q at a rate considerably higher than the background rate of approximately 5% for LOH on other chromosomes.[6,67] The locus identified on chromosome 16q may be involved in tumour progression since LOH on this chromosome occurs in addition to LOH at 11p13 and 11p15.[6]

A study narrowed the area of 16q LOH to a 6.7 Mb locus.[130] According to the National Center for Biotechnology Information (NCBI) database, this small consensus area contains 42 identified and 129 hypothetical genes. Of the described and hypothetical genes, there are three known tumour suppressor genes in this area: *E-cadherin*, *P-cadherin*, and *E2F4*.[130]

COX4, another potential Wilms tumour candidate gene, is also located within the isolated region of deletion and codes for the mitochondrial enzyme cytochrome c oxidase subunit IV.[14]

2.3.4.1 16q alterations

Cytogenetic abnormalities of chromosome 16 have been reported in 30% of Wilms tumour and predominantly involve the long arm.[4,131] Molecular studies have found LOH for polymorphic markers on 16q in 10-25% of Wilms tumours.[6,67,131-134]

One study found LOH 16q in 14 of 66 tumours (21.2%), including LOH of both alleles in two cases.[133] No LOH was found in 44 children (66.6%) and in eight cases the results were non-informative. The findings of Skotnicka-Klonowicz and co-workers[133] point to a significantly lower occurrence of LOH 16q in tumours removed in stage I than in stage IIN- or IIN+ and III.

In another study of 61 informative cases of nephroblastoma, 11 (18%) showed loss of heterozygosity for 16q.[67] In the cases where parental DNA was available, the maternal 16q allele was lost in four of the tumours while the paternal allele was absent in the other four cases. Thus, in contrast to the extensive studies demonstrating preferential maternal loss on 11p, there is no evidence for an imprinting mechanism or for preferential mutation of either allele at the 16q locus.[67]

Austruy and co-workers detected 16q LOH in seven tumours (25%) and in one case of nephroblastomatosis.[131] For 6 of the 7 tumours, the common region of LOH was distal to the 16q12-q13 marker, D16S419. This is consistent with earlier studies identifying either 16q21-qter,[67] 16q21-24-qter[4] or 16q24-qter corresponding to a cytogenetically identified deletion.[135] An identical translocation, der(16)t(1;16)q21;q13), has also been reported in four Wilms tumours,[68,135] and a der(16)t(1;16)(q12;q12) was reported by Kaneko *et al.*[136] Additional studies have suggested that the common region of allelic loss is localised to 16q12.1-16q21.[137] However, a slightly proximal locus at 16q11-13 has also been considered.[4] Overall, LOH studies for chromosome 16 in Wilms tumour clearly define the 16q24-qter region as the most common site of allelic loss.[134] The evidence for a tumour-suppressor gene in 16q21 is still based on only a limited number of observations, which may indicate that genes in these regions are less critical in establishing the tumour phenotype.

Grundy and co-workers[132] retrospectively analysed Wilms tumours from 232 patients registered in the National Wilms Tumour Study for loss of heterozygosity (LOH) on chromosomes 11p, 16q, and 1p. LOH for chromosome 16q occurred in 17% of 204 informative cases and for chromosome 1p in 12% of 175 informative cases and only one tumour harboured LOH at all three sites.[138]

Another study found unequivocal 16q LOH for at least 1 marker in 14 of 96 specimens evaluated.[14] Allelic imbalance (AI) was present in an additional 4 specimens. One specimen showed deletion at 16p12.3-q22.1 and 16q23.2-16q24.2. They also found MSI in two specimens. One of the specimens showed microsatellite instability for 14 of 16 markers and the other specimen for 8 of 11 markers. By adding two additional highly sensitive MSI markers Bat26 and hMLH-1 (exon 12), they confirmed MSI in the same two cases but discovered no new cases of MSI. The finding of the consistent presence of novel microsatellite alleles in two Wilms tumours provides presumptive evidence of an underlying defect in DNA mismatch repair in the tumour.[14] This was purportedly the first report of microsatellite instability in Wilms tumour.

2.3.4.2 Clinical significance of 16q loss of heterozygosity

There is strong evidence that 16q LOH affects relapse-free survival and prognosis in children with nephroblastoma.[132,134,138,139] LOH for 16q has been associated with a relapse rate 3.3 times higher than those with tumours retaining heterozygosity for 16q.[132] In a large study consisting of 2021 patients with favourable histology nephroblastomas enrolled in the fifth NWTS (NWTS-5), the role of 16q and 1p LOH was determined.[140] The incidence of 16q LOH was significantly increased in anaplastic tumours compared to favourable histology nephroblastomas (32.4% vs 17.4%)(p=0.001), however there was no association with outcome in this histological subtype. It is noteworthy that there were 182 anaplastic tumours in this series. Another interesting finding in this study was the variation in frequency of LOH by patient age, being less frequent in younger patients. In summary, this study found that tumour-specific LOH for both chromosomes 1p and 16q was found to identify a subset of favourable histology Wilms tumour patients who have a significantly increased risk of relapse (RR 2.59, 95%CI 1.62-4.15, p=0001) and death (RR 3.11, 95%CI 1.52-6.37, p=002). This result was maintained when

stratified for age at diagnosis in addition to stage. This finding for the combined 1p and 16q regions suggests that there is an interaction between these two chromosomal loci in nephroblastomas. However, an earlier study showed no statistically significant association between 16q LOH and either clinicopathological stage or histological classification of the tumour.[132]

In a study of 40 nephroblastomas, tumours from 2 of the 3 patients who died showed LOH for markers on 16q.[134] Two other patients relapsed but were cured by second-line therapy. One of these recurrent tumours also showed LOH for 16q. Although this tumour was stage I by clinicopathological staging, it had unfavourable histological features. This study found a tendency for LOH to occur in higher stage disease or with unfavourable histological features.[134]

The significant association of 16q LOH with an adverse prognosis suggests that the underlying genetic locus may be involved with tumour progression rather than initiation and that the genetic event may take place in an already established tumour, resulting in further growth advantage to the tumour cells or an increased ability to metastasise.[132,134,138] This would be consistent with the fact that no association has been reported between constitutional 16q deletion and the development of Wilms tumour and that linkage analyses of Wilms tumour families have excluded this region of chromosome 16 as the location of the inherited predisposition.[55]

Further evidence for 16q LOH being a relatively late event in nephroblastoma tumourigenesis is the observation that LOH at 16q does not normally occur in nephrogenic rests.[84] Even though fairly large series of tumours have been studied cytogenetically, no correlations between specific 16q chromosomal changes and any specific histologic subtypes have been found.[141]

LOH for these chromosomal regions may now be used as an independent prognostic factor together with disease stage to target intensity of treatment to risk of treatment failure. Loss of heterozygosity of DNA markers from certain chromosomal regions in the cells of a neoplasm means loss of a total/part of a

chromosome.[127] In the past the common belief was that the transformation of chromosome 16 (with or without trisomy 1q) may play a certain role in the development of a tumour. It was suggested that this is the place where suppressor gene *WT3*, responsible for the familial form of Wilms tumour, is located.[142] Since constitutional loss or deletions of chromosome 16q or 1p have not been described in Wilms tumours, and genetic linkage studies have excluded these regions in families segregating Wilms tumour, it is unlikely that these genes are involved in the development of Wilms tumours. It is more likely that these genes at 1p and 16q are involved with other phenotypic features of the tumour and later studies have revealed that LOH 16q is connected with the progression of the disease.[143,144]

The fact that the effect on outcome is greater for less intensively treated tumours (stage I and II) suggests that the function of these genes may relate to the ability of the cells to tolerate certain chemotherapy, in particular vincristine and actinomycin, or irradiation.[140] Patients with combined 16q and 1p LOH may benefit from the addition of doxorubicin to their therapy.[140] Likewise, patients with advanced stage disease, whose tumours harbour LOH for 1p and 16q, also have an inferior outcome, even when treated with three-drug therapy. These patients may benefit from intensification of their therapy with the addition of other chemotherapeutic agents that are active against Wilms tumour.[140]

2.3.5 Other genetic alterations in nephroblastoma

WT1 is the only cloned Wilms tumour gene at present. Other loci have been investigated to attempt to account for the development of the remainder of nephroblastoma cases. LOH over a range of 5-15% of cases are seen at the following chromosomal loci: 1p, 4p, 8p, 14q, 17p, 17q, and 18q.[7,67] It remains to be seen whether these losses reflect homozygosity of specific tumour suppressor gene mutations. The most commonly gained chromosomes are 6, 7, 8, 12, 13, and 18,[141] and the most common losses are 1p-, 16q-,11p-, 22q-, and –X.[145]

Mutations in β-*catenin* are common in many different types of cancer, and have been described in 10% of Wilms tumours.[61] Activating mutations in β-*catenin* disrupt its phosphorylation by CK1 (casein kinase 1) and GSK3 (glycogen synthase

kinase 3), normally facilitated by a 'destruction complex' containing APC (adenomatous polyposis coli) and Axin (axis inhibition protein). In the absence of phosphorylation, β-catenin is not targeted for proteosomal degradation and, mimicking normal wnt signalling, enters the nucleus where it forms a transcriptional complex with TCF/LEF (T-cell specific transcription factor and lymphoid enhancer factor) family members.[146]

Interestingly, there is a high degree of overlap between tumours that harbour mutations in β-*catenin* and in *WT1*. In one study, 19 out of 20 nephroblastomas with β-*catenin* mutations also had mutations in *WT1*.[96] *WT1* inactivation and β-*catenin* activation might therefore provide cooperative signals that drive renal tumourigenesis. It is conceivable that alterations in one pathway might actually trigger apoptotic signals that must be overcome by alterations in the second pathway before tumour formation can be initiated. Since β-*catenin* target genes, such as *c-myc*, are upregulated even in the absence of β-*catenin* mutations, activation of the wnt pathway might be a general feature of Wilms tumours with mutations in *WT1*.[147,148]

2.4 Nephrogenic rests

The existence of precursor lesions to Wilms tumour has been recognised for many years.[149] These lesions are referred to as nephrogenic rests (NRs) and they consist of abnormally persistent (after 36 weeks of gestation) embryonal nephroblastic tissue with small clusters of blastemal cells, tubules, or stromal cells.[150] Nephrogenic rests are seen in less than 1% of routine autopsies of infants, but are found in ~30% of sporadic nephroblastomas and in nearly 100% of patients with multifocal or bilateral Wilms tumour.[150] Nephroblastomas are assumed to develop from NRs by accumulating genetic alterations[31] and thus are considered subclones of NRs.[84]

Nephrogenic rests can be subclassified into two major categories according to their position within the renal lobe and histological appearance: perilobar (PLNR) and intralobar (ILNR). PLNRs, located at the periphery of the lobe, occur in 10-15% of all nephroblastoma cases and are strongly associated with synchronous bilateral

nephroblastoma, hemihypertrophy, and Beckwith-Wiedemann syndrome. ILNRs, observed in 15-20% of nephroblastomas overall, occur within the renal lobe and are more commonly found in metachronous bilateral nephroblastoma, aniridia, genitourinary anomalies and Denys-Drash syndrome.[31] These associations suggest a correlation between nephroblastoma phenotype and distinct molecular genetic events leading to the development of that tumour.[151] The term nephroblastomatosis is used to refer to the presence of multiple nephrogenic rests.

Mutations of the *WT1* gene have been detected in a few hyperplastic ILNRs and PLNRs.[83] LOH at chromosome 11p13 and 16q has been described in perilobar NRs, as well as LOH at chromosome 11p13, 11p15, and 16q in intralobar NRs.[84] Consequently, a possible genetic multistep model of the development of nephroblastomas via ILNRs or PLNRs has been proposed.[84]

2.5 Treatment of nephroblastoma

Treatment protocols have improved considerably over the past 25 years, and what used to be a uniformly lethal disease now has an excellent cure rate. Most cases are treated with a combination of surgery and chemotherapy, with radiation therapy reserved for advanced disease. However, there has been a long-running debate surrounding the relative merits of preoperative chemotherapy or immediate surgery in the treatment of Wilms tumours.[152,153] Preoperative chemotherapy has the potential to decrease morbidity and increase the survival rate in patients in three ways: (i) decreasing incidence of tumour rupture during surgery, (ii) downstaging the tumour,[154] and (iii) offering the potential of "nephron-sparing" surgery. Although the majority of tumours are sporadic and unilateral, 10% of tumours are bilateral or multifocal.[52,155] These children present earlier, and must undergo more complex surgical procedures that aim to preserve as much renal function as possible.

The management of nephroblastoma varies geographically. In Europe and many other countries, the protocols of Société Internationale d'Oncologie Pédiatrique (SIOP) are followed. Nephroblastomas have overall long-term survival rates approaching 90% in localised disease and over 70% for metastatic disease using current therapeutic protocols created by SIOP or the National Wilms Tumour Study

Group (NWTSG).[156,157] Treatment is based on the results of the initial imaging evaluation, and the protocols advocate preoperative chemotherapy followed by surgery and postoperative treatment, which is stratified according to histologic evidence of responsiveness to preoperative therapy, as reflected by post-therapy classification (low-risk, intermediate-risk, and high-risk histology). In the vast majority of centres in the United States and Canada, under the protocols of the National Wilms Tumour Study Group (NWTSG), primary nephrectomy is done for all but the largest unilateral tumours with extension into the inferior vena cava,[153] and subsequent adjuvant therapy is determined by the surgical and pathologic findings.[158]

Therapy for patients with favourable-histology (FH) Wilms tumour is based on the risk of relapse using such variables as age at diagnosis, lymph node involvement, local or intravascular tumour extension, and presence of metastatic disease. Further refinement of the therapy and improvement in the outcome will depend on more accurate stratification of patients using novel prognostic factors. Refined risk stratification will allow targeting intensified therapy to only those at higher risk of recurrence, whereas patients at lower risk may be cured with less therapy than they currently receive.

Because staging depicts the anatomic extent of the tumour and not necessarily its biological nature, treatment reduction in a downstaged tumour may not be of sufficient intensity to eradicate a biologically more aggressive tumour. There is a paucity of biological markers to complement the prognostic value of histological grading.

2.6 Prognosis of nephroblastoma

The assessment of the prognosis of nephroblastoma depends mainly on the histological classification and clinicopathological stage.[159-162]

Over the years, many clinical and pathological factors have been reported to influence prognosis. These include, amongst others, tumour weight,[163,164] tumour size,[165] renal vein invasion by tumour,[164,166] tumour capsule invasion, degree of epithelial

differentiation, presence of lymph node metastases,[163,167] tumour stage, and patient age.

The NWTS-1 identified four factors that independently predicted relapse-free survival. The factors were unfavourable tumour histology, positive lymph nodes, tumour weight more than 250 g, and patient age over 24 months.[163] The NWTS-2 suggested that tumour weight was of little value in predicting outcome.[168] The effects of age were due to the association of age with other factors like intrarenal vascular invasion, tumour thrombus in the renal vein, and operative spillage. The NWTS-3, however, reiterated the strong association of age at diagnosis and tumour size to relapse and death.[164] They suggested that the incorporation of both favourable and unfavourable histology nephroblastoma in the second NWTS and the use of double and triple agent chemotherapy may explain this discrepancy.

Histologic microsubstaging variables were described by Weeks *et al.*[169] These include (i) the presence of an inflammatory pseudocapsule, (ii) renal sinus invasion, (iii) intrarenal vascular invasion and (iv) tumour capsule invasion. The NWTS-4[170] found that patients with stage I/favourable histology nephroblastoma who were under 2 years of age at diagnosis and tumours weighing less than 550 g were more likely to have all negative microsubstaging variables. Many studies have identified age at diagnosis of less than 2 years as an important prognostic factor.[164] This study also found that tumour size was an important determinant of outcome especially in patients with localised disease - stage I.

In spite of these prognostic factors there is a subset of patients who have a poor outcome with current methods of management, used successfully in other apparently similar children. Studies are needed to determine whether responsive and resistant tumours differ at the molecular level. Many clinically relevant tumour markers have been put forward that may allow early diagnosis and identification of high-risk patients. Different tumour markers for nephroblastoma are being developed by different techniques.

Although the vast majority of children with Wilms tumour, particularly of blastemal type, respond well to standardized therapy, a small proportion of patients show non-

responsiveness to chemotherapy. In these cases, extensive residual blastemal tumour cells are frequently found following pretreatment. It seems that in addition to anaplasia as a well established predictive factor for poor responsiveness to chemotherapy, the persistence of large amounts of viable blastemal cells is also related to low response and reduced prognosis requiring intensified therapy. On the other hand, large amounts of necrosis and/or maturation into differentiated components are considered as evidence for responsiveness and may confer a more favourable prognosis.[157]

The multidisciplinary management of Wilms tumour has resulted in a striking improvement in survival from 30% in the 1930s to more than 90% nowadays and has become a paradigm for successful cancer therapy. The primary objective of clinical trials on Wilms tumour has shifted towards refinement of therapy for children with low-risk tumours so that they can be spared from unwanted long-term side-effects without compromising the excellent cure rates. At the same time, investigators continue to look for novel strategies, including treatment intensification, for patients with high-risk tumours for whom outcome might be further improved.

2.6.1 Molecular prognostic markers

There have been several studies attempting to identify novel markers of prognosis in nephroblastomas and recently, especially in favourable histology nephroblastomas. Specific molecular alterations apparently associated with poor prognosis have been reported from a number of studies. Some of these results are summarized in the following paragraphs. One of the problems encountered in some studies are the small number of cases analysed. It is important to note that larger studies are required to confirm whether these results (markers) have significant clinical value.

An interesting study using comparative genomic hybridisation (CGH) to screen for genomic imbalances in 58 favourable histology Wilms tumours found that gain of chromosome 1q was associated with adverse outcome.[145] Gain of 1q was more frequent in patients with relapse than in those without relapse (59% vs 24%). Furthermore, this genomic imbalance was associated with advanced disease stage with 70% of tumours harbouring 1q gain being either stage III or IV. A further

analysis of 12 paired tumour samples from the time of diagnosis and relapse showed no significant evidence of clonal evolution. This suggests that gain of 1q at diagnosis may be used to identify favourable histology Wilms tumours with an increased risk of relapse. A later study using comparative expressed sequence hybridisation found that gain of 1q was restricted to tumours that subsequently relapsed.[171] The results from a more recent study using expression profiling indicated close agreement with data obtained from genomic copy number analyses.[172] Based on the consistency of chromosome 1q results obtained using different methodologies, gain of 1q holds promise as an important molecular marker of adverse prognosis. A study looking at the differences between treated and untreated tumours found that high frequencies of +1q were maintained in pretreated tumours.[173] These observations suggest that Wilms tumour clones with +1q apparently are not obliterated by chemotherapeutic treatment, in line with a concept that clones with +1q are less responsive to chemotherapeutic treatment and therefore may be detected at a somewhat increased frequency in pretreated tumours.

A positive correlation between relative risk of relapse and levels of *TERT* mRNA and TERC expression was found in a univariate analysis of 243/244 patients with favourable histology nephroblastomas registered with NWTS-5.[174] The multivariate analysis adjusting for *TERT* mRNA, stage and patient age showed that the level of TERC expression remained a significant prognostic indicator. This study suggested that telomerase RNA level may assist with the risk stratification of patients with favourable histology nephroblastomas.

WT1 expression was evaluated in 61 patients with nephroblastomas who were treated with preoperative chemotherapy.[175] WT1 expression in blastema and epithelium was significantly associated with pathological stage ($p<0.05$). In this study however the tumours were staged according to the TNM classification system and not SIOP or NWTS thereby limiting comparison with other studies. Furthermore univariate analysis showed that blastemal WT1 expression correlated with clinical progression and tumour-related death. A multivariate Cox regression analysis stratified for stage identified blastemal WT1 expression as an independent prognostic marker for clinical progression Epithelial WT1 expression did not have

the same significance. There are other studies which have not found a similar association with WT1 protein expression and outcome.

The amino acid sequence in the zinc finger region of *WT1* shows a high degree of similarity with the zinc finger region of *EGR-1* (early growth response) gene.[74] *EGR-1* expression is stimulated during the G0-G1 transition and is thought to play a role in controlling cellular proliferation. A study also found that blastemal *EGR-1* expression correlated with clinical progression and tumour-related death.[175] However, in a multivariate analysis *EGR-1* did not retain its prognostic significance.

A study of 94 patients with nephroblastoma showed a significant association between fatty acid synthase (FAS) expression and risk group in a univariate analysis.[176] Kaplan-Meier analysis revealed an association between FAS and overall survival ($p=0.022$) as well as disease free survival ($p<0.0001$). A multivariate analysis using FAS expression, stage, risk type and pretreatment as independent variables identified a significant negative prognostic value with increasing FAS expression. These results were also seen with the intermediate risk group. This study suggests that FAS expression might be an independent prognostic factor in nephroblastomas.

In a small study of 25 nephroblastoma patients treated with preoperative chemotherapy VEGF-C immunoexpression had prognostic value for both clinical progression and tumour-related death.[177] Further analysis revealed that the probability of remission after 2 years was greater in VEGF-C negative nephroblastomas. Further evaluation of VEGF-C is required to reproduce these results and confirm its prognostic value. The statistical analysis was limited by the relatively small number of patients in this study.

In a study of 62 patients treated according to SIOP protocol 9, blastemal MIB-1 proliferation index and p27 expression correlated with clinical progression and tumour-related death in a univariate analysis.[178] A multivariate analysis including stage, MIB-1 and p27 expression confirmed these two markers as independent poor prognostic indicators for clinical progression.

A study of 30 patients with nephroblastoma treated according to NWTS-3 protocols suggested that HSP70 and HSP90α might be of prognostic value.[179] This study found that higher expression of these proteins was associated with survival.

High levels of topoisomerase IIα immunoexpression, investigated in 57 cases of nephroblastoma, correlated with tumour aggressiveness.[180] Topoisomerase IIα expression was highest in metastatic or recurrent tumours with lower expression in primary tumours and the lowest in the adjacent normal kidney. This study found that expression levels were higher in stage III and IV disease and anaplastic tumours but the results were not statistically significant.

It is possible that combined expression information of a set of different genes may provide more valuable information for prediction of prognosis than single gene expression.

2.6.2 Anaplasia in nephroblastoma and prognosis

One of the major objectives of The National Wilms Tumour Study Group (NWTS) in the United States, under the leadership of Dr J.B. Beckwith, was to search for histopathological markers of biologic behaviour in the Wilms tumour spectrum. The primary finding of their research (NTWS-1) was that the presence of anaplasia correlated with unfavourable outcome for nephroblastomas.[160]

They originally proposed the following diagnostic criteria for anaplasia:[160] (i) marked nuclear enlargement of stromal (except skeletal muscle cells), epithelial and blastemal cells to at least three times the diameter of adjacent nuclei of the same cell type, (ii) hyperchromatism of enlarged nuclei, and (iii) multipolar mitotic figures. These features may involve any of the three components of nephroblastomas.

The first criterion was later redefined as a threefold enlargement of the nucleus in two perpendicular axes, to avoid overinterpretation.[181] Nuclear hyperchromasia should be considered an expression of total nuclear chromatin and increased chromatin content is also implied when a markedly enlarged nucleus has normal density.[181] All three of the criteria had to be present in order to diagnose anaplasia.

Anaplasia was regarded as "focal" when present in fewer than 10% of high power fields, and "diffuse" when present in more than 10% of high power fields.[160] They found that the rates of relapse and death due to tumour were higher for "diffuse" anaplasia. However, any degree of anaplasia markedly worsened the prognosis. In the NWTS-2 no difference in treatment outcome was noted between "focal" and "diffuse" anaplasia.

Table 2: Revised definition of focal and diffuse anaplasia[162]

Focal anaplasia	**Diffuse anaplasia**
Localised to one or more discrete foci within the primary tumour	Non-localised anaplasia
No extension beyond primary tumour capsule	Extension beyond the primary tumour capsule
No other evidence of nuclear atypia (unrest) in the tumour	Marked nuclear atypia (unrest) within the tumour
If multiple foci, each must be surrounded by non-anaplastic tumour	Not clearly demarcated from non-anaplastic tumour
Not present in vascular extensions, or metastatic sites.	Anaplastic cells in vessels, renal sinus and metastatic sites
	Anaplasia in random biopsies
	If anaplastic cells are present at the edge of more than one section, and it cannot be determined whether these belong to a single discrete focus

A recent review of the definition of focal and diffuse anaplasia resulted in a revised definition based on topographical distribution (Table 2).[162] The introduction of the revised criteria has allowed further stratification of patients with anaplasia. Survival studies based on the new criteria showed that diffuse anaplasia has a significantly poorer prognosis than focal anaplasia.[162,182] Focal anaplasia has been redefined as one or a few well localised zones of anaplasia confined to intrarenal tumour sites.[162] Anaplasia confined to the kidney, i.e. stage I disease has been found to have no effect on prognosis. This finding and others support the theory that anaplasia is a marker of

increased resistance to therapy rather than a sign of increased tumour aggressiveness.[23]

Today, the histological feature of greatest clinical significance in untreated Wilms tumour is anaplasia. The frequency of anaplasia is about 5% and is correlated with patients' age.[181] It is rare in the first 2 years of life, then the frequency increases to about 13% in patients older than 5 years.[183] It has been strongly linked with the presence of *p53* mutations.[184] Anaplasia is judged to be a marker of resistance to chemotherapy,[185] but whether it also confers increased aggressiveness or tendency to disseminate is still uncertain. This recognition has resulted in a distinction between tumours showing anaplastic changes that are focal from those that are diffuse.[162] Anaplasia is also related to the ploidy of the tumour and appears to reflect tumour progression.[181]

2.7 Genes and Cancer

In principle, there are now two kinds of genes that prevent cancer, 'gatekeeper' genes that maintain a functional growth rate, comprising proto-oncogenes, as well as tumour suppressor genes, and 'caretaker' genes that maintain the integrity of the genome, either at the nucleotide or the chromosomal level.[186]

Genetic mutations in cancer fall principally into two categories: activation of proto-oncogenes and inactivation of tumour suppressor genes.[187] Mutations of numerous oncogenes and tumour suppressor genes have been discovered in human neoplasia. Tumour suppressor genes are probably not involved in the maintenance of cell survival, because they are recessive in nature, but they play a pivotal role in the regulation of cell growth and differentiation.

Several tumour suppressor genes have been identified, including the *Rb* and *p53* genes, which participate in the normal function of a cell, but which, when mutated or lost, contribute to cellular transformation.[188,189] Loss of function of a tumour suppressor gene is believed to contribute to carcinogenesis.[188] In 1971, Knudson postulated that the loss of both alleles of these tumour suppressor genes was necessary for the development of cancer.[190] Patients with familial cancer

syndromes inherited a germline change in one copy and, thus, required only one more genetic change (a "second hit") in the remaining copy.

In the current panel of oncogenes and recessive tumour suppressor genes, *p53* seems pivotal in understanding the basic molecular mechanisms of cancer.[191] Alterations in the structure of *p53* gene represent one of the most common genetic changes associated with human cancer, and experimental and clinical evidence have implicated *p53* in the development of a wide range of malignancies. There is not only a loss of function associated with *p53* mutations, but also a gain of functional oncogenic potential.[192] Experimental evidence suggests that the extended viability imparted by the proto-oncogene bcl-2 may render cells susceptible to the acquisition of alterations capable of complementing, or cooperating with bcl-2 during multistep carcinogenesis.[193]

The caretaker genes include a relatively new class of tumour-susceptibility gene that results in a generalized defect in the processes of DNA mismatch repair. Rapid and important advances in this field have provided new and significant insights into the biology and molecular genetics of cancer. The introduction of epigenetics involves an additional dimension.[194]

2.8 Genomic instability and the mutator phenotype

There are apparently two independent mechanisms of genomic instability, chromosomal instability (CI) and microsatellite instability (MSI), which have been identified in several types of carcinoma.[195] CI is the result of mutations in genes that are required for chromosomal segregation resulting in chromosomal aberrations. Chromosomal aberrations can be numerical, involving the loss and gain of whole chromosomes, or structural, such as deletions, amplifications, translocations and inversions. The CI phenotype found in some colorectal cancer cell lines is defective in a kinetocore-checkpoint function, and may harbour mutations in *hBUB1*, a gene encoding a component of the mitotic checkpoint.[195] MSI is characterised by the inactivation of the mismatch repair (MMR) mechanism resulting in an increasing rate of mutations in DNA molecules containing microsatellite repeats. MSI associated cancers appear to retain a diploid karyotype and do not develop gross chromosomal aberrations.

Both of these forms of genomic instability can lead to a mutator phenotype via altered protein expression, function or gene dosage effects. A progressive reduction in chromosome maintenance pathways appears to lead to an increase in the number of errors during carcinogenesis, and promotes the development of highly anaplastic tumours.

While a basal level of genetic alterations exists in all dividing cells, the force that accelerates the accumulation of genetic alterations still has to be clearly defined. The present dominating hypothesis, termed genomic instability, postulates that a 'mutator phenotype' promotes the initiation and progression of tumourigenesis by increasing the rate of new genetic alterations within the cancer.[197] Loeb coined the term 'mutator phenotype' based on the high frequencies of chromosomal abnormalities and mutations.[198] This hypothesis was based on the premise that the spontaneous mutation rate in normal cells is insufficient to account for the high frequency of mutations in human cancer cells.[198] Loeb and colleagues[199] have estimated that the background mutation rate in normal cells can account for only two or three mutations in each tumour. This is significantly lower than the larger number of mutations that are reported, or the even greater numbers that are likely to be found in cancers as the methods for detection become more sensitive. At some time during the life of a tumour, the mutation rate must be greater than in normal cells: i.e., cancer cells must exhibit or have exhibited a mutator phenotype.[198,200,201] Evidence for a 'mutator phenotype' in human cancers includes the frequent occurrence of gene amplification, microsatellite instability, chromosomal aberrations and aneuploidy. The hypothesis of the 'mutator phenotype' predicts that by the time cancers are detected clinically, they already have accumulated enormous numbers of mutations.

There is compelling evidence that DNA repair is critical for normal cellular function, and its dysfunction is associated with malignant transformation. MMR defects have been postulated to promote tumour development by allowing cells to exceed baseline rates of spontaneous mutation throughout the genome and achieve an altered phenotype with a growth advantage. Rather than directly causing malignant transformation, DNA mismatch repair deficiency creates the milieu that permits mutations to accumulate in other growth-regulatory genes. These defects lead to a

'mutator phenotype', which is manifested as MSI (the sentinel lesion of defective MMR). It is presumed that in eukaryotic cells, this mutator phenotype provides the appropriate milieu for the accumulation of mutations in known oncogenes, tumour suppressor genes, and other genes critical for oncogenesis, such as, growth regulatory genes.

It should be noted that the 'mutator phenotype' might be generated through mechanisms not directly related to defective mismatch repair. Alterations in the timing of cell-cycle phases provide another mechanism. During cell-cycle progression there are several regulatory pathways that function as checkpoints and monitor the repair of damage before proceeding to the next stage in the cell cycle.[202] If activated, these checkpoints function to transiently arrest cell-cycle progression so that damage can be repaired or that the proper assembly of cell components can be completed. In the presence of irreparable damage, apoptosis is triggered as a final means to halting the spread of mutations. Again, many cancers contain mutations that delay or prevent the apoptotic response and thus promote the survival of genetically unstable malignant cells.

2.9 The Cell Cycle

2.9.1 Evolution and Concept

Our present-day understanding of the cell cycle is founded on the early study of the DNA tumour viruses and the discovery of the p53 protein.[203,204] Cell division is dependent on the successful completion of many processes, which are temporally related and are confined to four discrete phases of the cell cycle. They are referred to as G1 (Gap 1), S (Synthesis), G2 (Gap 2) and M (Mitosis). The typical cycle of cultured cells lasts about 24 hours, although it can vary widely.

In the G1 phase the critical decision is made whether to proliferate or enter G0. In G1, growth occurs and a cell's metabolic requirements are at their peak. The duration of G1 is quite variable and is the major determinant of the overall length of the cell cycle. For example, in bowel epithelium G1 lasts around 11 hours out of a total cycle time of approximately 24 hours. Cells progress from G1 into the S phase which is characterised by DNA replication. This phase usually lasts for approximately 8 hours, but in the presence of DNA damage or if replication is

hindered in any way, this can be extended significantly. The cells then enter G2, during which the DNA replication process is completed by packaging chromosomes and sister chromatids. Cell growth continues, and proteins required for mitosis are synthesized. This phase lasts about 4 hours. The M phase usually lasts between 30 minutes and 1 hour, which is less than 5% of the total duration of the cell cycle. Dividing cells pass sequentially through these four phases and return to G1 after mitosis. A cell may also leave this cycle during G1 and pass to G0 to become a non-dividing cell.

The initiation of cell division is dependent upon growth factors and other external stimuli. Without these stimuli, G0 cells will not enter the cell cycle. In the absence of similar stimuli, daughter cells resultant from a completed cycle will leave G1 and pass into the quiescent G0 phase. However, once cell division is triggered the cycle is completed in full even if the initiating stimuli are removed.

An important component of the genetic programme of the cell cycle is the existence of diverse internal checkpoints at different stages. These checkpoints represent fail-safe mechanisms whose key role is to avoid the accumulation of genetic errors during cell divisions by preventing the cell from prematurely entering the next phase until appropriate conditions have been achieved, or to direct the cell towards fates other than division, such as, apoptosis.[202,205-208] Furthermore, the checkpoint genes are responsible for integrating the environmental signals and appropriate cell responses, thereby coordinating the progress through the cell cycle with cellular signals. Among the most important checkpoint pathways are the G1/S transition, G2/M transition[209] and the mitotic spindle checkpoint.[210] At the G1/S and G2/M transition checkpoints cells delay their cell cycle progress in response to DNA damage to allow for DNA repair before entering the next phase.[209] In G1/S, this prevents the perpetuation of damaged DNA and in G2/M this prevents against loss of genomic material.

2.9.2 The Cell Cycle regulators

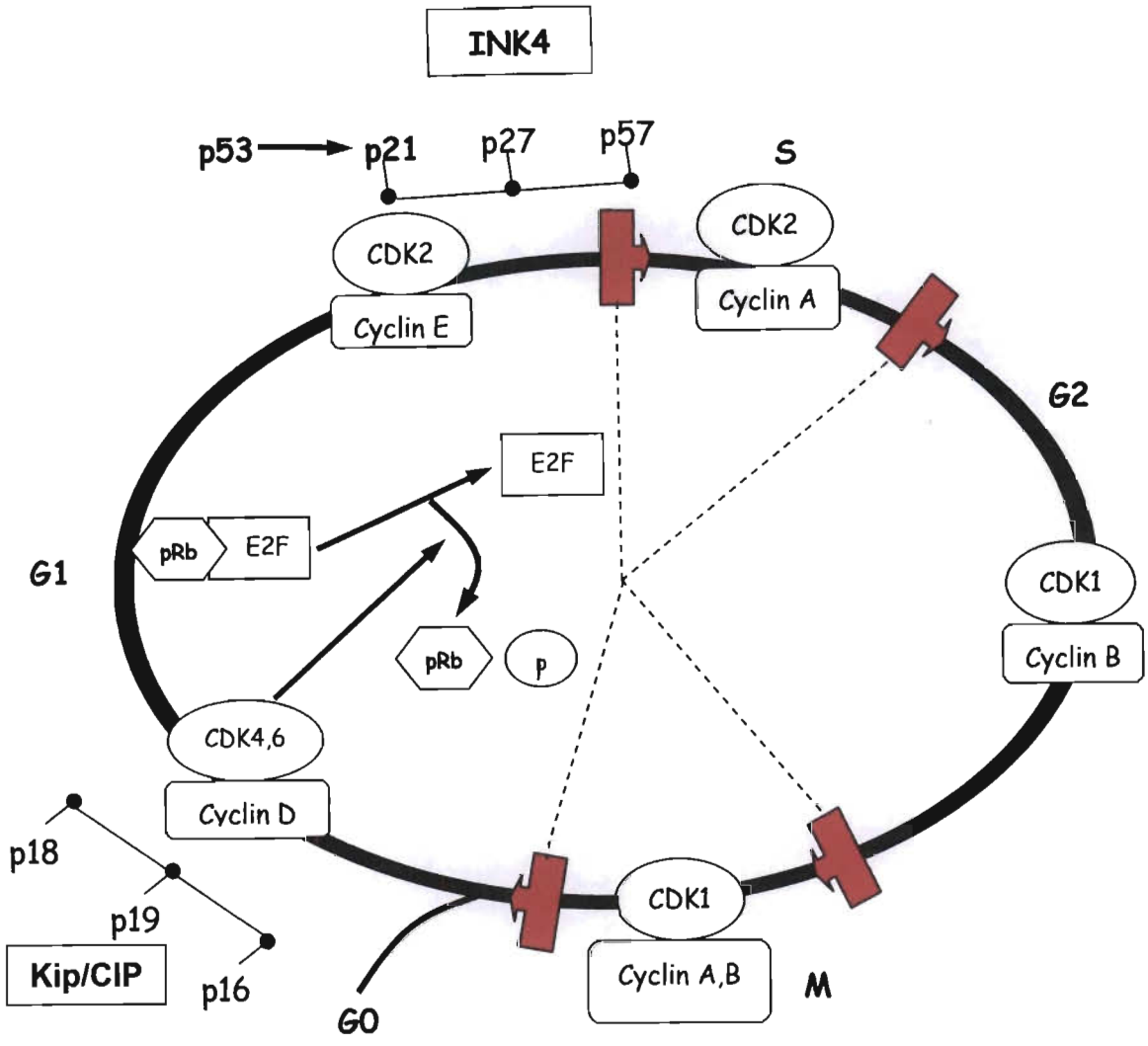


Figure 2: The basic regulation of the cell cycle including the role of p21, p53 and pRb.

2.9.2.1 The Cyclins

Cyclins are proteins which attain peak activity during different phases of the cell cycle due to cell cycle specific synthesis, for example, cyclins D1-3 and E reach their maximum activity during the G1 phase, and apparently regulate transition from G1 to S, whereas cyclins A and B1-2 are most active during the G2 and S phases when they regulate transition to the mitotic phase of the cell cycle (Figure 2).[212] Based on this cyclins can be divided into G1 cyclins and mitotic cyclins, which are involved in the G1/S and G2/M transition, respectively.[213,214] Cyclins, as positive regulators of cell-cycle progression, are also subject to rapid degradation after they have effected their target cell-cycle transitions, a mechanism that ensures that they are unable to 're-initiate' a given process once it has started.[215]

The D-type cyclins (D1, D2, and D3) interact with two distinct catalytic partners (CDK4 and CDK6) to yield at least six possible holoenzymes that are expressed in tissue-specific patterns. CDK4 and CDK6 are relatively long-lived proteins but the D-type cyclins are unstable, and their induction, synthesis, and assembly with their catalytic partners all depend upon persistent mitogenic signalling. In this sense, the D-type cyclins act as growth factor sensors, forming active kinases in response to extracellular cues.[216-218] The cyclin D-dependent kinases trigger the phosphorylation of pRb, thereby helping to cancel its growth-repressive functions.[219]

The E2Fs also induce the cyclin E and A genes. Cyclin E enters into a complex with its catalytic partner CDK2[213] and is responsible for completion of pRb phosphorylation.[220] Furthermore, cyclin D-dependent kinases sequester Cip/Kip proteins, thereby facilitating cyclin E-CDK2 activation.[221] This complements the pRb-E2F transcriptional programme and helps make the appearance of cyclin E-CDK2 activity contingent upon accumulation of cyclin D-CDK4/6 complexes. Transcription of cyclin-E represents a positive feedback loop, which results in ongoing expression of S-phase proteins, independent of cyclin-D levels and thus independent of ongoing growth factor stimulation. This shift in pRb phosphorylation from mitogen-dependent cyclin D-CDK4/6 complexes to mitogen-independent cyclin E-CDK2 accounts in part for the loss of dependency on extracellular growth factors at the restriction point. Amplification of the *CCND1* gene or overexpression of the cyclin D1 protein releases a cell from its normal controls and causes transformation to a malignant phenotype.

D-type cyclins are able to bind to pRb through an N-terminal LXCXE motif.[222] Interestingly, this LXCXE motif is common to the SV40 T antigen, adenovirus E1A, and human papillomavirus (HPV) E7 protein,[223] which may also bind to pRb and release E2F; a fact that in part explains the oncogenic potential of these viruses.

Accumulation of the wild-type p53 protein leads to induction of cyclin D1 expression.[224] This induction is mediated at least in part by the p21 gene product. Overexpression of cyclin D1 promotes cell progression and differentiation generally observed as shortened G1-S transition and oncogenesis.[216]

2.9.2.2 The cyclin dependent kinases

The CDKs, a family of protein kinases responsible for effecting the key transitions between phases in the eukaryotic cell cycle are activated by cyclins and inhibited by CDK inhibitors.[225-228] The most significant members of this group appear to be CDKs 1, 2, 4 and 6 (Figure 2). CDK1 (also known as cdc2) is responsible for initiating mitosis at the G2/M transition, whilst CDKs 2, 4 and 6 are involved in passage through the restriction point and the consequent initiation of S phase (G1/S transition).

The activity of the CDKs is dependent on their association with members of the cyclin family, which serve as regulatory subunits. In normal cells, CDKs exist predominantly in multiple quaternary complexes, each containing a CDK, cyclin, proliferating cell nuclear antigen (PCNA) and the p21 protein. However, in many transformed cells, PCNA and p21 are lost from these multiprotein enzyme complexes.

2.9.2.3 The cyclin dependent kinase inhibitors

One of the important discoveries in cell cycle research was that cyclin complexes had different constitutions in transformed and non-transformed cells.[229] The complexes in the non-transformed cells were associated with small proteins different from those found in transformed cells. Various investigations subsequently determined that these small proteins, the cyclin dependent kinase inhibitors (CDKIs), did not only bind to the cyclin complexes but were potent inhibitors of the associated CDKs.[230-233]

Two major classes of CDK inhibitors have been identified based on their function and structure.[234] Firstly, the Cip/Kip ('CDK interacting protein' or 'kinases inhibitory protein') family of CDKIs consists of related CDK inhibitors, $p21^{WAF1/Cip1/Sdi1}$, $p27^{Kip1}$ and $p57^{Kip2}$, that inhibit a broad range of cyclin-CDK complexes (Figure 2). Secondly, the INK4 (Inhibitor of CDK 4) family, unrelated to the Cip/Kip family, consists of $p15^{INK4b/MTS2}$, $p16^{INK4a/MTS1}$, $p18^{INK4c}$ and $p19^{INK4d}$ (Figure 2). The members of this family have a more selective inhibitory activity, affecting only CDK4 and CDK6.

Cip/Kip proteins clearly participate in unexpected regulatory pathways and should not be considered merely as CDK inhibitors. Cip/Kip proteins might act as cytoplasmic regulators of nuclear import, apoptosis and cell motility and they might function as transcriptional regulators in the nucleus. All three Cip/Kip family inhibitors contain a similar ~60-amino-acid domain in the N-terminal region and a nuclear localisation signal near the C-terminus.[235] The *p27* gene is located on chromosome 12p. The *p57* gene is on chromosome 11p15.5 and is a candidate tumour suppressor gene.[120] p57 is predominantly expressed in terminally differentiated cells and appears to be primarily involved in development and tissue patterning.[236]

INK4 proteins are differentially expressed in response to a variety of anti-mitotic stimuli. They specifically target the cyclin D-dependent kinases,[237] thus negatively regulating cell-cycle progression. In humans, the *p16* and *p15* genes are closely linked on the short arm of chromosome 9, *p18* gene maps to chromosome 1 and *p19* gene maps to chromosome 19.

There is often no difference in expression of CDKs and cyclins between different cell types, in contrast, the expression of individual CDKIs is often cell and tissue specific.

2.9.3 The G1/S Checkpoints

At the G1/S and the G2/M transitions as well as in S phase and mitosis cell cycle checkpoints exist which are complex signal transduction pathways that serve as fail-safe mechanisms to ensure the order of events in the cell cycle and integrate cell cycle progression with DNA repair. The integrity of these checkpoints is therefore considered pivotal in maintaining genetic stability.[202]

The point in late G1 phase, approximately 2-3 hours before the G1/S phase transition, at which a cell becomes committed to division independent of further stimulation is termed the restriction point.[226,234] The restriction point is a major determinant of the fate of the daughter cells resultant from mitosis, and represents a crucial mechanism in controlling the balance between differentiation and proliferation. Passage through the restriction point is regulated by multiple diverse

signals, including steroid hormones, transforming growth factor (TGF)-β1, myc and the MAPK pathways.[238] Cyclin D- and cyclin E-dependent kinases mediate restriction point control by providing an effector mechanism for these diverse pathways, which ultimately 'releases the brake' on the transcription of S-phase genes.

G1/S transition is the most frequently deregulated control in the cell cycle. Significantly, this step includes the so-called restriction point[217,239,240] and the onset of DNA replication. Cell cycle regulators critical for error free execution of these events encompass the D-type cyclins with their partner cyclin-dependent kinases CDK4 and CDK6, the CDK inhibitors of both the INK4 and the Cip/Kip families, the retinoblastoma tumour suppressor (pRb) and the pRb-controlled transcription factors such as E2F, the cyclin E-CDK2 kinase, CDC25A phosphatase and other regulators that control expression, stability, or activity of the above key elements.[217,239-241] Consistent with their positive versus negative effects on cell cycle progression, the G1/S regulators qualify as either proto-oncogenes (cyclins D1, D2 and E, CDK4/6, CDC25A) or tumour suppressors (p15, p16, p21, p27, and p57 CDK inhibitors and pRB), and each of them may become deregulated via several distinct molecular mechanisms.[217,239-243]

Cells commit to cell division in late G1, at the restriction point, following phosphorylation of pRb.[244] At least two cyclin-CDK complexes phosphorylate pRb, cyclin D-CDK4/6 and cyclin E-CDK2. While pRb appears to be an exclusive target of cyclin D-associated kinases, cyclin E most likely targets additional factors necessary for cell cycle progression.[222] Phosphorylation inactivates pRb and releases the transcription factor E2F from its complex with pRb. Free E2F transactivates numerous S phase gene promoters, including cyclins D and E, prior to the progression of cells into S phase, [245] forming an autoregulatory loop between G1 cyclins and pRb.[246] A cascade of events is triggered by cyclin D1 associated inactivation of pRb, release of E2F and increased expression of cyclin E, ensuring a sustained phosphorylation of pRb during G1, allowing cells to pass the G1/S checkpoint and continue DNA replication. The overexpression of E2F is sufficient to induce S phase entry in some situations.[245] Cyclin E-associated kinase activity

constitutes a second prerequisite signal for S phase entry. Overexpression of cyclin E can lead to progression through at least one cell cycle in the absence of detectable E2F function and overcome a G1 arrest by some G1 CDKIs while E2F cannot.

A related, potentially important, observation is that wild-type p53 can enhance transcription from the *Rb* gene promoter.[247] Thus, p53 may mediate G1 arrest not only by maintaining constitutive pRb activity, but also by increasing its concentration. *p53* also mediates growth arrest by its ability to induce expression of the *p21* gene, which inhibits cyclin dependent kinases and causes pRb to be maintained in its cell cycle-inhibitory form.[231,248,249]

Whether cycle arrest or apoptosis occurs may be related to the consequences of DNA damage and subsequent repair, i.e., if the damage can be repaired, only arrest is observed, whereas if the damage cannot be repaired then cell death ensues.[202] The status of the Rb family has a significant influence on this decision. If Rb proteins are functional, the cell will arrest and may sometimes re-enter the cell cycle when p53 activity returns to normal levels. In the absence of functional Rb family proteins, the cell will fail to arrest and apoptosis will ensue instead.[250] This model assumes that activation of wild-type p53 gives rise to two parallel signals: induction of p21 targets the cell for a G1 arrest, and activation of the apoptotic pathway, either through induction of genes such as *bax* or through an alternative mechanism. In summary, the presence of functional Rb family proteins results in G1 arrest while the absence of functional Rb family proteins leads to apoptosis.

2.9.4 G2/M checkpoint

The intrinsic linkage between DNA replication and mitosis ensures that once a cell has replicated its genome, further DNA synthesis is inhibited until mitosis has completed. Moreover, the replication checkpoint operates during S phase to ensure that entry into mitosis is restrained whilst DNA replication is ongoing.

2.9.5 Dysregulation of the Cell Cycle

If cells are repair deficient, e.g. in the absence of p21 cell inhibitory function, spontaneous mutation frequency is increased, and cells become more sensitive to

agents which induce DNA damage. McDonald *et al.*[251] further suggested that it is possible that the precise ratio of p21:PCNA, as well as their absolute amounts in normal cycling cells or cells exposed to DNA-damaging agents, may be critical *in vivo* to determine whether replication will continue or repair will be initiated.

After DNA damage, the levels of wild-type p53 protein increase and these levels induce either a growth arrest or an apoptotic response.[209,252,253] The inducing signals from the cellular environment are thereby eliminated and p53 levels return to normal in the surviving cells. In cells with mutant p53 and absent wt p53, there is neither growth arrest nor apoptosis.[209,252] Consequently, DNA-damaged cells continue to divide and create an environment wherein the p53 stability signal is constitutive and higher levels of mutant p53 are obtained.

An increase in the level of the wt p53 in response to DNA damage stimulates synthesis and activation of p21, an inhibitor of CDKs.[230-232,248] p21 is also able to directly block DNA replication by its inhibition of the proliferating cell nuclear antigen (PCNA),[254] an essential DNA replication protein. In addition to its role in DNA replication, PCNA is also required for nucleotide-excision repair of DNA, an intrinsic part of the cellular response to ultraviolet irradiation.

2.9.6 Neoplasia and the cell cycle

The vast majority of neoplasms, if not all, harbour one or more defects that derail the cell cycle machinery. These defects may either target: (i) components of the cell cycle apparatus,[217,239,240,242] including the checkpoint mechanisms that ensure fidelity and orderly progression through the cell cycle phases, thereby protecting genomic integrity[202,206-208] or, (ii) elements of the upstream signalling cascades[188,255] whose effects eventually converge to trigger cell cycle events.[243,256]

El-Deiry and co-workers[257] proposed a two-stage model for the abrogation of cell cycle control in neoplasia. The first stage involves a loss of the highly ordered spatial separation between proliferating and non-proliferating compartments. Since *APC* is known to influence the cadherin-catenin network linking cell-cell contacts to the cytoskeleton, they suggested that in the case of colonic adenomas this might be

due to mutations in the *APC* gene, which could result in disruption of cell contact signalling of growth as cells migrate up the crypts.[257] In the second stage, during progression to more malignant forms, other signals in the abnormal microenvironment may induce wt p53 expression and consequent p21 induction. The *p53* gene may function in these circumstances as a kind of "emergency brake". However, the consequence of this form of *p53* induction is that the stage is set for selective outgrowth of cells with *p53* mutations.

In addition to uncontrolled and uncoordinated cellular proliferation, malignant neoplasms demonstrate other characteristics that are not directly related to proliferation, such as, invasion and metastasis. Nevertheless, these latter processes are heavily dependent upon deregulated cell cycle control.

The cell cycle and the genetic alterations that drive tumourigenesis are inextricably linked. The discovery of p21 as an inhibitor of cyclin-CDK complexes and its ability to be transcriptionally activated by p53 provided a link between tumour suppression and cell cycle regulation.[205,230,231,248] Other examples include the amplification of cyclin and CDK4 genes, the phosphorylation of pRb by CDKs, and the tumour suppressor activity of the CDK inhibitor, p16.

2.10 The p53 gene

The p53 gene is located on the short arm of human chromosome 17, at 17p13,[258] and encodes a 53 kDa nuclear phosphoprotein composed of 393 amino acids. Five evolutionary conserved domains within the coding regions are considered to be essential to the functional activity of *p53*.[191,258] The highly conserved hydrophobic core is flanked at the N-terminus and C-terminus by more highly charged head and tail domains. The N-terminal region is acidic and contains a transcriptional transactivation domain, which allows *p53*, in the context of its specific binding to a target DNA sequence, to recruit the basal transcriptional machinery required for transcribing new mRNA and by so doing, activate the expression of target genes.[259] This region is also critically involved in regulating the stability and activity of p53 via interactions with MDM-2, which facilitates the degradation of p53 by the ubiquitin-mediated proteolytic machinery. The C-terminal region is more basic and contains motifs involved in the regulation of DNA binding activity and two oligomerisation

motifs. The binding of p53 to DNA is optimal when p53 is in a tetrameric state as a consequence of interactions of four separate p53 molecules via the tetramerisation domain in the C terminal region.[206] The central region of *p53* is conformationally flexible, and encodes the binding site for SV40 large T-antigen and for sequence specific dsDNA binding. In addition to those mentioned above p53 protein also associates with a variety of viral and cellular proteins,[252,260] including human papillomavirus (HPV) E6, the Epstein-Barr nuclear antigen, and adenovirus E1a.[261] Cellular proteins include the growth arrest and DNA damage protein ($GADD_{45}$), heat-shock protein 70, replication protein, the ubiquitin-ligase E6-AP, and transcription factor WT1.[191,252,260,262] Activated wt p53 promotes the transcription of proteins which can be placed into one of four categories:[263] cell-cycle inhibitors, proteins involved in apoptosis, DNA repair proteins and inhibitors of angiogenesis.

The functions of p53 in a cell are coordinated in a manner that integrates signals emanating from a wide range of cellular stresses, including inappropriate proliferation signalling (oncogenes), nucleotide depletion, hypoxia, and hypoglycaemia, and responds by activating a set of genes whose products facilitate adaptive and protective activities, such as, growth arrest, senescence and apoptosis.[264-267] Hypoxia may play a strong selective role in tumour progression.[268]

In particular, *p53* appears to play a pivotal role in the cellular response to genomic stress. This refers to any alteration to the integrity of the genome such as primary damage to DNA itself[269] and problems with the mechanisms responsible for the successful propagation and segregation of the genetic information. In this way, *p53* may prevent the propagation of genomic damage, which might otherwise promote the development of cancer. This feature of *p53* earned it the title of "guardian of the genome".[270]

The spectrum of genetic events involving *p53* differs among cancer types, but they include point mutations, deletions, allelic loss, and rearrangements.[271] Most of these mutations are missense point mutations clustered in the most highly conserved domains of the gene spanned by four to nine exons and the majority are found in the central conformational domain of p53.[272]

The presence of these mutations offer a reasonable explanation for the tumour suppressor function of *p53*, that is, a mechanism of *p53* loss of function similar to gene deletion. The nature of the p53 mutations encountered in neoplasms being mostly missense and retaining the intact original reading frame further supports the above explanation. In contrast to *p53*, most other tumour suppressor genes undergo mutations which give rise to truncated proteins (e.g. frameshifts, nonsense mutations, internal deletions) or to no protein at all (gross deletions).

Furthermore, mutated p53 protein is often much more stable than its wt counterpart resulting in a high concentrations of full length, mutant p53 protein in neoplasms. This led to the suggestion that the mutant protein confers a positive selective growth advantage on the tumour cells, thereby acting very much like a typical dominant oncogene (dominant negative effect).[192] In terms of function, this implied that some *p53* mutations may result in a gain of function, rather than a loss of tumour suppressor function.

p53 mutations are not confined to spontaneous or sporadic type cancers. Germline mutations of p53 have been found in association with a predisposition to the development of cancer, the Li-Fraumeni syndrome (LFS).[274,275].

Alterations of the *p53* gene and its encoded protein are the most frequently encountered genetic events in human cancer, having been reported in almost every type of sporadic neoplasm.[271] Over half of all malignant neoplasms harbour some *p53* gene alteration.[253] Mutations of *p53* are found in all major histogenetic groups, and approximately one half of adult cancers of the colon, lung, brain, oesophagus, stomach, liver, breast and cervix harbour *p53* gene mutations.[279,280] Furthermore, as a biological indicator, p53 mutations have been reported to be associated with aggressiveness or poor prognosis in some adult tumours.[281-283]

2.10.1 p53 mediated apoptosis

In addition to its role in the regulation of the cell cycle, wt p53 protein is also capable of inducing apoptosis or programmed cell death through both transcription-dependent and transcription-independent pathways.

It seems unlikely that *p53* itself is the determinant of whether apoptosis occurs or not. Other factors, including cell type, developmental stage, additional pathways triggered by the p53-inducing signal, growth factors, and the expression of other cellular and viral proteins which modulate cell death may play important roles in the cell death decision. Moreover, it is clear that both G1 arrest and apoptosis can occur without activation of either *p53* or *p21*.

The apoptotic inducing ability of wt p53 is supported by the rapid death of cells overexpressing wild-type p53.[284] The MDM-2 protein is known to bind p53 and incapacitate it. Therefore, cells lacking MDM-2 accumulate high levels of wt p53, which triggers apoptosis. The extreme toxicity of unchecked p53 function can be seen in the apoptotic death of MDM-2-null mouse embryos at the preimplantation stage.[285] Conversely, overexpression of MDM-2 was formally shown to inhibit p53-mediated apoptosis.

Furthermore, the stimulation of apoptosis by p53 may result, at least in part, from down-regulation of the survival factor bcl-2[286] and transcriptional up-regulation of another *p53* target gene, *bax* .[287] Evidence for transcriptional repression of *bcl-2* by p53 is provided by several investigations, including studies of breast cancer cell lines with wild-type and mutant p53,[288] co-transfection experiments of p53-deficient human lung cancer cells and murine lymphoblastoid cells,[289,290] and analysis of p53-deficient transgenic mice.[289] The bax protein, which is a member of the *bcl-2* family, directly promotes apoptosis.[291] Induction of bax expression is likely to play an important, albeit not exclusive, role in mediating the apoptotic effects of p53. p53-dependent apoptosis occurs normally in cells lacking p21.[249] These findings suggest that p21 protein normally protects cells from p53-mediated apoptosis by holding them in cell-cycle arrest.

Another suggestion is that p53 suppresses signal-mediated transport of proteins into the nucleus.[292] It had previously been shown to play an important role in apoptosis induced by DNA damage and the specific activating signal has now been found to be the presence of DNA breaks, induced either directly by the damaging agent or indirectly during attempts to repair or replicate damaged DNA.[293]

2.10.2 p53 protein stabilisation and accumulation

In normal unstressed cells, the concentration of wt p53 is maintained at a low level with a relatively short half-life of 20-30 min. p53 protein can be stabilised by means of mutations within the gene, sequestration of the normally nuclear p53 protein in the cytoplasm, inactivation of its tumour suppressor function or by binding with cellular proteins like WT1, p21 and bcl-2 family.[209,252,294,295] Activation and stabilisation of the p53 protein may also occur as a result of post-translational modifications, such as, phosphorylation, dephosphorylation, and acetylation.[206,267]

The characteristics of mutant p53 are considerably different from those of wt p53. Mutant p53 proteins involved in transformation usually have extended half-lives of 2-12 hours, thereby increasing intracellular concentrations.[252,271,279,296] Mutant p53 protein is mostly found in high-molecular weight aggregates, which are the result of an interaction with cellular- and virus-encoded proteins or self-oligomerisation.[192,252]

MDM-2 protein can block the ability of p53 to transcriptionally activate target genes.[297] Since MDM-2 is itself a target for downstream activation by p53, there exists an exquisitely sensitive feedback loop. Basal levels of p53 activate MDM-2 transcription and the resultant MDM-2 protein binds to p53, inactivating its transcriptional activating properties and targeting p53 for ubiquitin-mediated degradation.[298] This loop can be disrupted by events that inhibit the binding of MDM-2 to p53, such as, mutation of either gene or phosphorylation of either protein.[206] Mutant p53 also loses its transcriptional activation function, hence MDM-2 is not up-regulated. In the absence of the MDM-2-dependent stimulus to ubiquitin-mediated degradation, p53 protein levels accumulate.

In the absence of a complexing viral protein, detection of significant amounts of p53 in a cell has been generally taken to be indicative of *p53* gene mutation,[299] particularly when monoclonal antibodies that specifically recognize mutant epitopes are used. Most missense mutations of p53 prolong the half-life of the protein, permitting it to be immunohistochemically detectable in those tumours containing a p53 missense mutation.[271] Neoplasms with frameshift or nonsense (chain-terminating) mutations generally do not have immunohistochemically-detectable

p53. This is explained by the fact that the resultant protein is absent, truncated, or unstable. Harris and Hollstein[266] claim that these types of changes in the DNA base sequence make up <20% of the p53 mutations described in human tumours.

Immunohistochemical detection of p53 protein needs to be viewed with caution and some workers have suggested that p53 immunostaining is a somewhat imperfect reflection of the prevalence of p53 mutations.[300,301] Immunohistochemical results can be affected by degradation of antigen during tissue processing and by the specificity of the antibodies used.[302] Optimal fixation is essential for reliable immunohisto-chemical results. A study that evaluated various fixatives found that tissue fixed in phenol formol saline produced the best results.[303] This was followed by fixation in formalin saline at 4°C overnight. The worst fixatives were formol saline, heated to 55°C, and formol calcium, at any temperature. Borderline acceptable results were obtained with formol saline and neutral-buffered formol for 24 hours at room temperature. These workers also found that poorly fixed tissues not only yielded non-crisp nuclear staining, but also leakage of protein from the nucleus into the cytoplasm. False negative and positive results in immunohistochemical tests must always be considered. Some have stated that under optimal conditions, immunohistochemical methods are capable of detecting most missense mutations.[302] Importantly, as discussed earlier, some types of mutation do not induce p53 over-expression and may be undetected. In addition to the above, increased translation efficiency of p53 mRNA may also contribute to higher steady state protein levels.

2.10.3 Cytoplasmic p53

The location of p53 protein within a cell is of potential significance. In order for p53 to act as a transcription factor it has to be localized to the nucleus.[304,305] The C-terminal region has three nuclear localization signals that mediate migration of the protein into the cell nucleus.[304] There was uncertainty whether cytoplasmic localization of p53 was due to an artefact of fixation or processing and in the past is was regarded as false positive. It now appears to be a highly significant observation since cytoplasmic p53 cannot perform its role as a transcription factor.[306]

2.10.5 *p53* mutations in childhood embryonal neoplasms (including nephroblastomas)

Mutations in *p53* are infrequent in childhood tumours, with the exception of anaplastic nephroblastoma. There are a number of studies which investigated the frequency of *p53* mutations in hepatoblastomas. Four studies with a total of 74 hepatoblastomas found no evidence of *p53* mutations.[318-321] In a total of 108 hepatoblastomas investigated 15 showed *p53* mutations, a frequency of 13.9%.[318-324] One study of Japanese patients found *p53* mutations in 9 of 10 cases.[324] Eight of these cases had a common point mutation at codon 157 in exon 5. In another study of Japanese patients this frequency could not be reproduced.[320] It has been suggested that this may be explained by environmental factors based on geographic variation. Technical error may be another possible explanation.

Mutations in the p53 gene are also rare in neuroblastomas. Only 3 of 272 (1.1%) neurblastomas were found to harbour p53 mutations. In an interesting study which analysed *p53* at primary diagnosis and at relapse, a mutation was found in the relapse specimen but not in the primary specimen. In this situation *p53* mutation may be a mechanism for resistance to chemotherapy.

In retinoblastomas, p53 mutations were not seen in 23 primary tumours, however, a metastatic retinoblastoma displayed a point mutation in *p53* as well as LOH for chromosome 17.[325] This study concluded that *p53* mutations might not be associated with primary tumour development but might play a role in tumour progression.

Similar to other paediatric embryonal neoplasms, the frequency of *p53* mutations in medulloblastomas is low and varies from none to 11%. Seven of 93 (7.5%) medulloblastomas showed *p53* mutations.[326-329] LOH of 17p in contrast is a frequent finding in medulloblastoma leading to the suggestion that there is a novel tumour-related locus distal to *p53* on the short arm of chromosome 11.

In the rare pleuropulmonary blastoma *p53* mutations were identified in 2 of the 3 cases studied.[330] Both cases with mutations had a fatal outcome in comparison to

the patient without mutations who was disease free after 3 years. It appears that *p53* mutations are associated with poor outcome, but a larger study is warranted.

In a study of 82 childhood solid malignant neoplasms, including 44 neuroblastomas, 13 nephroblastomas, 11 hepatoblastomas, 10 rhabdomyosarcomas, 2 Ewing sarcomas and one each of an undifferentiated sarcoma of the liver and a fibrosarcoma, the overall incidence of *p53* gene mutations was low (2.4%, 2 of 82).[320] Specifically, no mutations were found in neuroblastomas, nephroblastomas, and hepatoblastomas. The 2 cases with mutations were an undifferentiated sarcoma of the liver and a rhabdomyosarcoma.

Takeuchi *et al.*[331] examined 66 nephroblastomas for alterations of the *p53* gene using PCR-SSCP analysis followed by direct sequencing and found mutations in 3 (5%) tumours. There were 2 missense mutations and 1 nonsense mutation. Of the 35 cases in which clinical information was available, one of four cases with anaplasia had a *p53* mutation and one of 31 (3%) non-anaplastic samples had a *p53* mutation.[331] Malkin *et al.*[332] reported finding mutations in 2 of 21 (10%) nephroblastomas. One of the patients had an advanced stage tumour with favourable histology and the other had focal anaplasia. Bardeesy *et al.*[184] identified mutations in 9 of 140 (6%) nephroblastomas, eight (8/11, 73%) of these occurred in anaplastic tumours. Defavery *et al.*[333] assessed the presence of p53 mutations in 10 cases of nephroblastomas using SSCP followed by sequencing. Although two cases showed altered electrophoretic migration by SSCP, mutations were not confirmed by DNA sequencing. Kusafuka *et al.*[320] studied 13 cases of nephroblastomas and detected no mutations of the p53 gene. In a study of 38 cases of nephroblastomas, Waber *et al.*[334] also did not detect any mutation of the p53 gene. Lahoti *et al.*[335] compared p53 mutations in two clinical groups. They found mutations in 4 cases, all belonging to the metastatic/recurrent group, whereas none of the 12 non-metastatic/non-recurrent tumours showed any mutations. Anaplasia was present in two of these four cases with mutation. El Bahtimi *et al.*[336] reported *p53* gene alterations in all three anaplastic tumours in their study but none in 10 favourable histology tumours.

In another study, Bardeesy *et al.*[337] analysed seven nephroblastomas with paired samples from nonanaplastic and anaplastic regions. Within this group of tumours, *p53* mutations were detected in six, 5 of which were restricted to anaplastic regions. The authors suggested that progression to anaplasia is associated with clonal expansion of cells that acquire *p53* mutations.[337]

Overall, only 22 tumours out of 325 nephroblastomas analysed in 9 different studies[184,320,331-336,338] carried *p53* gene mutations (6.8%). Of the 22 cases that showed *p53* mutations, at least 15 can be confirmed as anaplastic tumours. The number may be more but some of the studies did not provide information on tumour classification. Taken together, *p53* mutations do appear to be associated with anaplastic nephroblastomas.

Based on their observations, Malkin *et al.* suggested that *p53* gene mutations may be relatively unimportant in the genesis of nephroblastomas[332] but the genetic implications of the *WT1*/*p53* interaction need to be unravelled. Velasco *et al.*[338] postulated that the rare occurrence of *p53* mutations in nephroblastomas shows that they are much more complex than mutations occurring in the hot spots commonly seen in neoplasms in adults.

Mutations in *p53* are present in less than 5% of Wilms tumours, but they are of particular interest because they define a subset of Wilms tumours with poor prognosis. In fact, mutations in *p53* have been identified in 75% of Wilms tumours that have anaplasia, and strongly predict a poor clinical outcome.[184] Moreover, in tumours with focal anaplasia, mutations in *p53* are only present in areas with nuclear atypia, indicating that they might be a relatively late event in tumour progression, related specifically to this histological appearance.[337]

2.10.6 p53 Immunohistochemical studies in nephroblastomas

It is difficult to analyse p53 immunoexpression from the different studies because of a marked variation in what is regarded as positive. Some studies found p53 immunoexpression in all cases[339,340] or in the majority of cases.[335,341] In contrast to

these studies, other investigators found that a minority of cases showed p53 immunoreactivity.[336,342-345]

In a study by Skotnicka-Klonowicz *et al.*[340], positive immunoreaction was seen in all cases of nephroblastomas studied. The percentage of cells staining with anti-p53 antibody in all three components of the tumour ranged from 0% to 70% (mean 20.3%, median 16%). The expression of p53 was highest in the epithelial elements (70%) and in the blastema (68%) but lowest in the stroma (35%). Lemoine *et al.*[339] found that all 34 nephroblastomas showed immunoreactivity with at least one of three antibodies. In another study, 27 of 38 cases showed p53 immunoexpression for at least one of two antibodies.[341] In this study all 4 cases with favourable histology that also expressed p53 succumbed to metastatic disease. Another study detected p53 immunoreactivity in 7 out of 21 investigated tumours.[343]

A study of favourable histology nephroblastomas found that 8% of cases (5 of 63) had over expression of p53 protein. However, in contrast to other studies this study found that p53 expression in histologically favourable nephroblastomas is not related to tumour stage, recurrence or progression ($p > 0.3$)[342] and the authors concluded that evaluation of p53 immunopositivity in histologically favourable nephroblastomas before administration of chemotherapy has no clinical relevance. Furthermore, they inferred that some histologically favourable nephroblastomas that initially show p53 immunopositivity are chemosensitive to standard National Wilms Tumour Study therapeutic protocols but that the acquisition or persistence of p53 immunoexpression after chemotherapy is associated with a more biologically aggressive tumour.

It may be that anaplastic nephroblastomas show a more rapid and active production or slower elimination of mutant p53 protein. Mutant p53 may lead to loss of oncogenic suppression or increased oncogenic activity compatible with the increased aggressiveness. Cheah *et al.*[341] suggested that immunohistochemical detection of the mutated p53 in nephroblastomas may prove to be more useful in determining prognosis than the molecular identification of a mutation in the tumour suppressor gene.

2.11 The p21 gene

The gene for $p21^{Cip1/WAF1/sdi1}$, mapped to chromosome 6p21.2, has been cloned independently by a number of different routes. It was found to encode a 164 amino acid protein with a molecular mass of 21 kDa. This protein was variously identified as a senescent cell derived inhibitor (Sdi1)[346,347] and a cyclin dependent kinase interacting protein (Cip1).[230,231] In addition, it was cloned in a differential screen for genes activated by wild-type p53, wild-type p53 activated fragment (WAF1).[248]

p21 induction may occur under different physiological situations but appears to be regulated by two pathways, namely, p53-independent and dependent mechanisms.[230,231,348,349] When cells are treated with agents that cause DNA damage, such as radiation or chemotherapeutic drugs, p21 expression is induced through the p53-dependent pathway.[262] Besides p53 activation of p21, there are many other potential pathways of $p21^{cip1/WAF1/sdi1}$ induction that are totally independent of p53, such as, TGF-β1, high levels of toxic oxygen species, vitamin D, MyoD, nerve growth factor, platelet derived growth factor, fibroblast growth factor, and epidermal growth factor.[349,350] Since autocrine mechanisms involving growth factors are implicated in tumour growth, it is possible that p21 can be induced even in malignant cells either with mutant p53 or without wt p53. The expression of p21 is not significantly affected in cells with a mutation in only one allele of p53 with the other allele being intact. Therefore, loss of both functional copies of p53 is necessary to inhibit p21 transcription.[351] Accumulation of p21 protein may also result from posttranscriptional mechanisms.

Elevated levels of $p21^{cip1/WAF1/sdi1}$ protein irrespective of the mechanism of induction, appear to mediate cell-cycle arrest via CDK inhibition, predominantly at the G1 phase of the cell cycle but also at the G2/M phase.[216,223,230-232,248,249,254,270,352,353] Furthermore, p21 through its physical interaction with PCNA inhibits DNA replication.[251,254] but not PCNA-dependent DNA repair [354]

Cyclins, CDKs, and PCNA form quaternary complexes together with p21 in normal cells, whereas in transformed cells, neither p21 nor PCNA are associated with cyclin-CDK binary complexes.[229,348] However, more than one molecule of

$p21^{cip1/WAF1/sdi1}$ per complex results in kinase inhibition.[348] Although it is possible that in neoplasms with wt p53, alterations in p21 function may be responsible for tumour progression, this does not appear to be the case as p21 mutations are rare. No p21 mutations were detected in an investigation of 315 primary samples from 14 different types of human malignancies.[356] A possible explanation for the rarity of p21 mutations in neoplasia is that since p21 is a universal inhibitor of the CDKs, mutation and inactivation is catastrophic resulting in cell death. Furthermore, it has been shown that p21 null mice develop normally, with no increased incidence of neoplasia.[353]

An interesting suggestion is that cytoplasmic p21 has a role in the control of cell death.[357] If p21 inhibits cell death, its nuclear activity should be inactivated during the apoptotic process. In support of this, caspase 3 cleaves the p21 nuclear localization signal to relocalize the inhibitor to the cytoplasm.[358] This is followed by a substantial increase of CDK2 activity, which inhibits cell death by a dominant-negative effect.

2.12 The Retinoblastoma gene family

The retinoblastoma gene family is composed of 3 members, the retinoblastoma gene (*Rb*), which is one of the most studied tumour-suppressor genes, and 2 related genes *Rb2/p130* and *p107*. The proteins encoded by the two latter genes, *pRb2/p130* and *p107*, have been shown to be structurally and functionally similar to pRb[359] and play a pivotal role as negative regulators of cell proliferation.[360] These three retinoblastoma proteins have different growth-suppressive properties in specific cell lines, implying that they may complement each other but are not functionally redundant.[360]

The *Rb* gene is located on chromosome band 13q14 and encodes a 105 kDa nuclear cell cycle regulatory phosphoprotein.[364] Because of its size, it is difficult to identify individual point mutations of the *Rb* gene. However, it has been demonstrated that human tumours that possess *Rb* mutations usually exhibit LOH of the remaining allele. It has also been shown that mutations of *Rb* genes are common in a variety of tumours in adults, particularly carcinomas.[212,271]

The relationship between mutations in the *Rb* gene and altered Rb protein expression is not clear-cut since immunohistochemically detectable pRb protein may be present even in cases with mutated *Rb* gene.[365] Usually deletions result in total loss of pRb protein expression while point mutations may result in lowered expression intensity of the Rb protein.[365] Expression of pRb has been related to prognosis in some neoplasms,[366,367] while other studies could not confirm this.[368,369]

Other than its negative effect on the G1 phase of the cell cycle, pRb has been found to inhibit apoptosis induced by various stimuli such as ionising radiation,[370] TGF-β1,[371] interferon-γ,[372] and wild-type p53 overexpression.[373] However a study on invasive breast cancer did not show any evidence for any apoptosis inhibiting effects of pRb.[374]

The phosphorylation status of pRb is tightly regulated during the cell cycle.[375] During the G1 phase, pRb exists as an unphosphorylated or underphosphorylated form, whereas in late G1 through to the end of the M phase, it is extensively phosphorylated. It then undergoes rapid dephosphorylation during the anaphase. The underphosphorylated form of pRb is the functionally active, growth inhibitory form of pRb in G0/G1.[376] This form of pRb is preferentially localized to the nuclear matrix and binds to a family of cellular transcription factors known as E2F[377] and also recruits repressors, such as histone deacetylases, to E2F-responsive promoters on DNA.[378] The binding of pRb switches E2F from a transcriptional activator to a repressor. Phosphorylation of the retinoblastoma protein at G1/S, driven by the cyclin D1-CDK4/6, results in a release of the pRb bound E2F members and transcription of the S-phase genes.[244] These genes might be activated at the G1/S boundary as pRb is rapidly hyperphosphorylated, and as a result, E2F is freed from the constraints of pRb. E2F also exists associated with other Rb-related proteins, p107 and p130.

Underphosphorylated pRB molecules may also form complexes with viral transforming proteins, including the SV40 large T antigen, the adenovirus E1A protein, and the papillomavirus E7 protein resulting in inactivation of pRb function

and leading to cellular immortalisation. It is believed that the growth deregulation produced by pRb inhibition is counteracted by apoptotic cell death orchestrated by normal p53 function. Therefore, the loss of one tumour suppressor gene is compensated by the activity of the other, serving as a safeguard mechanism to protect against the emergence of neoplastic cell growth. Without both pRb and p53, it appears that E2F activation stimulates cell proliferation permitting tumour formation.[379] Nevertheless, if pRb is non-functional, due either to mutations in the gene or to the presence of inactivating viral proteins, a major component of the p53-mediated growth arrest is lacking. In this situation there is also overexpression of the cell cycle-promoting transcription factor, E2F.[380] Furthermore pRb's growth inhibitory function can be inactivated by phosphorylation of pRb by A-, D-, and E-type cyclin kinases in vivo.[219]

2.13 The bcl-2 gene family

This family of genes which share sequence homology,[291] can be further divided into two functionally antagonistic groups: cell death suppressors e.g. *bcl-2*, *bcl-X_L* and *MCL-1*; and cell death promoters e.g. *bax*, *bcl-X_S*, *bak*, and *bad*. The protein products of the gene family share two highly conserved domains BH1 and BH2 (bcl-2 homologue 1 and 2), which regulate heterodimerization.

The *bcl-2* gene is located at chromosome 18q21 and encodes a 26kD, putative membrane associated protein containing a hydrophobic carboxyl terminus that may locate it to the intracellular membranes and leaving the remainder of the protein in the cytosol.[382] Structurally, bcl-2 is an integral membrane protein localized to the intracellular membranes of the nucleus, mitochondria and endoplasmic reticulum. This gene was identified in 1984 while studying the t(14;18) chromosome translocations that occur frequently in B cell leukaemia and non-Hodgkin's follicular lymphoma.[383,384] The t(14;18) translocation juxtaposes *bcl-2* gene to the heavy chain immunoglobulin (IgH) locus at chromosome 14. The consequence of this translocation is a *bcl-2/IgH* fusion gene which results in high levels of bcl-2.

Bcl-2 gene expression inhibits apoptosis[385] and confers survival advantage to a variety of cell types.[385] This function of *bcl-2* appears to be highly evolutionarily

conserved.[386] Apoptosis is an important feature in many normal biological processes, such as, embryogenesis, morphogenesis, development of the immune system, and cell maturation and differentiation.[387-389] Bcl-2 does not seem to have a direct effect on cell proliferation because over expression of bcl-2 does not inevitably result in an increase in cell proliferation or alter the pattern of the cell cycle.[12]

The *bcl-X* gene through alternative splicing generates two messages, coding for two functionally distinct proteins:[392] a short version, designated bcl-X_S, which is a positive modulator of apoptosis, and a long version, bcl-X_L, which is a stronger inhibitor of apoptosis than *bcl-2*. *Bax*, via alternative splicing generates 4 messages coding for 3 proteins, designated bax-α, bax-β and bax-γ.[291] *Bax* is a positive modulator of apoptosis. The pro-apoptotic action of bax results from its ability to form inactive heterodimers with both bcl-2 and bcl-X_L [291] leading to abrogation of their ability to suppress apoptosis. The balance between bcl-2, bcl-X_L and bax ultimately controls apoptosis.

2.13.1 Bcl-2 and apoptosis

Apoptosis is a specific type of cell death that differs from necrosis by defined morphological and biochemical features. Nuclear and cytoplasmic condensation, subsequent formation of membrane bound apoptotic bodies, and oligonucleosomal DNA degradation without an inflammatory infiltrate characterise apoptosis and distinguish it from necrosis. Apoptosis results in physiological cell deletion during development in a variety of tissues and organisms, and in maintenance of the number of cells during the renewal of mature tissue.

The inhibition of apoptosis by overexpression of the *bcl-2* proto-oncogene causes a decrease in cell death, and the consequent increase in the number of cells is thought to play a role in the development of tumours.[393]

Bcl-2 is expressed in a wide variety of foetal tissues, including those of haematolymphoid, epithelial, neural, endocrine and mesenchymal type.[394] Some of these tissues also have been recently reported to show expression in adults,

supporting a role for bcl-2 in tissue homeostasis. Recently, studies have documented the expression of bcl-2 in human fetal kidney using immunohistochemical techniques and provided a potential role for bcl-2 during morphogenesis and differentiation in some tissues.[390,394-396]

Since bcl-2 deficient mice have prominent renal lesions that resemble polycystic kidneys and a reduced number of nephrons, it is evident that bcl-2 protein is essential for normal renal development.[397,398]

In the foetal kidney, the epithelial-like condensations of metanephrogenic mesenchyme comprising the nascent renal vesicles exhibit the most intense staining for bcl-2 protein.[394,395] In contrast, the undifferentiated metanephrogenic mesenchyme and the renal ampullae show no immunoreactivity for bcl-2 protein. The epithelium of the nascent glomerulus is initially strongly bcl-2 positive; however, with maturation and vascularisation bcl-2 staining becomes limited to the parietal layer of Bowman's capsule.[395] The upregulation of bcl-2 protein during metanephrogenic mesenchymal induction occurs before the acquisition of epithelial specific protein markers, such as low-molecular weight cytokeratins.[390] Therefore, it appears that the upregulation of bcl-2 occurs after the commitment of the undifferentiated mesenchymal cell to epithelial differentiation. This suggests that the survival advantage imparted by *bcl-2* gene expression may be necessary for the conversion of the induced metanephrogenic mesenchyme into the epithelial renal vesicle and development of the nephron.[390] It is also significant that the immunoexpression of bcl-2 protein within the developing kidney corresponds closely to that observed for the transcription factor, Pax-8,[399] and WT1,[43] suggesting that either one, or both, of these factors may play a role in the upregulation of bcl-2 expression.

The distribution of bcl-2 expression in normal adult tissues has been investigated immunohistochemically and shown to be expressed in a variety of tissues that either demonstrate extended viability, such as neurons, or in tissues that undergo self-renewal by mechanisms involving apoptosis, such as colonic epithelium.[400] In the adult kidney, the parietal layer of Bowman's capsule expresses bcl-2 protein

whereas the capillary tuft of the glomerulus does not show any immunoreactivity.[390,396] Henle's loop and collecting ducts of the normal adult kidney are also immunoreactive for bcl-2.

2.13.2 Bcl-2 immunohistochemical studies in nephroblastoma

Most clinically oriented studies on bcl-2 expression have found a positive correlation with adverse prognosis in a variety of solid tumours, including non-small cell lung carcinoma,[401] nasopharyngeal carcinoma,[402] prostatic carcinoma,[403] and neuroblastoma.[404] In contrast, bcl-2 expression was correlated with favourable prognosis in tumours arising from epithelia as in colon carcinoma[405,406] and breast cancer.[407,408]

For nephroblastomas, both the role and the prognostic significance of bcl-2 are unknown. A recent study although failing to demonstrate prognostic bcl-2 significance in Wilms tumour because of a limited number of cases studied,[409] found that classical nephroblastomas expressed significant amounts of cytoplasmic bcl-2 and measurable levels of *bcl-2* mRNA, whereas anaplastic nephroblastomas consistently did not.[409] This study investigated 10 classical and 3 anaplastic nephroblastomas, a total of 13 primary nephroblastomas. In another study, Tanaka *et al.*[396] showed that preoperative chemotherapy did not significantly influence either the occurrence of apoptosis or expression of bcl-2 in a group of treated Wilms tumour patients. Furthermore, there was no correlation between bcl-2 expression and tumour stage or outcome. Ghanem *et al.*[410] found that blastemal bcl-2 immunoexpression was of prognostic value for clinical progression. Another study found weak bcl-2 expression limited to some epithelial cells arranged in tubules and to rhabdomyoblasts detected in the stromal component of some tumours.[411] This study also found that bcl-2 expression and MIB appeared complementary, i.e. areas with strong bcl-2 expression were MIB negative and vice versa. Wünsch *et al.*[411] also found that bcl-2 was strongly expressed in microscopic but not in macroscopic NRs, and they suggested that bcl-2 may play a role in NR persistence and involution.

Nephroblastoma is a genetically heterogeneous neoplasm but regulation of bcl-2 by the WT1 gene is of particular interest[102] and requires investigation.

2.14 Drug resistance

The recognition of drug resistance as a major reason for the failure of chemotherapy has stimulated efforts to identify mechanisms of drug tolerance. Success in this search offers the possibility that therapies may be designed to overcome mechanisms of resistance. The so-called MDR phenotype is characterised by decreased intracellular drug uptake and enhanced drug efflux, against a broad category of chemotherapeutics, resulting in a net reduction of the intracellular drug concentration.

Five to 10% of nephroblastomas are fatal due to metastases and drug resistance.[412] Only a small number of these untreatable tumours are distinguished by anaplasia. Data on relapse in South African Wilms tumour patients has not been published but the experience in the referral hospital in Durban is similar to Western data (personal communication, Prof GP Hadley). Anaplasia is frequently associated with drug resistance,[161,413] *p53* mutations[184,331,332] and attenuated apoptosis.[337] Diffuse anaplasia, in particular, has been found to bear the most unfavourable prognosis.[161,162]

Ischaemia, radiation therapy, cytotoxic drugs, or expression of oncogenes might induce deregulation of apoptosis in neoplasms.[414] Generally in neoplasms, a high apoptotic index (AI) is associated with a good response to chemotherapy, whereas a low AI is found in neoplasms that are less sensitive to chemotherapy.[415] As a result of its role in the regulation of cell death, *bcl-2* is being implicated in the process of multidrug resistance of cancer. In prostatic carcinoma bcl-2 expression is associated with tumour progression after hormone therapy, suggesting that bcl-2 may confer resistance to androgen ablation treatment.[403] In hormone refractory tumours, there are always two epithelial populations, bcl-2 negative and bcl-2 positive. It is suggested that while bcl-2 negative cells are sensitive to androgen withdrawal and eliminated by the treatment, bcl-2 positive cells may survive and

continue to proliferate, and eventually metastasize. Furthermore, the cell-cycle may decisively influence the outcome of therapy.[207]

Apart from its role in growth arrest, DNA repair, and apoptosis, p53 also affects the expression of drug resistance genes. Wild-type p53 down-regulates the expression of drug resistance genes, e.g. *MDR1*, *MRP1* and *DNA topoisomerase IIα*.[416-418]

2.14.1 The MDR1 gene

Several transmembrane transport proteins, which share similar macrostructures including ATP binding domains, and transmembrane regions, have been identified, belonging to the superfamily known as the ATP binding cassette (ABC). Genes encoding proteins in this group include MDR1 and the cystic fibrosis gene.[419] The bacterial transport proteins, as well as the most recently described multidrug resistant related protein (MRP1), are also members of this group.[420]

The MDR1 gene maps to chromosome 7q21.1 and encodes a 170 kDa cell surface protein called p-glycoprotein.[421-424] P-glycoprotein is composed of 1,280 amino acids. Based on sequence analysis, the N- and C-terminal regions have approximately 43% homology, and each have six transmembrane domains with an adenosine triphosphate (ATP) binding site. P-glycoprotein functions as an energy dependent drug efflux pump.[425,426] It is highly conserved and in normal human tissues is detected predominantly on epithelial cells with excretory function and/or transport function. Expression was found in the cell membranes of normal tissues in colon, liver, adrenal glands, kidney, small intestine, pancreas, capillaries of the brain and testis.[427-429] Tumours derived from these tissues often inherently express the *MDR1* and are drug resistant.[418,430] However, tumours that do not inherently express the *MDR1* gene such as acute leukaemias, neuroblastoma, phaeochromocytoma, ovarian cancers, and breast cancers can show induction of expression of p-glycoprotein after relapse post chemotherapy.[418,430] In the normal kidney, the glomeruli, distal tubules and collecting ducts are negative.[429]

The recognition of the role of p-glycoprotein as a drug efflux pump[426,431] and the subsequent demonstration of the reversal of drug tolerance by verapamil has

stimulated efforts to antagonize the function of this membrane phosphoglycoprotein.[432]

Previously reported results of immunohistochemical studies on p-glycoprotein detection using paraffin sections after formalin fixation are contradictory.[429,433-436] In one comprehensive study, p-glycoprotein could not be detected using traditional indirect immunoperoxidase and avidin-biotin complex methods applying three monoclonal antibodies against p-glycoprotein.[429] However, other investigators have reported the detection of p-glycoprotein in paraffin wax embedded tissue sections.[437-441] The discrepancies among these conventional immunohistochemical studies can be explained by significant differences in the methodologies used in the various laboratories and by major differences in quality control criteria.

The overexpression of MDR1 was previously shown to impart broad resistance to a variety of chemotherapeutic drugs.[430] Studies in nephroblastomas did not demonstrate significant p-glycoprotein expression.[413,418,430] In contrast, Volm *et al.*[442] found no overexpression of p-glycoprotein in untreated nephroblastomas but 12 out of 23 patients treated with actinomycin D and vincristine showed increased p-glycoprotein expression. Re *et al.*[413] suggested that *p53* inactivation is necessary for *MDR1* up-regulation, but that it is not sufficient because they could not demonstrate p-glycoprotein expression in a nephroblastoma with a *p53* mutation.[336,413] They speculated that this specific *p53* mutation, although crippling to other effects, does not prevent the long-lived p53 from still acting as a transcriptional repressor over the *MDR1* promoter, and that *p53* mutations alone might not be sufficient to impart the up-regulated *MDR1* phenotype.[413] In conclusion, they suggested that the multidrug resistance phenotype of anaplastic nephroblastomas may result from mutations of several genes.

2.15 Molecular techniques

The use of molecular procedures to study disease processes in the human genome has increased rapidly over the past several years. Until recently, however, these methods relied on the use of fresh or frozen tissues, excluding the study of conventionally prepared formalin-fixed, paraffin-embedded tissues.

Recently, several reports have described the extraction of DNA from paraffin wax embedded tissue blocks, demonstrating that this technical problem has largely been overcome.[444,445] The DNA obtained using these extraction methods is suitable for analysis by molecular techniques, thus allowing the vast amount of archival tissue to be exploited. Furthermore, this approach makes possible the study of statistically significant numbers of relatively rare disease conditions. An added advantage of using archival tissues is that the clinical outcome is often known.

Currently, most tissue samples available for analysis have been fixed in formaldehyde before being embedded in paraffin wax. Both under- and over-fixation are undesirable. It is has been shown that the DNA in fixed material displays some fragmentation and damage caused by the formation of covalent adducts. Paradoxically, incomplete fixation can result in greater loss of DNA as a result of autolysis or bacterial degradation. Despite the limitations of DNA extracted from fixed and embedded tissue, it is possible to work around these limitations. One way would be to avoid attempting to amplify DNA fragments greater than around 300-400 bp in PCR.[446,447].

Recent technological advances in molecular genetics have significantly improved our ability to dissect the morbid anatomy of the human genome and locate disease genes. Despite the introduction of molecular methods, such as the polymerase chain reaction, and the discovery of highly polymorphic microsatellite markers, genotyping remains a rate-limiting step in our ability to localise disease genes by linkage. These methods remain highly technical, time-consuming, and expensive.

2.16 Microsatellites

Present within the genome are large numbers of seemingly unimportant DNA segments arranged in repetitive units. The family of repetitive noncoding DNA sequences can be classified as follows:[448] (i) Satellite sequences: arrays with repeat sizes ranging from 5 to 100 bp, characteristically organised in clusters up to 100 megabases (Mb). These are located in the heterochromatin near chromosomal centromeres and telomeres and are not as variable in size within populations as the other members of this family, (ii) Minisatellite sequences: arrays with repeat sizes of 15-70 bp, which range in size from 0.5 to 30 kilobases (kb). These are found in

euchromatic regions of the genome and are highly variable in repeat size within populations and (iii) Microsatellite sequences: arrays with a repeat size of 1-6 bp, highly variable in size but ranging around a mean of 100 bp.

Microsatellites are widely distributed throughout the human genome, usually within non-coding introns and are estimated to occur in between 55 000 and 100000 copies in the human genome, providing a marker density of one microsatellite every 100 000 base pairs, even by the most conservative estimates.[448] However, although widely distributed, microsatellites are not regularly spaced in the chromosome. In humans, the most common repeat sequences are $(A)_n/(T)_n$ and $(CA)_n/(GT)_n$. A common microsatellite repeat motif is CA, and there are estimated to be 100 000 CA microsatellites scattered throughout the human genome.[449] The number of repeats in a microsatellite varies, and alleles of differing size are stably inherited. The length of the microsatellites is unique to each individual and varies between individuals, but there is no variation between different cells in the same individual. These variations or polymorphisms result in a unique DNA fingerprint for each individual. DNA polymorphisms are sequences that vary between the two homologous chromosomal alleles, and such a distinction allows one to trace the inheritance of each allele within a kindred. Based on their high degree of polymorphism, microsatellites have become invaluable in genetic linkage studies. Furthermore, the chromosomal location of many microsatellites is known. The combination of variable size and accurate mapping in relation to genes of interest make microsatellites ideal to study loss of heterozygosity (LOH). In addition, microsatellites can also be used to detect defective DNA mismatch repair.

Even though microsatellite variations might not affect the phenotype of the cell, they are by definition mutations.[199] The variation in the length of microsatellite repeats in tumour tissue compared to constitutional tissue represents a mutational process of insertions or deletions within the tumour DNA. Microsatellite repeats are likely to be hot spots for mutagenesis, and mutations within these sequences might be a marker of increased replicative errors throughout the genome of cancer cells. As microsatellites are scattered throughout the genome, such mutations must be widespread.

Most microsatellite sequences in the genome are located within noncoding, or intronic, sequences and mutations in these introns were believed to be silent and inconsequential. However, tumours with MSI have been found to potentially inactivate certain target genes by permitting an increased frequency of mutations in short repeat tracts in the DNA encoding the expressed portions of these genes. Some of these genes that have been identified thus far include receptors for growth factors, such as, transforming growth factor β receptor II (*TGFβ RII*),[450] insulin-like growth factor II receptor (*IGF2R*), regulators of the cell cycle (e.g. *E2F4*),[451] regulators of apoptosis (e.g. *Bax*),[452] and even the mismatch repair genes (*hMSH3* and *hMSH6*) themselves. Most of these microsatellite alterations result in frameshifts that truncate proteins, presumably leading to inactivation of the affected allele. The transformation to malignancy thus occurs when these target genes are mutated.

In some colorectal cancers mutations were observed in microsatellites.[453] However, it is becoming increasingly apparent in colorectal cancer that microsatellites are also present within genes and also undergo expansion or contraction specifically in cancer cells at very high frequencies.[450,452] Contraction or expansion of repeat sequences within genes provides an attractive mechanism for inactivation of tumour suppressor genes during tumour progression.

2.17 DNA damage and errors

Maintenance of genomic stability is one of the key criteria that govern the survival of species. DNA is a reactive molecule and as such is modified continuously by a broad range of agents. DNA is damaged by both environmental and cellular (endogenous) sources. Chemical carcinogens or ultraviolet radiation can directly induce structural alterations, such as thymidine dimers, single-or double-stranded breaks, and covalent cross-linking. The thymidine dimers can block normal replication of DNA. DNA may also be damaged from within: through hydrolysis, methylation or active oxygen species.

Maintaining the integrity of DNA is essential for normal cellular function.[454] There seems to be a homeostatic equilibrium in which extensive DNA damage is counterbalanced by multiple DNA repair pathways. In normal cells, only rarely do

errors in DNA escape the screen provided for by DNA repair. In other words, most DNA damage is repaired without error in normal cells. In eukaryotic cells there is the emerging concept of checkpoints in which cells sterilise the DNA immediately prior to the onset of DNA replication.[202] However, in cancer cells this balance may be tilted and the consequence is an accumulation of multiple mutations. Among the genes mutated are those that maintain the stability of the genome.

The DNA excision repair system recognizes and excises such structurally altered nucleotides.[455] Mechanisms have evolved to repair DNA damage, but considering the high frequency at which they occur and the compact and inaccessible structure of human chromatin, it is likely that a significant portion of damaged DNA escapes DNA repair and produces mutations during replication of the damaged DNA. Overall, it seems that the nucleotide sequence of DNA is maintained at a homeostatic equilibrium, such that an increase in DNA damage or a reduction in DNA repair results in an increased frequency of mutations.

DNA duplication occurs with every mitotic cell division, and this process must have an extremely precise proofreading system because errors could introduce mutations that would be transmitted to subsequent generations of cells. Certain strategies must be adopted to preserve the overall fidelity of DNA.

Errors of DNA replication are observed primarily during the elongation phase. The most common error is a simple mispairing of nucleotides. Patterns of hydrogen bonding govern the pairings of nucleotides. An adenine (A) is always paired with a thymidine (T), and a cytosine (C) is always paired with a guanine (G). The DNA polymerase may incorrectly pair an adenine with a guanine. There can also be spontaneous deaminations of cytosine residues into uracil residues. Because uracil is not one of the four bases ordinarily in DNA, transcription is once again blocked when the polymerase enzyme encounters one of these altered residues. Such mispairings are predicted to occur once every 103 to 104 base pairs.[454] Repair mechanisms keep the actual error rate much lower.

Another type of error can occur during DNA replication. This unique situation involves repetitive sequences of DNA (such as a dinucleotide CA repeat) that may

be more prone to replication infidelity. There can be "slippage" of the DNA polymerase complex, thereby leading to daughter chromosomes with either fewer or more copies of the repeat.[456] DNA slippage occurs when the normal base pairing between two complementary strands is altered by staggering of the repeats on the two strands, leading to incorrect pairing of repeats.[457] The system responsible for correcting such defects is the DNA mismatch repair system and with intact mismatch repair mechanisms, these errors of slippage are quickly corrected, and microsatellite DNA sequences are considered stable. However, in the presence of deficient mismatch repair function, these errors are not corrected, resulting in microsatellite instability (MSI).

2.18 DNA repair systems

Replication is the process by which DNA is copied in preparation for cell division. DNA replication is a complex process, whose fidelity, estimated to be in the range of one error per 1010 nucleotides synthesized,[458] depends on three factors: DNA polymerase(s), exonucleolytic proofreading and mismatch repair (MMR). Errors of replication are inevitable, but specialized repair systems have evolved to prevent the accumulation of harmful mutations in the genome. DNA repair encompasses a multitude of metabolic processes, which can reverse the damage either directly (photolyases, alkyltransferases), or indirectly by removing damaged bases (base excision) or oligonucleotides (nucleotide excision) from DNA, and resynthesizing the removed patch. The main task of DNA repair is to ensure that the DNA molecule is free of modifications or mutations, such that it can be transcribed efficiently and, most importantly, that it can be replicated faithfully and passed on to progeny cells. Microsatellites are prone to DNA replication errors because of the highly repetitive nature of the sequences, but these errors are usually rapidly repaired by highly robust mechanisms.

The DNA polymerase complex itself provides the first line of defence against errors of replication. DNA polymerase can immediately recognize a mismatched pair of nucleotides. An intrinsic enzyme subunit with 3 to 5 exonuclease activity excises the mispaired nucleotide from the newly synthesized strand, and the excised sequences are replaced with the correct nucleotides.

Another repair system is called nucleotide excision repair or short-patch repair. The proteins responsible for nucleotide excision repair have been studied in detail in *Escherichia coli*.[459] The uvrA and uvrB proteins bind to structurally damaged DNA in an adenosine triphosphate-dependent manner (Figure 3). The uvrC protein then binds and cleaves the damaged strand at two sites 12 base pairs apart, flanking the damaged nucleotides. Helicase II unwinds the DNA, and then polymerase I both excises the nucleotides between the two nicks and resynthesizes the strand with intact nucleotides. Finally, DNA ligase seals this newly synthesized strand. In eukaryotes, the nucleotide excision repair system is more complex. For errors that are not immediately corrected by DNA polymerase, the DNA mismatch repair system provides a secondary system of proofreading. This system functions as a critical "spell checker" that identifies and then corrects not only single base-pair mismatches but also small mispaired loops of DNA that result from replication errors of microsatellite tracts.

DNA mismatch repair has been studied and characterised most extensively in prokaryotes.[459] The mutS protein recognizes and binds to mismatched DNA sequences (Figure 4). Such DNA exists as a heteroduplex structure owing to the lack of complementarity of bases. The mutL and mutH proteins then function in concert with the bound mutS protein. MutH finds a single-stranded nick in the strand containing the incorrect nucleotide, which is followed by helicase II unwinding the DNA. Thereafter, a bidirectional exonuclease removes the bases between the nick and the mismatched pairs, and DNA polymerase fills in the gap with the correct sequence. Finally, DNA ligase seals the newly synthesized strand.

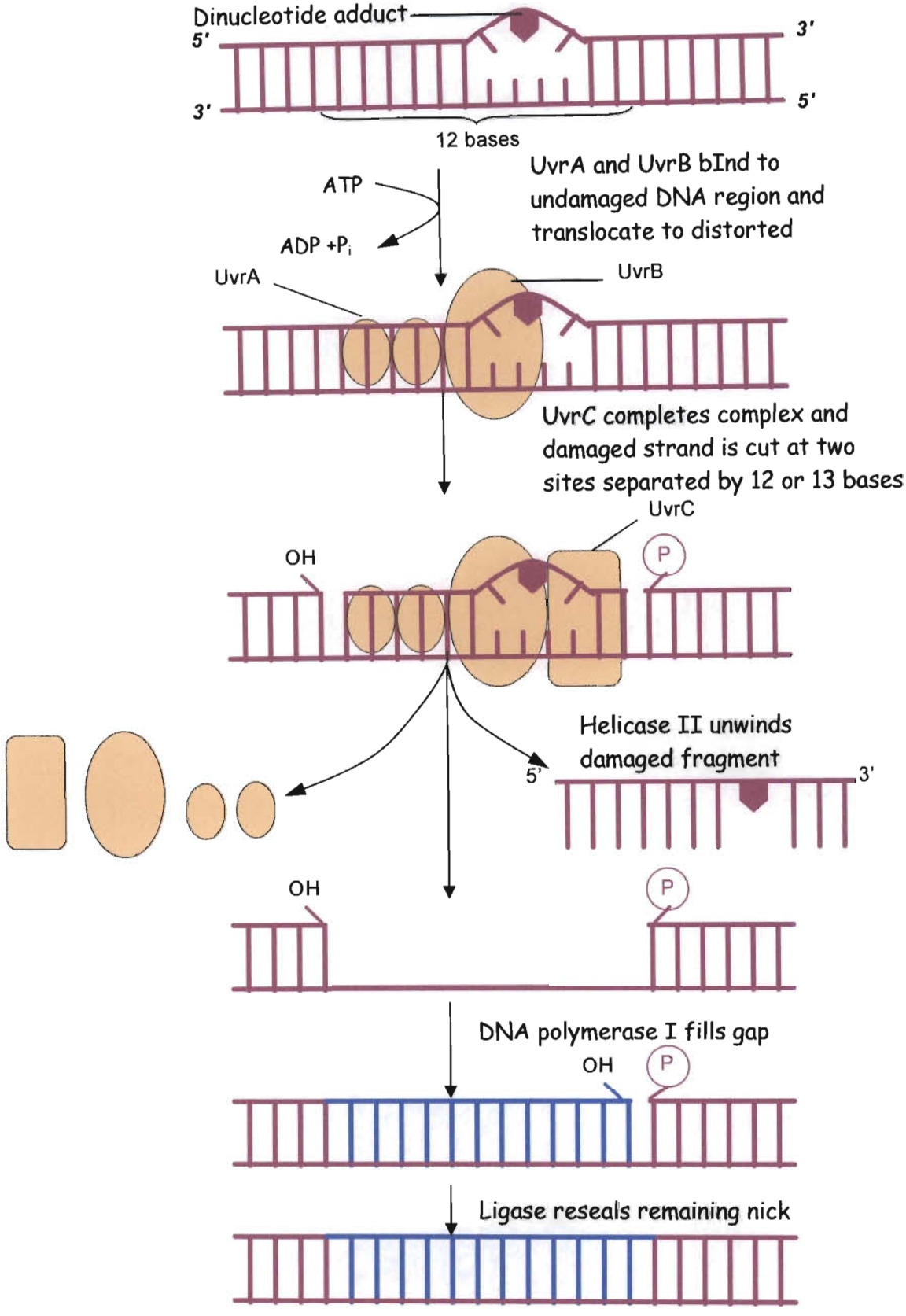


Figure 3: Nucleotide excision repair (adapted from Stryer[460])

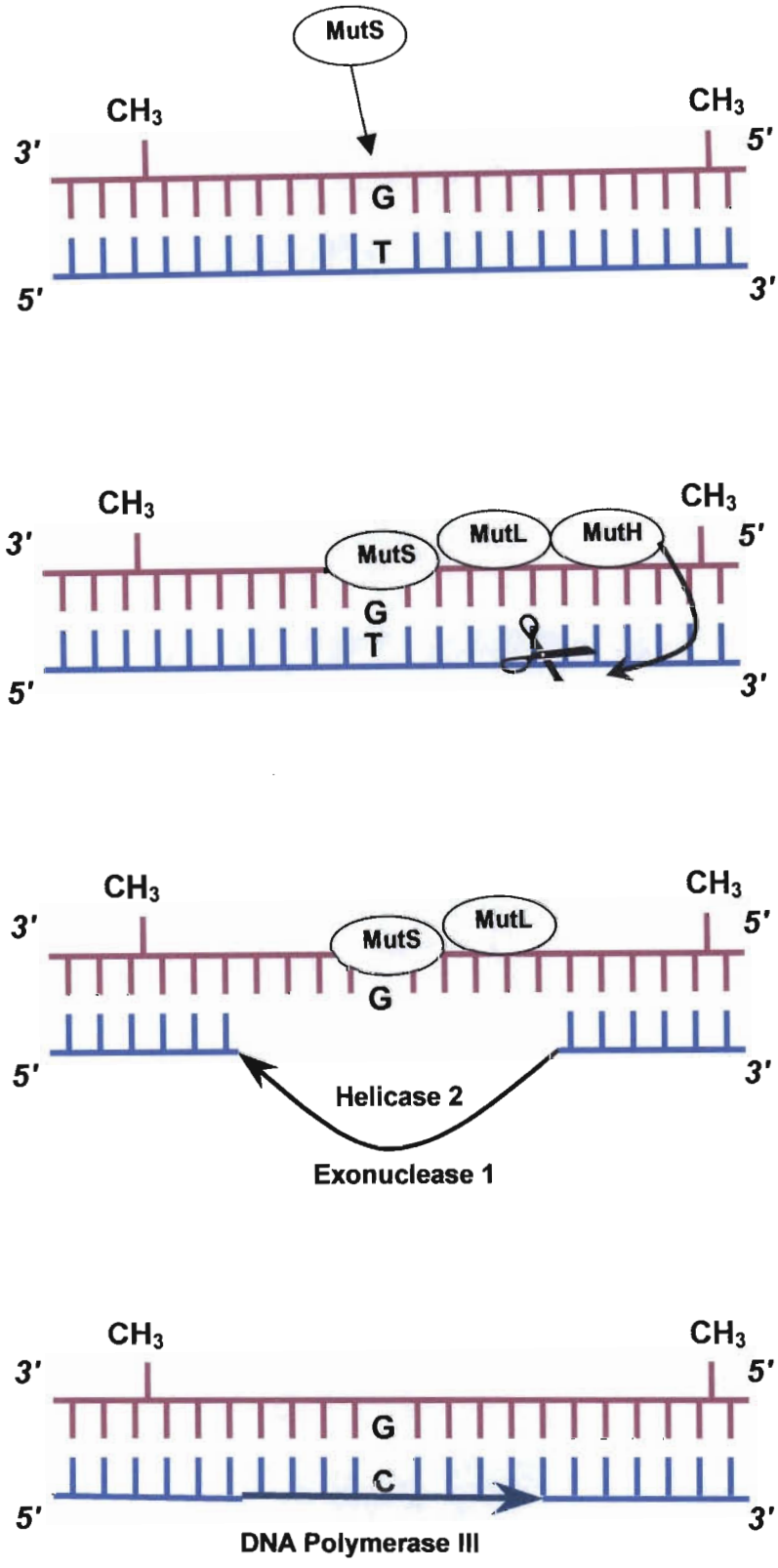


Figure 4: DNA mismatch repair (modified from Chung and Rustgi[461])

Investigators working on bacterial and yeast DNA repair were the first to recognize the pattern of microsatellite instability and the mismatch repair genes. Consequently, the human homologues of many of the mismatch repair genes described in bacteria and yeast, have been uncovered. The DNA mismatch repair system is composed of at least six genes: human *mutL* homologue 1 *(hMLH1)*, *hMLH3*, *hPMS1*, *hPMS2*, human *mutS* homologue 2 *(hMSH2)*, *hMSH3*, and *hMSH6*.[457,462] The most important proteins are conserved from bacteria to man. In the first step of the repair process in humans, DNA mismatches are recognised by protein heterodimers containing hMSH2. In hMutSa, the most abundant complex, hMSH2 is partnered by hMSH6. The quantitatively more minor hMutSb recognition factor comprises hMSH2 and hMSH3.[463]

DNA MMR is a highly conserved repair system that corrects mismatches arising during DNA replication and safeguards genomic integrity.[464-466] Several studies have implicated MMR function in other cellular processes, such as DNA recombination,[467] meiosis,[468] transcription-coupled repair,[469] and damage responses to chemical and physical agents.[470,471]

An interesting hypothesis has emerged in ulcerative colitis, in which DNA repair enzymes are no longer seen as guardians of the genome but instead can go wrong and create unintended consequences.[472] The conceptual basis of this idea was introduced a decade ago, when Xiao and Samson first proposed and demonstrated that imbalanced base excision repair (BER) with broad substrate-specific 3-methyladenine DNA glycosylases (AAG) could affect spontaneous mutation rates.[473] While the mismatch repair pathway has frequently been the focus of MSI studies, Hofseth and co-workers[474] instead considered the BER pathway, which includes the DNA repair enzymes AAG and apurinic/apyrimidinic endonuclease (APE1).

hMLH1 is localised to chromosome 3p21, contains 19 exons, and is predicted to encode a 756 amino acid protein, whereas *hMSH2* is localised to chromosome 2p21-22 and contains 16 exons encoding a 935 amino acid protein.[475-478]

A mutation in one of several mismatch repair genes results in deficient DNA mismatch repair activity. The extent of the mismatch repair deficiency depends on the specific gene that is altered. Mutations in mismatch repair genes may occur in germline or somatic DNA.[454] Of interest, an inherited germline alteration does not result in widespread developmental anomalies. One possible explanation for this is that the second wild-type allele may provide sufficient DNA mismatch repair function. The observed phenotype is the predisposition to early-onset tumours, primarily of the colon and endometrium.

Germline mutations of *hMLH1* or *hMSH2,* resulting in loss of protein function, account for 80% to 90% of observed mutations in HNPCC patients with mismatch repair-deficient cancers.[475-478] During tumour development, the second (wild-type) allele of these genes is also inactivated. The resultant mismatch repair deficiency leads to an increased rate of spontaneous point mutations and a high frequency of microsatellite instability (MSI-H).[453,457,462,479-481] Cells with mutated MMR genes replicate DNA errors more frequently than normal cells.[196,453]

2.19 Microsatellite instability

Considerable variability has arisen in the literature as to how to best describe alterations in the length of microsatellites discovered in certain tumours when compared to normal tissue. Proposed terms included: MSI, MIN, or MI (for MSI); RERs, USM (ubiquitous somatic mutations), and MMP (microsatellite mutator phenotype). Thibodeau *et al.*[481] referred to the process as microsatellite instability (which they abbreviated MIN), and recognised that tumours with MSI predominantly occurred in the proximal colon, were associated with an enhanced survival, and were notable for the absence of "LOH". Peltomaki *et al.*[482] used the term RER phenotype, and the same international collaborating group, linked this to a locus that would later yield the first of several DNA MMR genes responsible for HNPCC.[196] Ionov *et al.*[453] referred to the process as ubiquitous somatic mutations at simple repetitive sequences, linked the process to familial cancer, and made other observations on this novel tumour progression pathway. MSI has been accepted as the most appropriate term to describe this phenomenon.[17]

MSI is defined as a change of any length due to either insertion or deletion of repeating units in a microsatellite within a tumour when compared to normal tissue. It is important to stress that MSI, as defined, does not describe a particular tumour phenotype but refers only to the observation of instability at a given marker. Furthermore, it is important to recognise that MSI refers solely to novel length alleles and is distinct from the observation of LOH or allelic imbalance, in which one of the pre-existing alleles has been lost in the tumour. Studies aimed at characterising microsatellite stability serve as surrogate functional markers for defective mismatch repair function and are not indicators of specific mutations in a particular mismatch repair gene. Microsatellite instability (MSI) is a marker of defective MMR, hence MSI is used to identify human cancers with MMR defects. Surveys of the frequency of MSI among various tumour types identified MMR defects in 10–20% of sporadic colorectal carcinomas.[16,483]

The discovery of MSI in colorectal cancers and its linkage to HNPCC opened new chapters in tumour biology. Over the past 10 years, the scope of MSI has been expanded to encompass a unique form of genomic instability broadly involved in the genesis of cancer and limited to neither HNPCC nor colorectal cancer.

The literature on MSI is growing rapidly, and the need for development of uniform criteria for its detection and definition is recognized. The "Amsterdam Criteria" were developed in 1991 to permit the uniform identification of familial clusters of colorectal cancer so that HNPCC families might be recognised and studied.[484] The Amsterdam criteria strictly defined HNPCC as occurring when colon cancer is diagnosed in at least three family members, one a first-degree relative of the other two. The cases must span two generations, and one case must be diagnosed before 50 years of age.[484] Less stringent criteria, including the Amsterdam II and modified Amsterdam, have subsequently been developed. In 1996, a second workshop was held, which led to the development of the "Bethesda guidelines" for testing of colorectal tumours for MSI.[485] A third workshop entitled the "International Workshop on Microsatellite Instability and RER Phenotypes in Cancer Detection and Familial Predisposition" was held in 1997.[479] This workshop noted that although dinucleotide repeats have been used most frequently to study MSI, instability has also been observed with mono-, tri-, and tetranucleotide repeats.

The likelihood that a microsatellite will be susceptible to instability may therefore relate to the inherent mutation rate at that locus. Several mononucleotide repeats, such as the ones defined by Bat25, Bat26, and Bat40,[479] had proven to be very useful for the identification of the MSI-H group of tumours. Instability at these loci appeared in the majority of tumours with MSI-H but rarely in tumours defined as MSI-L. This meeting recommended that the panel for colorectal cancer be composed of two mononucleotide repeats (Bat26 and Bat25) and three dinucleotide repeats (D5S346, D2S123, and D17S250).[479]

Consensus has emerged regarding the number of loci that should be examined and how many abnormal loci are necessary to be designated as RER positive, in the setting of colorectal carcinoma. It is conceivable that evaluation of an insufficient number of loci may result in the underestimation of the frequency of MSI. The Revised Bethesda Guidelines recommend that a panel of 5 or more microsatellite loci be screened.[17] Based on the microsatellite status, cancers can be subdivided into three designations: high frequency of MSI (MSI-H), low frequency of MSI (MSI-L) and microsatellite stable (MSS).[17] MSI-H is defined as MSI involving two or more of the five loci or ≥30% of all microsatellite loci studied. MSI-L is defined as MSI occurring in less than two of the five loci or <30% of all microsatellite loci studied. MSS is defined as no MSI detected at any of the loci studied.

The finding that MSI affects repeat tracts within some but not all coding regions, may represent chance events rather than specific gene targeting, led to the setting of certain criteria to determine whether an affected gene is a true target of inactivation involved in tumourigenesis. These include: (i) a high frequency of inactivation, (ii) biallelic inactivation by simultaneous alteration of the other allele's repeat tract, point mutation, or loss, (iii) involvement of the candidate MSI target gene in a *bona fide* growth suppressor pathway, (iv) inactivation of the same growth suppression pathway in MSI-negative tumours through inactivation of the same gene, or of another gene within the same pathway, and (v) functional suppressor studies in *in vitro* or *in vivo* models, such as cell lines or animals.

There are several mechanisms for inactivation of MMR genes including germline mutations, genetic gene silencing, and allelic loss. Mutation and allelic loss are the

predominant mechanisms of MMR inactivation in HNPCC, but in sporadic colorectal carcinoma methylation appears to be responsible.[486]

There are probably additional MSI-independent mechanisms through which deficient DNA mismatch repair may lead to tumour formation. For example, a link between defects in mismatch repair and proliferation has been described in yeast cells that lack telomerase.[487] The telomerase enzyme preserves the telomeric ends of chromosomes, and this activity has a crucial role in the immortalisation of cancer cells. The ability of these cells to proliferate in the absence of telomerase may be mediated by enhanced chromosomal recombination in the setting of deficient mismatch repair.

MSI is usually assayed by using PCR methods to amplify specific microsatellite sequences in paired DNA samples of normal and tumour tissue from an individual. The electrophoretic pattern of bands of the PCR products using normal and tumour DNA extracts are then compared using gel electrophoresis. Altered electrophoretic band patterns reflect changes in the lengths of microsatellites in the tumour. With microsatellite markers, the difference between the two alleles is not a base pair substitution but rather a difference in the number of repeat sequences. The significance of MSI is that it may indicate a general deficiency in DNA repair mechanisms and other critical processes, even though the specific sequences analysed may not involve a critical coding region. Significantly, since the genetic alterations resulting in MSI are known, individuals can be tested for inactivation of MMR genes and for frequently occurring alterations in known microsatellite loci.[197,488]

Factors that may influence the detection of MSI include: variability in histopathological diagnosis, differences in purity of normal and tumour DNA, differences in the number and specific loci examined for the type of tumour under study, and variation in the number and degree of alterations in microsatellites required to score a lesion as MSI.[489] Scoring for mutations in only a few microsatellite sequences may not be adequate for determining whether or not a specific tumour exhibits microsatellite instability. There are over 2000 polymorphic microsatellite loci identified throughout the human genome, and these will continue

to increase in number.[490] Furthermore, various combinations of mono-, di-, tri-, tetra- and penta-nucleotide repeats employed in determining MSI also influence the results obtained.

The major technical problem in the analysis of microsatellites is the resolution and detection of the amplified DNA. This is due to the presence of additional bands that appear in addition to the microsatellite band, creating ambiguity in the analysis of the results. These bands are referred to as "stutter bands".

Another potential problem is PCR failure, in which a locus simply does not amplify. In situations in which one or more PCR failures are present, the need to reanalyse is dependent upon the information obtained from the remaining markers that successfully amplified. For example, a case of colorectal carcinoma having PCR failure for one marker, no MSI for one marker, and the remaining three markers demonstrating MSI could be scored as MSI-H, regardless of whether the PCR failure would have been positive or negative for instability (i.e., three or more of five markers).

A further innovation is the concept of 'allelic imbalance', developed by Cawkwell *et al.*[491] for the analysis of tissue microdissected from histopathological sections, where the quantitation of the minute amounts of DNA extracted is not possible. Since, in such circumstances, the differentiation of allelic amplification from allelic loss is not feasible, the neutral term 'allelic imbalance' (AI) is preferred. It is calculated as a ratio of ratios, the numerator and denominator being ratios of the intensities of the two allelic peaks in the tumour and constitutional DNA.

Investigating multiple loci by a semi-automated fluorescence-based technique is highly accurate, efficient, and likely to be cost effective as compared to conventional techniques. The use of fluorescent-based technology was shown to be a sensitive and useful tool for the detection and analysis of microsatellite PCR products.[492] The advantages of using fluorescent-based DNA technology for the analysis of microsatellites are very clear. The computer package calculates areas under the peaks and ratios comparing normal and tumour DNA can be determined. In addition, it allows for interpretation of novel peaks as compared to artefactual stutter

bands. It also allows for easy interpretation of the data, which can be captured, and stored using the software linked to the sequencer. A high throughput of samples is another advantage.

2.19.1 Microsatellite instability in non-HNPCC kindreds

MSI has been described across a spectrum of microsatellites and at varying frequencies in other tumour types. Some of these tumours with MSI occur sporadically in non-HNPCC kindreds and in other tumours not part of the usual HNPCC spectrum. The classification of these tumours is difficult because the absolute background frequency of a given microsatellite alteration in somatic tissue is largely unknown. Background rates of microsatellite alterations in germline tissues have been obtained from germline linkage studies and analysis of single sperm.[494,495] In these cells, approximately one new allele is generated at a dinucleotide repeat every 1000 divisions (0.1%) and at tetranucleotide repeats every 30-100 divisions (1-3%).

These non-colonic non-HNPCC tumours that display elevated frequencies of MSI may be divided into two groups.[479] The first group, commonly gastric and endometrial neoplasms, has a similar phenotype to MSI in colorectal cancer and displays instability at dinucleotide and mononucleotide markers and, to a lesser degree, at larger repeats. Like colorectal cancer, these tumours with a high frequency of MSI are mainly seen in sporadic cases. Furthermore, they have not been commonly found to have mutations in the known MMR genes, a finding similar to sporadic cases of colorectal cancer with MSI-H. The second group of non-colonic non-HNPCC tumours displays elevated frequencies of instability only at highly selected tri- and tetranucleotide repeats. The mechanisms underlying these phenotypes are yet to be elucidated. It is possible that environmental factors, in addition to underlying deficiencies in repair mechanisms, may play a role in generating some microsatellite alterations.[479] The influence of the microenvironment has been raised, with a particular emphasis on DNA damage from oxygen free radicals or lipid adducts leading to MSI.[479] Other environmental factors, such as smoking and diet, were also raised as potential generators of increased microsatellite alterations in tumours, regardless of the proficiency of

endogenous repair pathways. These exposures could act alone or in concert with DNA repair pathways to generate the specific phenotypes.

A study that examined secondary paediatric malignant neoplasms for MSI, identified MSI at five to seven loci in the secondary tumours of all nine patients.[496] These authors suggested that the development of a secondary tumour is due to a mutator phenotype that predisposes these children to the evolution of multiple tumours. They proposed the following sequence for the development of secondary, therapy-related tumours: (i) patients have inherited germline mutations in a MMR gene, (ii) this leads to a 'mutator phenotype', (iii) treatment given for the primary malignancy accelerates DNA instability in many additional cancer-associated genes, and (iv) this contributes to the development of secondary malignancies. Alternatively, treatment given for the primary neoplasm causes acquired mutations in DNA MMR genes.

A recent study investigated mutations of the MMR genes in 15 children who had more than one malignant tumour.[497] Mutations of MMR genes were found in 2 cases. One patient had a glioblastoma and nephroblastoma at 4 years of age. The glioblastoma was MSI stable but the nephroblastoma showed high frequency MSI. Both tumours lacked expression of hMLH1 and hPMS2.

Elucidation of the mechanisms of MSI will broaden our understanding of tumourigenesis in general. In the future it seems likely that other genes involved in mismatch repair will be discovered. Moreover, aside from patients with HNPCC, it may be possible that not all microsatellite instability is caused by defects in mismatch repair genes.[498]

2.20 AIMS OF THIS STUDY

(i) To determine whether there is an association between p53, bcl-2, pRb, p21, cyclin A and p-glycoprotein expression and prognosis in nephroblastomas

(ii) To determine the frequency of loss of heterozygosity and microsatellite instability at 11p, 16q and mismatch repair genes

(iii) To determine whether these genetic alterations were associated with prognosis.

CHAPTER THREE

MATERIALS AND METHODS

3.1 ETHICS APPROVAL

This study was approved by the Research Ethics Committee of the Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa. The study reference number is H059/99.

3.2 STUDY DESIGN AND SAMPLE SELECTION

This is a retrospective, single institutional study. During the study period 1984 to 1998, 224 cases of nephroblastoma were admitted. This included cases diagnosed at other peripheral hospitals and referred for further management. Patients that were diagnosed at other hospitals, did not reach nephrectomy, died before investigation and refused western medical treatment were excluded because of the lack of clinical data and tumour tissue. The total number of nephrectomies during this period was 177. Patients who had nephrectomies at other hospitals and where tissue was not available for study were excluded. Slides and blocks of the remaining cases that underwent nephrectomy at King Edward VIII hospital were retrieved from the archives of the Department of Pathology, Nelson R Mandela School of Medicine, University of KwaZulu-Natal and allocated case study numbers (n=158). All slides were reviewed by light microscopy and cases that had sufficient well-fixed, viable neoplastic tissue and normal kidney tissue were selected for the study. Cases with complete necrosis or insufficient tissue for further analysis were excluded and two cases were reclassified as clear cell sarcoma of the kidney. As a result 138 nephroblastoma cases formed the study cohort. All cases selected had a minimum of 20 paraffin embedded tissue blocks available but in many cases there were limited number of tumour blocks with viable tissue. Patients' clinical records were available from the Department of Paediatric Surgery.

Immunohistochemical expression and the results of the microsatellite analysis were correlated with clinicopathological features using statistical methods described later. The clinicopathological data available for analysis included, age, gender, race/ethnic group, use of preoperative chemotherapy, disease stage, SIOP histological classification, SIOP risk group, and patient outcome at last follow up.

3.2.1 Treatment groups

Patients were treated according to two main treatment options.

- Administration of chemotherapy before nephrectomy (Treatment group A for the immunohistochemistry study and A1 for the microsatellite study)
- Primary nephrectomy, no preoperative chemotherapy (Treatment group B for the immunohistochemistry study and B1 for the microsatellite study)

3.3 TISSUE PROCESSING

All tissues were fixed in 10% buffered formalin, followed by routine processing in the Shandon Hypercentre. The practice was for the pathology laboratory to prepare and supply fixative to the operating theatre in the academic hospital, King Edward VIII hospital, therefore it can be confirmed that the fixation method was constant during the study period.

3.4 HISTOLOGICAL REVIEW

All haematoxylin and eosin (H & E) stained slides were retrieved from the archives. Slides that were faded, broken or lost were recut from stored paraffin wax embedded tissue blocks. Routine H & E stained sections were then reviewed by light microscopy. The histopathological diagnosis, tumour classification, risk classification and staging criteria were reviewed. Appropriate tissue blocks were selected for the immunohistochemical study. All slides were reviewed under the supervision of Professor R Chetty.

3.4.1 Histopathological Diagnosis and Classification

Nephroblastomas are characterised by extreme histological diversity, which sometimes leads to diagnostic problems. There is not only diversity of cell types and tissue patterns but also varying degrees of cellular differentiation. The classical triphasic nephroblastoma is composed of three tissue elements, all found in the developing kidney: blastema, epithelium, and stroma (Plate 1a). The blastemal cells are polygonal in shape with scanty cytoplasm and round to oval nuclei. They may be arranged in diffuse, nodular, serpentine (Plate 1c) and/or basaloid patterns. The epithelial component is composed mainly of nephrogenic tubules (Plate 1b) and glomeruloid structures in varying stages of maturation. The stroma is usually a spindle cell fibrous or myxoid stroma but differentiated elements may be present (Plate 1d). The proportion of these three primary elements may differ in a single tumour and between tumours.

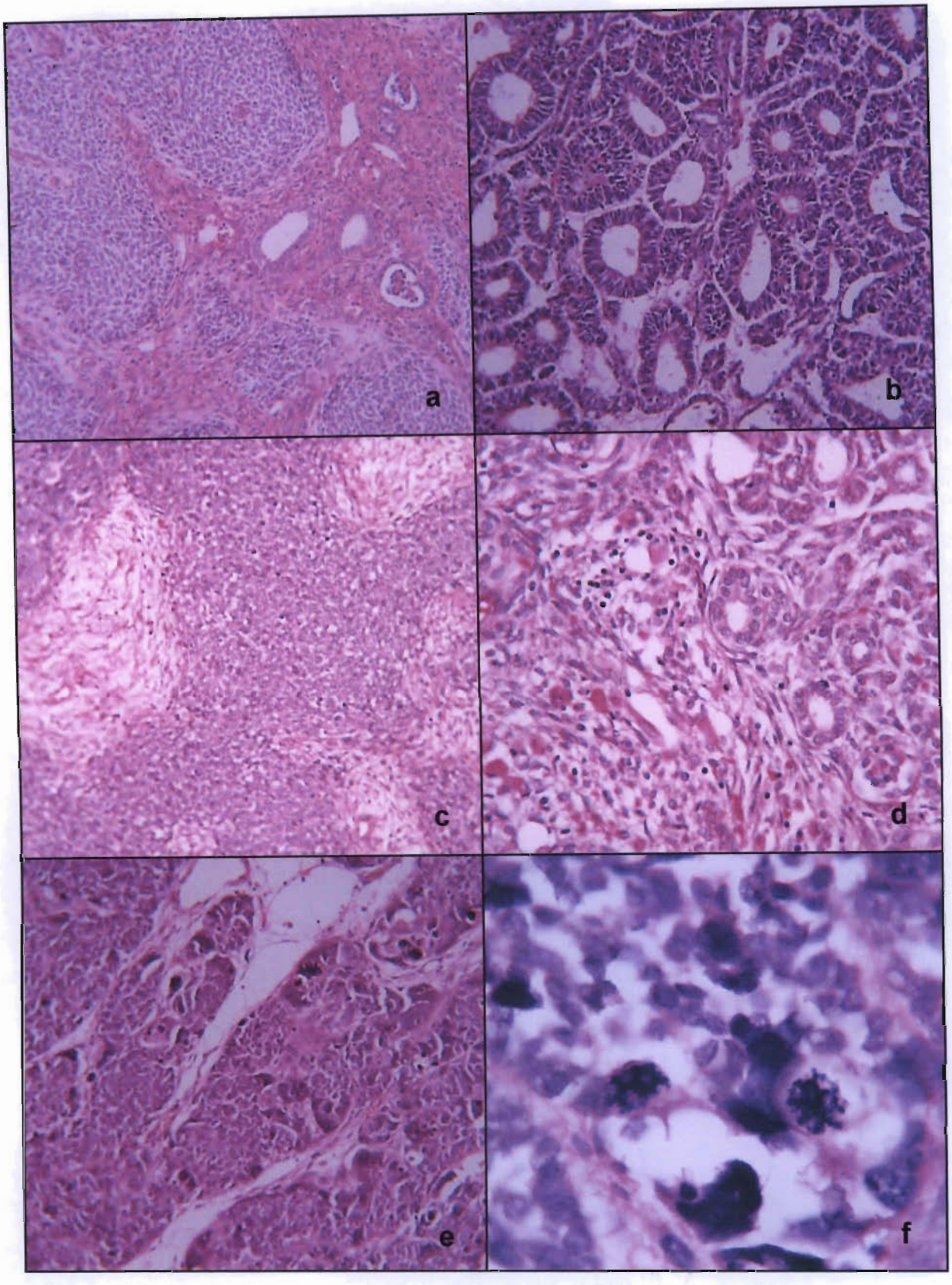


Plate 1: Histological features of nephroblastoma, **a**. Mixed nephroblastoma composed of blastema, epithelium and stroma, **b**. epithelial lined tubules in an epithelial nephroblastoma, **c**. interconnecting broad sheets of blastema surrounded by stroma, **d**. differentiated stroma consisting of striated skeletal muscle fibres; note focal well differentiated tubules, **e**. anaplastic blastemal cells with enlarged, hyperchromatic nuclei and abnormal mitoses, and **f**. high power showing anaplastic nuclei and multipolar mitotic figures.

The tumours were classified according to the Revised SIOP working classification of renal tumours of childhood.[502] Anaplasia was diagnosed when all three histological criteria were present. These were marked nuclear enlargement of stromal (except skeletal muscle), epithelial or blastemal cells to at least three times the diameter of adjacent nuclei of the same cell type, ie. a threefold enlargement of the nucleus in two perpendicular axes; hyperchromatism of enlarged nuclei, and multipolar mitotic figures (Plate 1e,f).[160,162] Anaplasia was further subclassified as focal or diffuse according to the recent criteria.[162,502]

The Revised S.I.O.P. Working Classification of Renal tumours of childhood (2001)[502]

A. For pretreated cases

Low risk tumours

Mesoblastic nephroma

Cystic partially differentiated nephroblastoma

Completely necrotic nephroblastoma

Intermediate risk tumours

Nephroblastoma - epithelial type

Nephroblastoma - stromal type

Nephroblastoma - mixed type

Nephroblastoma - regressive type

Nephroblastoma - focal anaplasia

High risk tumours

Nephroblastoma - blastemal type

Nephroblastoma - diffuse anaplasia

Clear cell sarcoma of the kidney

Rhabdoid tumour of the kidney

B. For primary nephrectomy cases

Low risk tumours

Mesoblastic nephroma

Cystic partially differentiated nephroblastoma

Intermediate risk tumours

Non-anaplastic nephroblastoma and its variants

Nephroblastoma - focal anaplasia

High risk tumours

Nephroblastoma – diffuse anaplasia

Clear cell sarcoma of the kidney

Rhabdoid tumour of the kidney

Tumours in italic type are not nephroblastomas and are considered to be distinct entities and were excluded from the study. Cases with complete necrosis were excluded from the study since there was no viable tumour tissue for immunohistochemical and molecular analysis.

3.4.2 Clinicopathological Staging

Histopathological features for staging were reviewed according to the following staging criteria (SIOP).[502]

Stage I

a) The tumour is limited to kidney or surrounded with a fibrous pseudocapsule if outside of the normal contours of the kidney. The renal capsule or pseudocapsule may be infiltrated with the tumour but it does not reach the outer surface, and it is completely resected (resection margins 'clear')
b) The tumour may be protruding ('bulging') into the pelvic system and 'dipping' into the ureter (but it is not infiltrating their walls)
c) The vessels of the renal sinus are not involved
d) Intrarenal vessel involvement may be present
(Fine needle aspiration or percutaneous core biopsy do not upstage the tumour. The presence of necrotic tumour or chemotherapy induced changes in the renal sinus/hilar fat and /or outside the kidney does not upstage the tumour.)

Stage II

a) The tumour extends beyond kidney or penetrates through the renal capsule and/or fibrous pseudocapsule into peri-renal fat but is completely resected (resection margins 'clear')

b) Tumour infiltrates the renal sinus and/or invades blood and lymphatic vessels outside the renal parenchyma but it is completely resected

c) Tumour infiltrates adjacent organs or vena cava but is completely resected

Stage III

a) Incomplete excision of the tumour which extends beyond resection margins. (gross or microscopical tumour remains post-operatively)

b) Any abdominal lymph nodes are involved

c) Tumour rupture before or intra-operatively (irrespective of other criteria for staging)

d) The tumour has penetrated through the peritoneal surface

e) Tumour implants are found on the peritoneal surface

f) The tumour thrombi present at resection margins of vessels or ureter, transected or removed piecemeal by surgeon

g) The tumour has been surgically biopsied (wedge biopsy) prior to pre-operative chemotherapy or surgery.
(The presence of necrotic tumour or chemotherapy induced changes in a lymph node or at the resection margins should be regarded as stage III.)

Stage IV

Haematogeneous metastases (lung, liver, bone, brain, etc.) or lymph node metastases outside the abdomino-pelvic region.

Stage V

Bilateral renal tumours at diagnosis. Each side should be substaged according to the above classifications.

3.5 IMMUNOHISTOCHEMICAL STUDY

3.5.1 Slide Preparation

- 2µm tissue sections were cut from paraffin wax embedded tissue blocks.
- Sections were floated on a water-bath and picked up on poly-L-lysine (Sigma Diagnostics, St Louis, USA) coated glass slides (Appendix 1).
- Slides were placed on a hot-plate at 60°C for 10 minutes.

- Sections were dewaxed in two in 2 changes of xylene, followed by immersion in decreasing concentrations of ethanol and finally washed in tap water.

3.5.2 Antigen Retrieval

3.5.2.1 Antigen retrieval solution – 0.01 M citrate buffer (pH 6)

- 2.46g of tri-sodium citrate ($C_6H_5O_7Na_3$) were dissolved in 950ml of distilled water in a 1000ml measuring cylinder.
- 400µl of concentrated HCl was added to this solution.
- The solution was topped up with distilled water to 1000ml mark and mixed.
- Since it was vitally important that the pH of this solution remained at pH 6 at all times, the pH of the solution was checked prior to commencing staining, and titrated with NaOH or HCl as required.

3.5.2.2 Heat induced epitope retrieval (HIER) techniques

3.5.2.2.1 Microwave technique

- Slides were placed in a thermoresistant plastic coplin jar, filled with 0,01 M sodium citrate solution at pH 6, and incubated at 85°C for 10 minutes in the H2500 Microwave Processor (Energy Beam Sciences, Inc, Agawam, Massachusetts).
- The slides were allowed to cool down in the coplin jar for approximately 5 – 10 minutes.
- When cooled, then staining procedure was continued.

3.5.2.2.2 Pressure cooking technique

- 2 litres of 0.01 M sodium citrate solution were poured into the pressure cooker and heated on a hot-plate. The lid was not locked.
- The slides were stacked in a clean stainless steel rack, which was immersed into the pressure cooker ensuring all slides were completely immersed in buffer and the lid was locked.
- When maximum pressure was attained (indicated by the pressure indicator valve), the timer was set for 1 minute.
- The pressure cooker was then removed from the hot-plate and placed under running cold water.

- When the pressure subsided completely (indicated by the pressure indicator valve), the lid was gently opened.
- The slides were allowed to cool in the pressure cooker with tap water.
- When cooled, after approximately 15-30 minutes, the staining procedure was continued.

3.5.3 Primary antibodies

The details of the primary antibodies are shown in Table 3.

Table 3: Details of Primary Antibodies

Primary Antibody	Clone	Dilution	Supplier	Antigen retrieval	Kit used
p21 IgG1, kappa	SX118 Mouse MAb	1:25	Dako, Copenhagen, Denmark	Citrate buffer Pressure cooker	EnVision™, Dako
p53 IgG2b, kappa	DO-7 Mouse MAb	1:100	Dako, Copenhagen, Denmark	Citrate buffer Microwave	LSAB, Dako
bcl-2 oncoprotein IgG1, kappa	124 Mouse MAb	1:55	Dako, Copenhagen, Denmark	Citrate buffer Microwave	LSAB, Dako
Retinoblastoma gene product IgG1, kappa	Rb1 Mouse MAb	1:50	Dako, Copenhagen, Denmark	Citrate buffer Microwave	CSA, Dako
Cyclin A IgG1, kappa	6E6 Mouse MAb	1:50	Novocastra	Citrate buffer Microwave	CSA, Dako
p-glycoprotein IgG 2a	C219 Mouse MAb	1:20	Signet Laboratories, Dedham, MA.	Citrate buffer Microwave	LSAB, Dako

MAb: Monoclonal antibody

3.5.3.1 Controls

Positive controls (Table 4) and negative reagent controls were run simultaneously on all batches of immunohistochemical stains. In the negative reagent controls the primary antibody was omitted and substituted with phosphate buffered saline.

Table 4: Positive controls for each primary antibody

Primary antibody	Positive control
p21	Tonsil
p53	Colonic adenocarcinoma
bcl-2	Tonsil
pRb	Normal retina
Cyclin A	Tonsil
p-glycoprotein	Liver (bile canaliculi)

3.5.4 Reagents and Kits for immunohistochemical stains

3.5.4.1 Phosphate buffered saline (PBS), pH 7.4

- 10 PBS tablets (Dako, Copenhagen, Denmark) were dissolved in 1 litre of distilled water.
- The solution was stored at room temperature and shaken before use.
- The pH was checked before use.

3.5.4.2 Bovine serum albumin (BSA)

- 100ml bovine serum albumin (Delta Bioproducts, Johannesburg, South Africa) aliquoted into 5ml tubes and stored at –20°C.

3.5.4.3 3% Hydrogen peroxide

- 3ml of liquid hydrogen peroxide assay (Associated Chemical Enterprises Ltd, Southdale) was added to 97ml of distilled water.
- Stored at 4°C.

3.5.4.4 Mayer's haematoxylin

- In a 2 litre conical flask 1g of haematoxylin (Merck Chemicals) was dissolved in approximately 950ml of distilled water.
- 50g of aluminium ammonium sulphate (Merck Lab. Supplies, Gauteng, RSA), 0.2g of sodium iodate (BDH Chemicals, England, UK), 1g citric acid (Saarchem Halpro Analytic, Krugersdorp, RSA) and 50g chloral hydrate (Associated Chemical Enterprises Ltd., Southdale) were added.
- The solution was mixed well and topped up to 2 litres, and left overnight to dissolve.

- The following morning, the solution was boiled for 10 minutes and then left to cool.
- The solution was filtered and stored at room temperature.

3.5.4.5 37 mM Ammonium hydroxide (Ammoniated Water)

- 2.5ml of 15M ammonium hydroxide solution was diluted in distilled water to a total volume of 1000ml.
- Stored at room temperature.

3.5.4.6 Kits for immunohistochemical stains

See Appendix 2

3.5.5 Immunohistochemical method

3.5.5.1 LSAB staining method

- Following microwave antigen retrieval, the slides were washed gently in water for 2 minutes.
- Slides were then transferred to a coplin jar of PBS at pH 7,4 to prevent slides from drying.
- Slides were transferred to a coplin jar of 3% H_2O_2 for 5 minutes at room temperature in order to block endogenous peroxidase activity.
- Slides were then washed in three changes of PBS and the excess PBS was removed.
- A circle was drawn around the tissue section with a Dako pen (Dako A/V, Copenhagen, Denmark) to create a well for the reagents.
- Approximately 200µl of diluted primary antibody (p53, bcl-2, p-glycoprotein) was applied to the sections, ensuring that the sections were covered by the reagent and incubated for 1 hour at room temperature. The antibodies were diluted immediately prior to use.
- Slides were rinsed well in three changes of PBS and excess PBS was removed. Sections were incubated in 1 to 3 drops of Biotinylated Link reagent for 10 minutes at room temperature.
- Slides were rinsed in 3 changes of PBS and excess PBS was removed.
- Sections were incubated in 1 to 3 drops of labelling Streptavidin-HRP reagent for 10 minutes at room temperature.

- Slides were rinsed in 3 changes of PBS and the excess was removed.
- Sections were then incubated in 1 to 3 drops of prepared 3, 3-diaminobenzidine solution for approximately 5 minutes at room temperature.
- Slides were rinsed well in running tap water for 3 minutes.
- Sections were counterstained with Mayer's haematoxylin for 1-3 minutes.
- Slides were then rinsed in tap water.
- Ammoniated water was used to develop the haematoxylin counterstain.
- Slides were rinsed in running tap water.
- Slides were dehydrated in one change of 95% ethanol and then two changes of 100% ethanol, followed by two changes of xylene.
- Finally, the slides were permanently mounted using DPX.

3.5.5.2 Catalyzed Signal Amplification (CSA) staining method

- Following microwave antigen retrieval, the slides were allowed to cool and washed well in water.
- Slides were transferred to a coplin jar of PBS to prevent drying of sections.
- Slides were incubated in 3% H_2O_2 for 5 minutes in a coplin jar at room temperature.
- Slides were washed in three changes of PBS, and excess PBS was removed.
- A circle was drawn around the tissue section with a Dako pen (Dako A/V, Copenhagen, Denmark) to create a well for the reagents.
- Protein Block (1–3 drops) was added to each slide and allowed to incubate for 5 minutes. Protein block was tapped off slides.
- Diluted primary antibody (cyclin A, pRb) (approximately 200 µl) was added to sections and allowed to incubate for 1 hour at room temperature. The antibodies were diluted immediately prior to use.
- The sections were then washed well in PBS and the excess removed.
- Link antibody (1–3 drops) was added to each slide and allowed to incubate for 15 minutes at room temperature.
- Slides were washed in PBS, and the excess was removed.
- Sections were incubated in 1-3 drops of Streptavidin-Biotin Complex, which was prepared 30 minutes prior to use, for 15 minutes at room temperature.
- Sections were washed in PBS, and the excess removed.

- Sections were then incubated in 1-3 drops of Amplification Reagent for 15 minutes at room temperature.
- Slides were washed in 3 changes of PBS, and the excess removed.
- Sections were incubated in 1-3 drops of Streptavidin-Peroxidase for 15 minutes at room temperature.
- Slides washed well in PBS, and the excess removed.
- Sections were incubated in 1-3 drops of Substrate-Chromogen Solution for approximately 5 minutes at room temperature
- Slides were examined microscopically for chromogen staining.
- Slides were washed in tap water.
- Sections were counterstained in Mayers haematoxylin for 1-3 minutes.
- Slides were washed in tap water.
- Ammoniated water was used to develop the counterstain.
- Slides were washed gently in tap water again.
- Slides were then dehydrated through one change of 95% ethanol, two changes of 100% ethanol and two changes of xylene.
- Finally, the slides were mounted using DPX.

3.5.5.3 EnVision™ Method

- Following microwave antigen retrieval, the slides were washed gently in water for 2 minutes.
- Slides were transferred to a coplin jar of PBS to prevent drying of sections.
- Slides were incubated in 3 % hydrogen peroxide for 5 minutes at room temperature in a coplin jar.
- Slides were washed in three changes of PBS, and excess PBS was removed.
- The excess PBS was removed from the slide and a circle was drawn around the tissue section with a Dako pen (Dako A/V, Copenhagen, Denmark) to create a well for the reagents.
- Diluted primary antibody (p21) (approximately 200µl) was added to sections and allowed to incubate for 1 hour at room temperature. The antibody was diluted immediately prior to use.
- The sections were then washed well in PBS and the excess removed.
- Sections were incubated in Dako EnVision™ Peroxidase for 30 minutes at room temperature.

- The sections were then washed well in PBS and the excess removed.
- Sections were incubated in Substrate-Chromogen solution for approximately 5 minutes at room temperature.
- Slides were examined microscopically for chromogen staining.
- Slides were washed in water.
- Sections were counterstained in Mayers haematoxylin for 1-3 minutes.
- Slides were washed in tap water.
- Ammoniated water was used to develop the counterstain.
- Slides were washed gently in tap water again.
- Slides were then dehydrated through one change of 95% ethanol, two changes of 100% ethanol and two changes of xylene.
- Finally, the slides were mounted using DPX.

3.5.6 Immunohistochemical Assessment

For p53, p21, pRb and cyclin A, only crisp nuclear staining was accepted as positive. For bcl-2 cytoplasmic staining was accepted as positive and for p-glycoprotein cell membrane staining or accentuation was mandatory for acceptance as positive.

The quantity of staining was graded as follows:

<5% positive nuclei	0
6-25% positive nuclei	1+
26-50% positive nuclei	2+
51-75% positive nuclei:	3+
>75% positive nuclei:	4+

The above scoring system has been used in other studies.[341,343] A score of 0 and 1+ (≤25%) was regarded as low expression and a score of 2+, 3+ and 4+ (>25%) as high expression. The number of tumour cells that were immunopositive was expressed as a percentage of the total number of tumour cells per high power field (HPF). Fifty HPFs were counted per slide and the final percentage was an average of the 50 HPFs. The quantitation was performed manually using an Olympus BH2 microscope.

3.6 MICROSATELLITE STUDY

3.6.1 Reagents for DNA extraction

3.6.1.1 TE buffer (10mM Tris, 1mM EDTA) pH 7.4

- 0.16 g Tris-HCl and 0.04 g EDTA were added to a 100ml Schott bottle containing 95ml of sterile water.
- The solution was mixed well and topped up to 100ml with sterile water.
- The pH was checked and the solution stored at room temperature.

3.6.1.2 Proteinase K (20mg/ml)

- 100mg of proteinase K (Roche Diagnostics, Mannheim, Germany) was dissolved in 5ml of sterile water in a sterile 10ml disposable tube.
- 200µl were aliquoted into smaller tubes and stored at –20°C.

3.6.1.3 Phenol-choloroform-isoamyl alcohol

- Saturated phenol (25ml) was added to 25ml of chloroform in a sterile dark bottle.
- 330µl isoamyl alcohol was added.
- The solution was mixed and allowed to clear.
- This was overlaid with 5ml of TE buffer and stored at 4°C.

3.6.1.4 Lysis buffer

- To approximately 80 ml of distilled water in a clean 100ml Schott bottle, the following were added:

  1ml 1M Tris-HCL (pH 7.5)
  1ml 0.5M EDTA (pH 8)
  8ml 5M NaCl.

- The solution was topped up to 100ml with distilled water and stored at 4°C.

3.6.1.5 3M Sodium acetate

- Sodium acetate (12.3g) was dissolved in 40ml of distilled water in a 100ml Schott bottle.
- Glacial acetic acid (7.5ml) was added and the solution topped up to 50ml with distilled water.
- The solution was autoclaved and stored at room temperature.

3.6.1.6 Ethanol (80%)

- 60ml of sterile distilled water was added to 240ml absolute ethanol (Merck Chemicals). The solution was mixed and stored at -20°C.

3.6.1.7 Glycogen (20mg/ml)

- 200mg of Glycogen Type II (Sigma-Aldrich, St Louis, Missouri, USA) was dissolved in 10ml of sterile water.
- 200µl aliquots were made and stored at –20°C.

3.6.2 Tissue preparation for DNA extraction

During histological review of the H & E stained slides, appropriate blocks with adequate viable neoplastic tissue and blocks with normal tissue from each case were selected for DNA extraction. Normal tissue was either kidney tissue or lymph node without metastases. In slides that did not contain 100% neoplastic tissue a minimum of 80% viable neoplastic tissue was present. A number of cases were excluded from the molecular study because of insufficient viable tumour tissue per block.

Once the slides were selected the corresponding paraffin wax embedded tissue block was re-cut. Five sections were cut from each block on a microtome with a sterile disposable blade. The first and last section were cut at 2µm and stained with H & E. The viable neoplastic tissue or normal tissue was demarcated with a Sharpie pen on the H & E stained glass slides. The second, third and fourth sections were cut at 6µm. They were picked up on glass slides, and baked on a hot-plate at 60°C.

The marked H & E slides were superimposed on the unstained slide and marked out on the reverse side using a Sharpie pen. Normal or neoplastic tissue was then scraped away from the slide using a sterile scalpel blade and transferred into separate prelabelled 1.5ml eppendorf tubes.

3.6.3 DNA extraction method

- 500µl of warm xylene was added to 1.5ml eppendorf tubes containing tissue scrapings.

- The tubes were vortexed and incubated at 65°C for 10 minutes.
- The tubes were then centrifuged for 10 minutes at 10 000 rpm and the supernatant was discarded.
- The above steps were repeated twice.
- 500ml of ethanol was added and allowed to dry on a heating block.
- 40µl of proteinase K and 360µl of lysis buffer were added to each tube.
- The tubes were sealed with parafilm, vortexed and incubated at 55°C for 48 hours. The tubes were shaken intermittently.
- Following incubation, the tubes were heated at 95°C for 20 minutes on a heat block to inactivate the proteinase K.
- 400µl of phenol-chloroform-isoamyl alcohol was added, and the tubes were vortexed briefly.
- The tubes were centrifuged at 12 000 rpm for 5 minutes to separate the two phases.
- The supernatant (upper aqueous phase) was transferred to a correspondingly labelled, new, sterile, eppendorf tube. The remaining phenol-chloroform mixture was disposed in a waste bottle.
- 1µl glycogen, 40µl 3M sodium acetate and 800µl cold absolute ethanol (-20°C) were added to each new eppendorf tube containing the supernatant.
- The tubes were placed in a deep freeze (-20°C) for 1 hour.
- The tubes were then centrifuged at 14 000 rpm for 20 minutes and the supernatant carefully removed without disturbing the pellet (DNA) at the bottom.
- The pellet was washed with 75µl of 80% ethanol (-20°C), by centrifuging at 12 000 rpm for 3 minutes.
- The supernatant was removed and the pellet allowed to air-dry.
- The dry pellet was resuspended in 60µl of sterile distilled water, and stored at –20°C.

In cases where DNA extraction failed, extraction was repeated. If after 3 attempts amplifiable DNA was not obtained the case was excluded.

3.6.4 Assay for DNA concentration and purity

DNA concentration was determined using ultraviolet spectrophotometry.[503]

- The quartz cuvette was washed in distilled water and dried.
- 100µl of blank solution (distilled water) was transferred to the cuvette.
- The cuvette was inserted and the ultraviolet spectrophotometer (Gene Quant II DNA/RNA calculator, Pharmacia Biotech) calibrated at 260nm and 280nm against the distilled water blank.
- The cuvette was removed, rinsed thoroughly with distilled water and dried.
- 2µl of DNA sample was mixed with 98µl of distilled water, giving a dilution of 1:50.
- The test solution was transferred into an acid washed quartz cuvette.
- The cuvette was inserted and the OD_{260}, OD_{280}, OD_{260}/OD_{280} ratio, and DNA concentration were recorded.
- The concentration was determined by reading at 260nm where the estimate of 50µg/ml is equal to an absorbance of 1.
- An OD_{260}/OD_{280} ratio of greater than 1.8 denoted DNA of good purity.[504]

3.6.5 PCR using the insulin primers

In order to establish whether the DNA extracted from the paraffin-wax embedded tissues was amplifiable, PCR was carried out on all extracted DNA, both normal and tumour DNA, using primers for the *insulin* gene.

3.6.5.1 Insulin primers

The insulin primers were synthesised by the Department of Biochemistry, University of Cape Town, South Africa. The oligonucleotide sequences for the primers to exon 2 region of the insulin gene were as follows:

(F) 5' ACC CAG ATC ACT GTC CTT CTG CC 3'

(R) 5' AGG GGC AGC AAT GGG CGG TTG 3'

The expected PCR product size was 236 base pairs.

3.6.5.2 Reagents

The PCR was carried out using the PCR Core Kit (Roche Diagnostics, Mannheim, Germany) (Appendix 3).

3.6.5.3 Insulin PCR

The master mix for the insulin PCR is shown in Table 5.

Table 5: Preparation of master mix for insulin PCR (Total PCR volume = 25µl)

Reagents	Volume	Concentration
Sterile water	17.85 µl/sample	
PCR reaction buffer, including 1.5mM $MgCl_2$	2.50 µl/sample	10x
dNTP	0.50 µl/sample	10mM
Forward primer	1.00 µl/sample	10pmol
Reverse primer	1.00 µl/sample	10pmol
Taq DNA polymerase	0.15 µl/sample	5U/µl

23µl of the master mix was transferred to each 200µl reaction tube and the tubes were held on ice. 2µl of template DNA was added to the tube to make a total reaction volume of 25µl. A control DNA sample was also included. The tubes were held at 4°C until they were loaded into the thermocycler. The PCR conditions are shown in Table 6.

Table 6: Insulin PCR conditions (Techne Progene Thermocycler)

Step	Temperature	Time	Cycles
Denaturation	94°C	5 min	1
Denaturation	94°C	1 min	30
Annealing	64°C	1 min	30
Extension	72°C	2 min	30
Final extension	72°C	10 min	1

The PCR products were then analysed on a 2% agarose gel.

3.6.5.4 Reagents for agarose gel electrophoresis

3.6.5.4.1 TBE buffer (10x)

- 108g Tris base, 55g boric acid and 9.3g EDTA were dissolved in 900 ml of distilled water using a magnetic stirrer.
- The solution was topped up to 1 litre, filtered and stored at room temperature.

TBE buffer (1x)

- 100 ml of 10x TBE was added to 900 ml of distilled water.
- Stored at room temperature.

3.6.5.4.2 Ethidium bromide

- Ethidium bromide (10mg) was added to 1ml of sterile water.
- The mixture was vortexed, covered with aluminium foil and stored at room temperature.

3.6.5.4.3 Bromophenol blue loading dye (1ml)

- Bromophenol blue (0.02%), xylene cyanol (0.02%), 500µl glycerol (50%) and 500µl sterile water were mixed and stored at room temperature.

3.6.5.5 Preparation of 2% Agarose gel

- Agarose (1.4g) was added to 70ml of 1x TBE buffer in a glass beaker.
- The mixture was heated in a microwave oven until completely dissolved, approximately 90 seconds.
- When cooled to approximately 50°C, 3µl of ethidium bromide was added.
- The combs were positioned into the grooves of the tray approximately 10mm from one end of the tray.
- The gel was carefully poured into casting tray and bubbles were removed.
- The gel was allowed to polymerise for 30-45 minutes at room temperature.

3.6.5.6 Preparation of samples

- 2µl of bromophenol blue loading dye was added to 15µl of insulin PCR product.

3.6.5.7 Preparation of molecular weight marker

- 1µl of the 100 base pair ladder molecular weight marker (Roche Diagnostics, Mannheim, Germany) was added to 8µl of sterile water and 2µl of bromophenol blue loading dye.

3.6.5.8 Procedure

Once this gel was completely polymerised, the comb was gently removed and the gel was placed into the electrophoretic tank. TBE buffer (1x) was added to the

buffer chamber until the gel was completely submerged. The DNA samples and the molecular weight marker were carefully loaded into the wells. Electrophoresis was carried out at 80V for 1 hour at room temperature. After the electrophoretic run, the gel was visualised using a UV transilluminator.

3.6.5.9 Photography of the gel

The gel was then scanned using the Syngene gel capture system (Vacutec). The appropriate light and contrast adjustment was made using Genesnap software and the picture was either printed and/or saved onto a disk.

3.6.6 Microsatellite PCR

3.6.6.1 PCR Core Kit (See Appendix 3)

3.6.6.2 Cy5 labelled microsatellite primers

Primer sequencers for 11p and 16q were obtained from Gyapay *et al.*[490] and confirmed on the GDB Human Genome Database website (http://gdbwww.gdb.org).

Six polymorphic microsatellite markers were selected from the short arm of chromosome 11, focussing on 11p13 and 11p15. These markers, each with heterozygosity of over 70%, were selected from linkage maps published by Gyapay *et al.*[490] and Weissenbach *et al.*[505] Three microsatellite markers were selected for the 11p13 region (D11S904, D11S907 and D11S935), one for 11p15 (D11S922) and two markers covered the 11p13 to 11p15 region (D11S902 and D11S930). D11S922 was used as a 11p15 marker and D11S907 was used as a 11p13 marker in previous studies.[138,506] Five polymorphic microsatellite markers from 16q were selected, covering the region from 16q12.1 to 16qter. These markers, each with heterozygosity of over 70%, were selected from linkage maps published by Gyapay *et al.*[490] One microsatellite marker was selected for the 16q12.1 region (D161S411), two for 16q21-22 (D16S496 and D16S503) and two markers covered the 16q24-qter region (D16S413 and D16S520). D16S413, D16S503 and D16S520 were used to investigate LOH of 16q in previous studies.[14,131,134] Six polymorphic microsatellite markers for the mismatch repair loci were selected. Three of these markers, bat25, bat26 and D2S123, are in the recommended panel for assessment of MSI in colorectal carcinoma.[17,479]

The forward primer (F) of all primer pairs for 11p (Figure 5 & Table 7), 16q (Figure 6 & Table 8) and mismatch repair genes (Table 9) were labelled with Cy5. The primers were made and supplied by Roche Diagnostics, Penzberg, Germany. The recommended amplification conditions were obtained from the "Dr Jean Weissenbach protocol" on the GDB Human Genome Database website (http://gdbwww.gdb.org). The markers were chosen for their high heterozygosity rates.

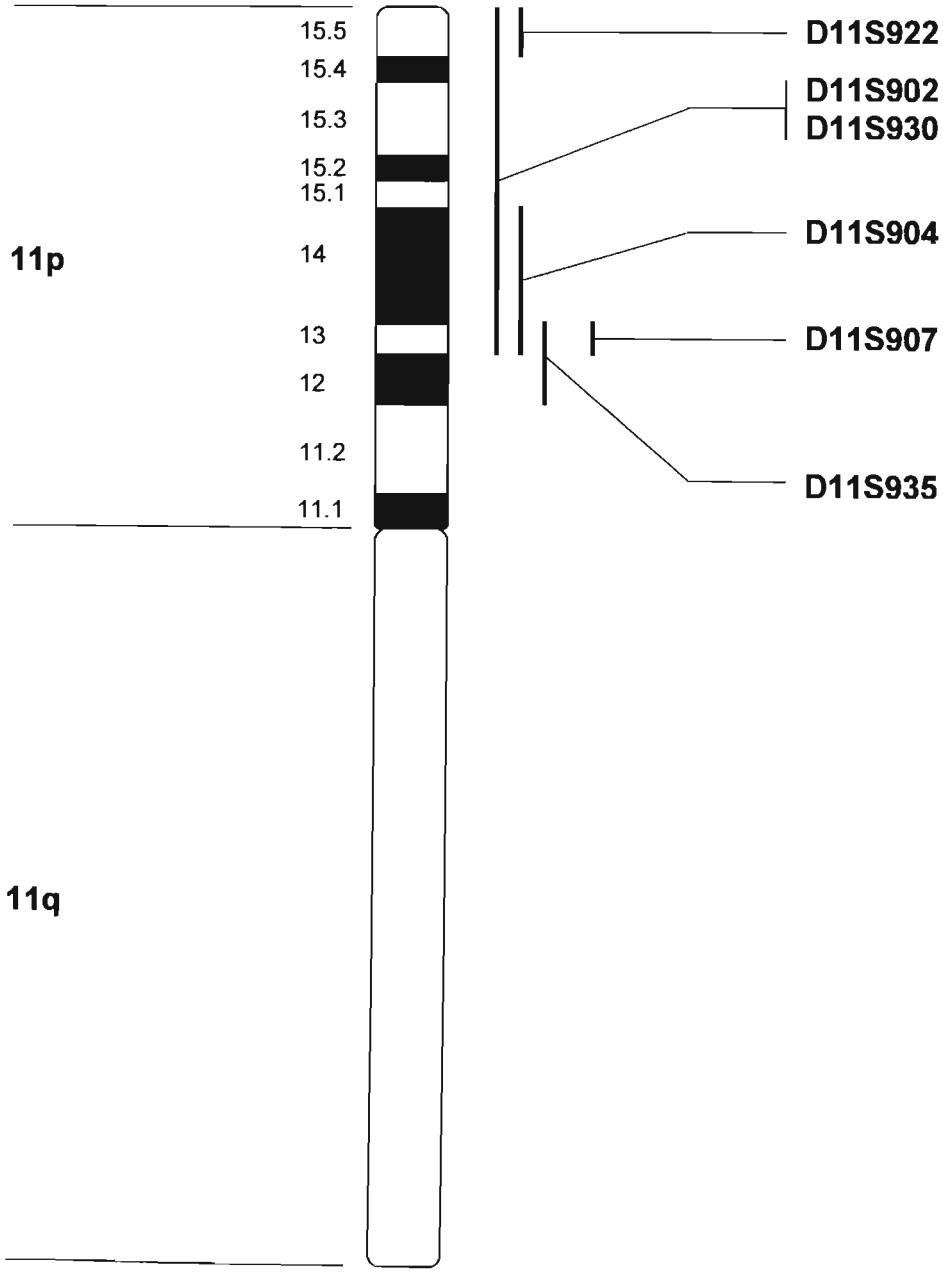


Figure 5: Ideogram of chromosome 11 showing location of 11p markers

Table 7: Details of the microsatellite markers for chromosome 11p

Microsatellite Marker	Primer name	Primer sequence	Microsatellite type	Physical localisation	Map position (cM)	Maximum Heterozygosity (%)	Product Size (bp)
D11S922	AFM217yb10a AFM217yb10m	(F) 5'-GGGGCATCTTTGGCTA-3' (R) 5'-TCCGGTTTGGTTCAGG-3'	Dinucleotide repeat	11p15.5 – 11p15.5	34.7	93.5	88 – 138
D11S930	AFM238xe5a AFM238xe5m	(F) 5'-AAAGAGATTGTGGATATGGC-3' (R) 5'-GTTTTCTGACACTGCTTCCT-3'	Dinucleotide repeat	11p15 – 11p13	41.0	68.4	225 – 237
D11S902	AFM072yd3a AFM072yd3m	(F) 5'-CCCGGCTGTGAATATACTTAATGC-3' (R) 5'-CCCAACAGCAATGGGAAGTT-3'	Dinucleotide repeat	11p15 – 11p13	41.0	80.5	145 – 163
D11S904	AFM081za5a AFM081za5m	(F) 5'-ATGACAAGCAATCCTTGAGC-3' (R) 5'-CTGTGTTATATCCCTAAAGTGGTGA-3'	Dinucleotide repeat	11p14 – 11p13	45.6	83.4	185 – 201
D11S907	AFM109ya1a AFM109ya1m	(F) 5'-GCTTATTGTCCATACCCAAA-3' (R) 5'-AAAGNACCTTAATTTCAGGC-3'	Dinucleotide repeat	11p13 – 11p13	47.8	73.5	163 –173
D11S935	AFM254zb9a AFM254zb9m	(F) 5'-TACTAACCAAAAGAGTTGGGG-3' (R) 5'-CTATCATTCAGAAAATGTTGGC-3'	Dinucleotide repeat	11p13 – 11p12	52.1	74.5	196 – 208

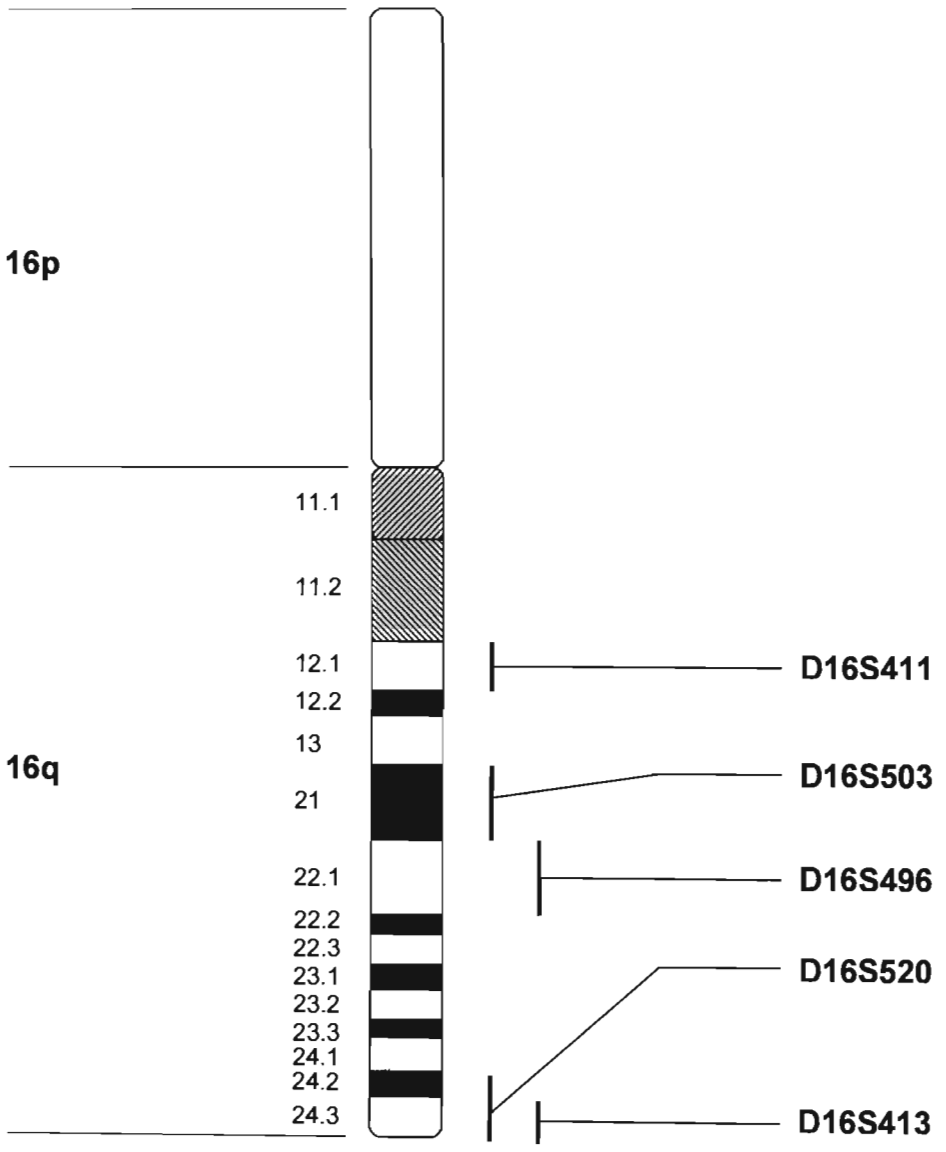


Figure 6: Ideogram of chromosome 16 showing location of 16q markers

Table 8: Details of the microsatellite markers for chromosome 16q

Microsatellite Marker	Primer name	Primer sequence	Microsatellite type	Physical localisation	Map position (cM)	Maximum Heterozygosity (%)	Product Size (bp)
D16S411	AFM186xa3a AFM186xa3m	(F) 5'-TCATCTCCAAAGGAGTTTCT-3' (R) 5'-GTGCATGTGTTCGTATCAAC-3'	Dinucleotide repeat	16q12.1 – 16q12.1	51.5	79.4	209 – 229
D16S503	AFM274ya5a AFM274ya5m	(F) 5'-AGTGCTCTGGAATGATGTG-3' (R) 5'-TTGCTAGGTAGTTGTCTCCC-3'	Dinucleotide repeat	16q21 – 16q21	59.6	81.0	232 (min)
D16S496	AFM214zg5a AFM214zg5m	(F) 5'-GAAAGGCTACTTCATAGATGGCAAT-3' (R) 5'-ATAAGCCACTGCGCCCAT-3'	Dinucleotide repeat	16q22.1 – 16q22.1	78.8	74.2	217 (min)
D16S520	AFMa135xg5a AFMa135xg5m	(F) 5'-GCTTAGTCATACGAGCGG-3' (R) 5'-TCCACAGCCATGTAAACC-3'	Dinucleotide repeat	16q24.2 – 16qter	77.1	84.0	181 – 197
D16S413	AFM196xg1a AFM196xg1m	(F) 5'-ACTCCAGCCCGAGTAA-3' (R) 5'-GGTCACAGGTGGGTTC-3'	Dinucleotide repeat	16q24.3 – 16q24.3	77.7	84.6	131 – 149

Table 9: Details of the microsatellite markers for mismatch repair genes

Microsatellite Marker	Primer name	Primer sequence	Microsatellite type	Physical localisation	Map position (cM)	Maximum Heterozygosity (%)	Product Size (bp)
Bat25	BAT-25.1 BAT-25.2	(F) 5'-TCGCCTCCA AGAATGTAAGT-3' (R) 5'-TCTGCATTTTAACTATGGCTC 3'	Mononucleotide	4q12 – 4q12	-	-	± 90
Bat26	BAT-26.1 BAT-26.2	(F) 5'-TGACTACTTTTGACTTCAGCC-3' (R) 5'-AACCATTCAACATTTTTAACCC-3'	Mononucleotide	2p16 – 2p16	-	-	130 - 160
Bat40	BAT-40.2.1 BAT-40.2.2	(F) 5'-ACAACCCTGCTTTTGTTCCT-3' (R) 5'-GTAGAGCAAGACCACCTTG-3'	Mononucleotide	1p13.1 – 1p13.1	-	-	130 - 160
D2S123	AFM093xh3a AFM093xh3m	(F) 5'-AAACAGGATGCCTGCCTTTA-3' (R) 5'-GAACTTTCCACCTATGGGAC-3'	Dinucleotide repeat	2p16 – 2p16	58.1	77.3	197 - 227
D3S659	D3S659.PCR1.1 D3S659.PCR1.2	(F) 5'-ATTCCAGGGACAAGTTCCCC-3' (R) 5'-CTGCAAGGTCTGTTTAACAG-3'	Dinucleotide repeat	3p13 – 3p13	74.6	73.0	103 - 140
D3S1255	R7K110F R7K110R	(F) 5'-CTCACTCATGAACACAGATGC-3' (R) 5'-AACCCATCTTGTATTCTTGCAG-3'	Dinucleotide repeat	3p25 – 3p24.2	31.5	86.0	145 - 168

3.6.6.3 Microsatellite PCR

Only cases with amplifiable normal and tumour DNA were selected for microsatellite PCR (n = 70). The polymerase chain reaction was carried out in 200µl thin-walled reaction tubes.

3.6.6.3.1 PCR for 11p markers

Initially the following PCR mix was used for all 11p primer pairs in a total reaction volume of 25µl (Table 10).

Table 10: Initial mix used for all 11p markers

Reagents	Volume	Conc
Sterile water	17.85 µl/sample	
PCR reaction buffer, including 1.5mM $MgCl_2$	2.50 µl/sample	10x
dNTP	0.50 µl/sample	10mM
Forward primer	1.00 µl/sample	10pmol
Reverse primer	1.00 µl/sample	10pmol
Taq DNA polymerase	0.15 µl/sample	5U/µl
Template DNA	2.00 µl/sample	

There were many samples that were not amplifiable with the initial protocol. $MgCl_2$ titrations were done to determine the optimal $MgCl_2$ concentration required for each primer pair (Table 11). Control DNA was used in each reaction.

Table 11: Reaction mix for $MgCl_2$ titration used for all 11p markers

Reagents	Volume					Concentration
	A	B	C	D	E	
Sterile water	16.85	15.85	14.85	13.85	12.85	
PCR buffer	2.5	2.5	2.5	2.5	2.5	10x
$MgCl_2$	1	2	3	4	5	25mM
dNTP	0.5	0.5	0.5	0.5	0.5	10rnM
Forward primer	1	1	1	1	1	10pmol
Reverse primer	1	1	1	1	1	10pmol
Taq DNA polymerase	0.15	0.15	0.15	0.15	0.15	5U/µl
Control DNA	2	2	2	2	2	

Following the magnesium titration, the following mixes were used for the 11p markers (Table 12).

Table 12: PCR mixes after $MgCl_2$ titrations for 11p markers

Reagents	**Volume**						**Conc.**
	S902	S904	S907	S922	S930	S935	
Sterile water	16.85	15.85	14.85	15.85	15.85	14.85	
PCR buffer	2.5	2.5	2.5	2.5	2.5	2.5	10x
$MgCl_2$	1	2	3	2	2	3	25mM
DNTP	0.5	0.5	0.5	0.5	0.5	0.5	10mM
Forward primer	1	1	1	1	1	1	10pmol
Reverse primer	1	1	1	1	1	1	10pmol
Taq DNA polymerase	0.15	0.15	0.15	0.15	0.15	0.15	5U/µl

All volumes in µl per sample.

23µl of the master mix was transferred to each 200µl reaction tube and held on ice. 2µl of template DNA was added to the tube to make a total reaction volume of 25µl. The tubes were held at 4°C until loaded onto the thermocycler set at the following conditions (Table 13)

PCR conditions

Amplification conditions were obtained from the Dr Jean Weissenbach protocol available on the GDB Human Genome Database website (http://gdbwww.gdb.org).

Table 13: PCR conditions for all 11p primer pairs

Step	**Temp (°C)**	**Time**	**Cycles**
Initial	96	5 min	1
Denaturation	96	1 min	30
Annealing	55	1 min	30
Extension	72	1 min	30
Final extension	72	10 min	1

The PCR products were held at 4°C until prepared for loading on the sequencer.

3.6.6.3.2 PCR for 16q markers

Initially the following mix was used for the 16q markers (Table 14).

Table 14: Initial mix for 16q markers

Reagents	Volume	Conc
Sterile water	17.85 µl/sample	
PCR reaction buffer, including 1.5mM $MgCl_2$	2.50 µl/sample	10x
DNTP	0.50 µl/sample	10mM
Forward primer	1.00 µl/sample	10pmol
Reverse primer	1.00 µl/sample	10pmol
Taq DNA polymerase	0.15 µl/sample	5U/µl
Template DNA	2.00 µl/sample	

There were many samples that were not amplifiable with the initial protocol. This was noted mainly with the D16S411, D16S413 and D16S496 primers. $MgCl_2$ titrations were done to determine the optimal $MgCl_2$ concentration required for each primer pair (Table 15). Control DNA was used in each reaction.

Table 15: Reaction mix for $MgCl_2$ titration for all 16q markers

Reagents	Volume					Concentration
	A	B	C	D	E	
Sterile water	16.85	15.85	14.85	13.85	12.85	
PCR buffer	2.5	2.5	2.5	2.5	2.5	10x
$MgCl_2$	1	2	3	4	5	25mM
DNTP	0.5	0.5	0.5	0.5	0.5	10mM
Forward primer	1	1	1	1	1	10pmol
Reverse primer	1	1	1	1	1	10pmol
Taq DNA polymerase	0.15	0.15	0.15	0.15	0.15	5U/µl
Control DNA	2	2	2	2	2	

Following the magnesium titration, the following mixes were used for the 16q markers (Table 16).

Table 16: PCR mix after $MgCl_2$ titrations for 16q markers

Reagents	Volume					Concentration
	S411	**S413**	**S496**	**S503**	**S520**	
Sterile water	12.85	13.85	14.85	15.85	15.85	
PCR buffer	2.5	2.5	2.5	2.5	2.5	10x
$MgCl_2$	5	4	3	2	2	25mM
DNTP	0.5	0.5	0.5	0.5	0.5	10mM
Forward primer	1	1	1	1	1	10pmol
Reverse primer	1	1	1	1	1	10pmol
Taq DNA polymerase	0.15	0.15	0.15	0.15	0.15	5U/µl

All volumes in µl per sample.

23µl of the master mix was transferred to each 200µl reaction tube and held on ice. 2µl of template DNA was added to the tube to make a total reaction volume of 25µl. The tubes were held at 4°C until loaded onto the thermocycler set at the following conditions (Table 17).

PCR conditions

Amplification conditions were obtained from the Dr Jean Weissenbach protocol available on the GDB Human Genome Database website (http://gdbwww.gdb.org).

Table 17: PCR conditions for all 16q markers (Techne Progene Thermocycler)

Step	Temp (°C)	Time	Cycles
Initial	96	5 min	1
Denaturation	96	1 min	30
Annealing	55	1 min	30
Extension	72	1 min	30
Final extension	72	10 min	1

The PCR products were stored at 4°C until preparation for loading onto the sequencer.

3.6.6.3.3 Mismatch repair gene markers

The PCR conditions and master mix for the mismatch repair gene markers (Table 18) were previously established in this laboratory.[507]

Table 18: PCR mix for mismatch repair gene markers

Reagents	Volume						Concentration
	Bat25	Bat26	Bat40	S123	S659	S1255	
Sterile water	16.85	15.85	14.85	15.85	15.85	14.85	
PCR buffer	2.5	2.5	2.5	2.5	2.5	2.5	10x
$MgCl_2$	1	2	3	2	2	3	25mM
DNTP	0.5	0.5	0.5	0.5	0.5	0.5	10mM
Forward primer	1	1	1	1	1	1	10pmol
Reverse primer	1	1	1	1	1	1	10pmol
Taq DNA polymerase	0.15	0.15	0.15	0.15	0.15	0.15	5U/µl

All volumes in µl per sample.

23µl of the master mix was transferred to each 200µl reaction tube and held on ice. 2µl of template DNA was added to the tube to make a total reaction volume of 25µl. The tubes were held at 4°C until loaded onto the thermocycler set at the following conditions (Table 19).

PCR conditions

Table 19: PCR conditions for MMR markers (Techne Progene Thermocycler)

Step	Temp (°C)	Time	Cycles
Denaturation	95	5 min	1
Denaturation	94	30 sec	35
Annealing	55	30 sec	35
Extension	72	40 sec	35
Final extension	72	10 min	1

The products of this reaction were stored at 4°C, before analysis on a sequencing gel.

3.6.6.4 Preparation of 6% Longranger sequencing gel

Firstly, a 10% ammonium persulphate (APS) solution was prepared by adding 1g of ammonium persulphate to 10ml of sterile water in a sterile disposable tube. Aliquots of 600µl were stored at –20°C.

To prepare the gel, 25.2 g of ultra-pure urea was weighed in a clean 250ml beaker to which 40ml of sterile water was added. This was dissolved on a magnetic stirrer at low speed. TBE buffer 10x (7.2ml) was added to the urea solution followed by 6ml of Longranger (FMC Bioproducts, Rockland, Maine, USA) gel. The volume was topped up to 60ml with sterile water and mixed well. The gel was divided into two, 30ml each, and stored at 4°C in foiled plastic bottles.

Just prior to preparing the sequencing gel, 300µl of APS (10%) and 30µl of TEMED were added to 30ml of the gel in a special pour bottle.

3.6.6.5 Preparation of gel plate (Short Thermoplate)

The short thermoplate (Pharmacia Biotech, Uppsala, Sweden) was used to cast the gel. Disposable gloves were used during this procedure. The plates, glass spacers (0.3mm) and the comb (0.3mm) thick were thoroughly cleaned with sterile water and 70% alcohol. All the parts of the gel plate were carefully examined under reflected light before assembly, to ensure there were no dust particles present, which may affect band migration and broadening during the electrophoretic run. This cleaning procedure was adopted because the ALFexpress DNA Sequencer is highly sensitive and capable of detecting trace contaminants. The spacers were carefully positioned along the indented edges on the right and left sides of the thermoplates, and gentle pressure was applied to secure them into the silicone-rubber seals. The glass coverplate was positioned over the thermoplate and bound together with clamps. The comb was positioned between the plates and the gel was cast from the lower edge. The gel solution was allowed to polymerise for approximately 90 minutes.

3.6.6.6 Preparation of samples

3µl of PCR product was mixed with 3µl of STOP solution (Blue dextran 2000, deionized formamide) (Pharmacia Biotech, Uppsala, Sweden) and held on ice. Just before loading the samples on the gel, the samples were denatured at 96°C for 3 minutes in a Techne Progene Thermocycler and held on ice until loading.

3.6.6.7 Preparation of size marker

A 50-500 base pair Cy5-labelled standard (Pharmacia Biotech, Uppsala, Sweden) was used as the external size marker. 1µl of marker was mixed with 3µl of STOP solution (Blue dextran 2000, deionized formamide) (Pharmacia Biotech, Uppsala, Sweden) and denatured at 96°C for 3 minutes in a Techne Progene Thermocycler.

3.6.6.8 Programming and Running Conditions for the ALFexpress DNA sequencer

The DNA sequencer was switched on and the running conditions, sample data and filename were entered using the ALF Manager Software (Pharmacia Biotech). The following running parameters were used for the microsatellite analysis:

Sampling interval	-	1 second
Voltage	-	1600 V
Power	-	25 W
Current	-	60 mA
Temperature	-	55°C
Running time	-	135 min

3.6.6.9 Attachment of the gel cassette/plate on to the ALFexpress DNA sequencer

The outer surfaces of the glass plates were cleaned, especially around the area of the detectors. The lower buffer chamber was placed in front of the instrument before attaching the gel cassette to the sequencer. The push-fit connectors of the sequencer's thermocirculator were attached to the slots on the thermoplate. One litre of TBE buffer (1x) was added to both the upper and lower buffer chambers. Once the temperature reached 45°C, the comb was removed and the wells cleaned thoroughly using a syringe with buffer. When the temperature reached 55°C the samples which were held on ice were loaded. Special elongated gel loading tips

were used to load 6µl of sample in each well. Normal and tumour DNA from each case were loaded in consecutive lanes on the gel.

3.6.6.10 Analysis of electrophoretograms

The peaks were visualised directly on the screen using the Fragment Manager software version 1.0 (Pharmacia Biotech, Uppsala, Sweden) and the results were recorded. Each run had a unique filename. Once the run was completed the file was stored on computer. The software detects the number of peaks, peak area and size in base pairs provided an external size marker is loaded.

3.6.6.10.1 Calculations of ratios for allelic imbalance

The presence of allelic imbalance was determined by calculating the allelic ratio (R) using the method suggested by Canzian *et al.*[508]

In the following ratio, T1 and T2 refer to the first and second peak on the tumour sample respectively, and N1 and N2 refer to the first and second peak in the normal sample respectively.

The $R = \frac{T2 \times N1}{T1 \times N2}$ case was regarded as showing allelic imbalance if:

$R < 0.6$, where the larger allele was lost, and

$R > 1.67$, where the smaller allele was lost.

3.6.6.10.2 Microsatellite instability

The detection of a new, novel allele in the tumour sample compared to the normal sample was interpreted as microsatellite instability (MSI).[509] A new peak within 2 bp of the original allele(s) was regarded as a "stutter band".[448]

3.7 STATISTICAL ANALYSIS

Test for association between patient characteristics and SIOP measures were done using the chi-square test and Fisher's Exact test for cross-tabulations with spares data.

Spearman correlations were calculated to test for associations between ordinal variables and a significance level of 5% was used throughout.

The mean age of different sub-groups were compared by means of analysis of variance.

Survival analysis was used to investigate the individual markers for association with time to death. To account for the observational nature of the sample the Cox regression model included confounders such as age, gender, cinicopathological stage and pre-operative treatment status. Each marker was evaluated independently in a model since the sample size of the study – especially the microsatellite sub-group has a very small sample size which could not accommodate an extensive model with all the markers or regions in one model.

Kaplan-Meier curves of the survival distribution within each of the levels of a marker or region were estimated to obtain a graphical representation of the time to death in each of the groups.

Patients lost to follow-up was substantial in this cohort and although the survival analysis utilized the information up to the time of lost to follow-up the extent of this feature can bias the result and should be considered a limitation of the study.

CHAPTER FOUR

RESULTS

4.1 IMMUNOHISTOCHEMICAL STUDY

4.1.1 CLINICAL DATA

Table 20: Clinical data for the 27 patients not treated with preoperative chemotherapy (Treatment group B)

Case No	Age (months)	Gender	Race/ Ethnic	SIOP Classific	SIOP Risk group	Stage	Outcome	Follow up (months)
1	22	F	African	Mixed	Intermed	1	A	195
2	60	F	African	Mixed	Intermed	4	C	0.8
3	24	F	African	Mixed	Intermed	1	L	53.9
4	40	M	African	Mixed	Intermed	3	L	15.6
5	48	F	African	Blastemal	Intermed	4	C	9.8
6	12	F	African	Mixed	Intermed	2	L	33.3
7	12	F	African	Stromal	Intermed	3	L	35.1
8	9	F	African	Blastemal	Intermed	1	A	152.9
10	10	M	African	Mixed	Intermed	2	C	3.3
11	48	F	African	Mixed	Intermed	3	L	7.7
12	36	F	African	Mixed	Intermed	4	C	34.6
13	16	M	African	Blastemal	Intermed	3	A	165.9
14	36	F	African	Mixed	Intermed	2	L	7.7
15	18	F	African	Mixed	Intermed	3	A	72.2
16	20	M	African	Mixed	Intermed	1	L	13.1
17	24	M	Indian	Mixed	Intermed	3	A	146.5
18	36	M	African	Mixed	Intermed	3	L	4.5
19	24	M	African	Mixed	Intermed	3*	L	4.5
20	60	F	African	Blastemal	Intermed	4	C	14.8
21	4	F	Indian	Mixed	Intermed	1	A	90.5
22	6	F	African	Mixed	Intermed	1	L	1.8
23	14	F	African	Mixed	Intermed	1	L	4
25	36	M	African	Mixed	Intermed	1	L	16.2
35	72	F	African	Blastemal	Intermed	4	C	12.4
142	15	M	African	Mixed	Intermed	4*	B	14.2
147	24	M	African	Stromal	Intermed	4	A	9.1
148	48	F	African	Mixed	Intermed	2	L	5.5

Table 21: Clinical data for the 111 patients treated with preoperative chemotherapy - treatment group A

Case No	Age (months)	Gender	Race/ Ethnic	SIOP Classific	SIOP Risk group	Stage	Outcome	Follow up (months)
24	48	F	African	Mixed	Intermed	3	L	8
26	48	F	African	Blastemal	High	4	C	12.1
27	19	F	African	Regressive	Intermed	4	C	19.8
28	23	F	African	Regressive	Intermed	2	A	173.7
29	60	F	African	Stromal	Intermed	4	C	9
30	24	M	African	Stromal	Intermed	2	L	58.7
31	24	F	African	Mixed	Intermed	2	C	4.2
32	96	F	African	Blastemal	High	3	C	2.1
33	48	M	African	Mixed	Intermed	4	C	100.5
34	27	F	African	Mixed	Intermed	3	C	3.4
36	108	F	African	Stromal	Intermed	3	L	15.4
37	120	F	African	Regressive	Intermed	4	C	7.9
38	36	M	African	Regressive	Intermed	1	A	142.5
39	72	F	African	Blastemal	High	4	C	19.4
40	108	M	African	Epithelial	Intermed	4	C	7.8
41	36	F	African	Stromal	Intermed	4	L	69.7
42	24	F	African	Mixed	Intermed	2	C	11
43	36	M	African	Mixed	Intermed	4*	C	13.3
44	120	M	African	D. Anaplastic	High	3	C	13.2
45	60	M	African	Regressive	Intermed	3	L	50.3
46	84	F	African	Mixed	Intermed	2	L	11.9
47	66	M	African	D. Anaplastic	High	4	C	19.4
48	36	F	African	D. Anaplastic	High	4	C	13.5
49	50	M	African	Epithelial	Intermed	2	L	5
50	17	F	African	Blastemal	High	4	B	14
51	21	F	African	Blastemal	High	4	C	15.5
52	84	M	African	Regressive	Intermed	2	L	2.5
53	36	M	African	Regressive	Intermed	4	B	8.4
54	16	M	African	Mixed	Intermed	2	L	13.7
55	9	F	African	Regressive	Intermed	1	L	21.6
56	132	F	African	Mixed	Intermed	4	C	32.2
57	72	M	African	Mixed	Intermed	3	C	1.7
58	28	M	African	Regressive	Intermed	3	L	31.4

Case No	Age (months)	Gender	Race/ Ethnic	SIOP Classific	SIOP Risk group	Stage	Outcome	Follow up (months)
59	48	F	African	Mixed	Intermed	2	A	104.4
60	26	M	African	Mixed	Intermed	4	B	19
61	12	M	African	Regressive	Intermed	1	L	6.8
62	14	F	African	Regressive	Intermed	1	A	111.8
63	36	F	African	Regressive	Intermed	3	L	22.2
64	54	M	African	Regressive	Intermed	3	L	19.9
65	24	M	African	Mixed	Intermed	2	L	8.4
66	84	F	African	Mixed	Intermed	4	B	25.4
67	22	F	African	Epithelial	Intermed	2	A	106.9
68	60	F	African	Regressive	Intermed	2	L	14.1
69	17	M	African	Stromal	Intermed	2	L	6.9
70	9	F	African	Epithelial	Intermed	1	A	114
71	11	F	African	Epithelial	Intermed	1	L	8.6
72	13	M	African	Mixed	Intermed	1*	B	65.7
73	168	M	African	Mixed	Intermed	3	L	26.5
74	109	F	African	Blastemal	High	3	L	14.4
75	9	M	African	Mixed	Intermed	1	L	19.2
76	19	F	African	Mixed	Intermed	2	A	102.2
77	14	M	African	Mixed	Intermed	1	L	7.4
78	10	M	African	D. Anaplastic	High	4	C	10.1
79	12	F	African	Mixed	Intermed	1	A	102.7
80	12	M	African	Epithelial	Intermed	2	L	12.4
81	42	M	African	D. Anaplastic	High	2	L	11.7
82	16	F	African	Mixed	Intermed	1	A	72
83	48	F	African	Stromal	Intermed	3	L	25.7
84	12	M	African	Regressive	Intermed	3	L	8.2
85	24	M	African	Regressive	Intermed	3	L	8.7
86	36	F	African	Blastemal	High	4	C	3
87	12	F	African	Regressive	Intermed	3	A	87
88	24	M	African	Mixed	Intermed	4	C	16.6
89	12	M	African	Stromal	Intermed	1	L	18.8
90	21	M	African	D. Anaplastic	High	3	L	22.6
91	46	M	African	D. Anaplastic	High	3	C	12.6
92	48	F	African	D. Anaplastic	High	4	C	4
93	36	M	African	D. Anaplastic	High	4	C	18

Case No	Age (months)	Gender	Race/ Ethnic	SIOP Classific	SIOP Risk group	Stage	Outcome	Follow up (months)
94	96	M	African	Regressive	Intermed	2	A	64.7
95	15	M	African	Mixed	Intermed	1	A	90.8
96	48	M	African	D. Anaplastic	High	4	C	13
97	36	F	African	Blastemal	High	4	B	29
98	48	F	African	Blastemal	High	4	C	13.6
99	72	M	African	Blastemal	High	4	C	2
100	36	F	African	Regressive	Intermed	2	A	76.7
101	72	F	African	Blastemal	High	4	C	6
102	48	F	African	Regressive	Intermed	1	A	74
103	36	M	African	Blastemal	High	4	B	5.8
104	24	M	African	Blastemal	High	2	C	20.1
105	120	M	African	Blastemal	High	4	C	9
106	24	M	African	Blastemal	High	3	C	19.8
107	36	F	African	Mixed	Intermed	2	L	17.4
108	18	M	African	Stromal	Intermed	1	A	69.9
109	48	F	African	Mixed	Intermed	2	L	14
110	48	F	African	Mixed	Intermed	1	A	70.9
111	36	M	African	Regressive	Intermed	3	L	12
112	15	M	African	Stromal	Intermed	1	L	8
113	60	M	African	Mixed	Intermed	1	L	9
114	30	F	African	Stromal	Intermed	3	L	17.6
115	72	F	African	D. Anaplastic	High	4	C	13.5
116	24	F	African	Mixed	Intermed	4	C	27
118	58	M	African	Mixed	Intermed	2	C	68.5
137	12	F	African	Mixed	Intermed	2	C	1.9
138	88	M	Indian	Mixed	Intermed	4	C	34.3
139	56	M	African	Stromal	Intermed	4	B	14.8
140	108	F	African	Regressive	Intermed	3	C	12.8
141	36	F	African	Stromal	Intermed	2	A	130.2
143	96	F	African	Regressive	Intermed	3	B	34.5
144	13	M	Indian	Mixed	Intermed	2	C	3.9
145	50	M	African	Regressive	Intermed	4	A	122.3
146	34	M	African	Regressive	Intermed	3	A	107.2
149	24	F	African	Mixed	Intermed	2	A	7.6
150	24	F	African	Mixed	Intermed	4	C	6.1

Case No	Age (months)	Gender	Race/ Ethnic	SIOP Classific	SIOP Risk group	Stage	Outcome	Follow up (months)
151	108	M	African	Blastemal	High	4	B	9.3
152	36	M	African	Mixed	Intermed	2	A	13.2
153	72	F	African	Mixed	Intermed	3	A	9.8
154	12	M	African	Regressive	Intermed	2	A	7.5
155	96	M	African	Regressive	Intermed	2	A	10.7
156	36	F	African	Regressive	Intermed	2	A	46.1
157	48	F	African	Regressive	Intermed	3	A	8
158	48	F	African	Regressive	Intermed	2	A	104.8

Key:

* four cases with stage V disease – substaged as above.

A – Alive and disease free

B – Residual/recurrent disease

C – Dead

L – Lost to follow-up

M – Male

F – Female

N – No

Y – Yes

Note: Cases 9, 117 and 119 – 136 were excluded from the study (see 3.2 Sample selection)

4.1.1.1 Patient age

Figure 7 demonstrates the age distribution of the 138 patients in the immunohistochemical study. The ages ranged from 4 months to 14 years. The mean age was 42.3 months and the median age was 36 months. Sixty three (45.7%) patients were between 12 and 36 months of age. One hundred and twenty (87.0%) patients were 6 years or younger and 18 patients were older than 6 years (13.0%). The mean age for treatment group A was 45.6 years and that for treatment group B was 28.7 years.

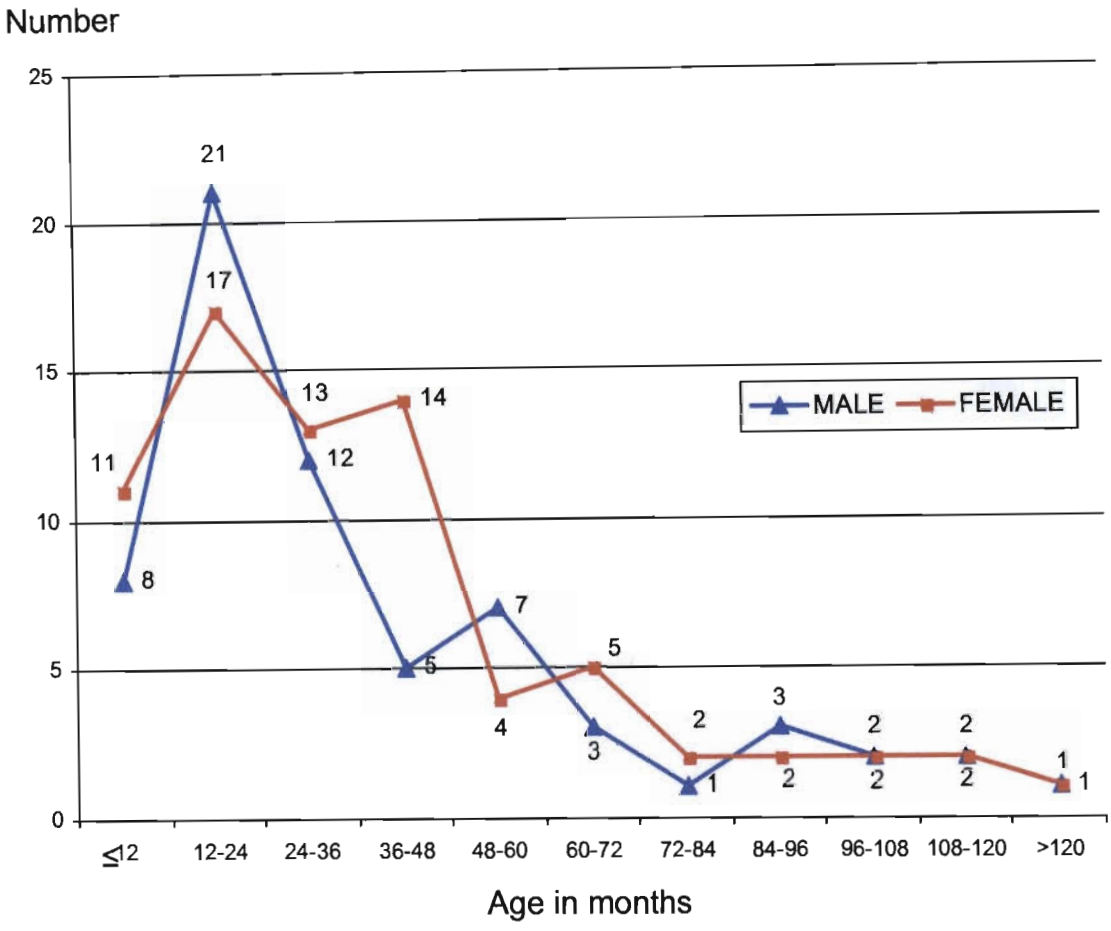


Figure 7: Age and gender distribution (n=138)

4.1.1.2 Gender

The gender distribution and its relationship to age are shown in Figure 7. The female:male ratio was 1.12:1.0 (73:65). The mean age and median age for male patients was 41.8 months and 36 months, respectively. For female patients the mean age and median age was 42.7 months and 36 months, respectively.

For treatment group A, the mean and median ages for male patients were 45 months and 36 months and for female patients these were 46.3 months and 36 months, respectively.

4.1.1.3 Race/Ethnic group

Of the 138 patients enrolled in the immunohistochemical study 134 (97.1%) were African and 4 (2.9%) were Asians of Indian descent. There were 2 Asians in treatment group A and two in treatment group B.

4.1.1.4 Preoperative chemotherapy

Preoperative chemotherapy was administered to 111 (80.4%) patients (treatment group A) (Table 21). Since this is a retrospective study, patients were recruited over a period of many years during which the chemotherapeutic regimens varied. Chemotherapy induced changes consisted of necrosis, haemorrhage, cystic change, accumulation of foam cells, fibrosis with hyalinisation, chronic inflammation, vascular sclerosis, focal granulomas, calcification and haemosiderin and haematoidin pigment deposits.

The treatment groups A and B were analysed separately, however, treatment group B (n=27) was too small for any significant statistical analysis.

4.1.1.6 Clinicopathological stage

Table 22: Clinicopathological stage

Stage	No preop chemo	With preop chemo	Total	%
I	8	18	26	18.8
II	4	31	35	25.4
III	8	26	34	24.6
IV	7	36	43	31.2

The stages for the two patient groups are shown in Table 22. Overall, 77 patients (55.8%) had either stage III or IV disease, while 61 patients (44,2%) had stage I or II disease. Four patients had stage V disease (2,9%). These were substaged according to SIOP guidelines as one stage I/I, one stage III and two stage IV.

In treatment group A there were 18 patients with stage I disease, 31 with stage II disease, 26 with stage III disease and 36 with stage IV disease (Table 22).

Patients with advanced disease (stage III and IV) were on average older than patients with low stage disease (stage I and II). There was a positive association between mean age and clinicopathological stage (Table 23). An increase in clinicopathological stage was associated with an increase in mean patient age ($p<0.0001$, χ^2).

Table 23: Mean patient age for clinicopathological stage

SIOP stage	Mean age (months)	Standard deviation
I	19.5	14.3
II	36.9	24.0
III	51.6	37.6
IV	53.2	30.6

$p<0.0001$, χ^2

In treatment group A the death rates for stage I to IV disease were 0, 19.4%, 26,9% and 72.2%, respectively ($p<0.0001$, Pearson χ^2).

4.1.1.5 SIOP histological classification and SIOP risk group

Table 24: Histological classification (SIOP)

Classification	No preop chemo	With preop Chemo	Total
Blastemal	5	16	21
Diffuse anaplastic	0	11	11
Epithelial	0	6	6
Mixed	20	36	56
Regressive	0	30	30
Stromal	2	12	14
Total	27	111	138

All 27 cases in treatment group B were classified in the intermediate SIOP risk group. There were no cases of diffuse anaplasia in this cohort. Of the 111 cases in treatment group A, 27 had high risk tumours and 84 had intermediate risk tumours. The 27 high risk tumours consisted of 16 blastemal type and 11 with diffuse anaplasia (Table 24). There were no low risk histology tumours since all cases with complete necrosis were excluded from the study because of the lack of viable tumour for immunohistochemical and molecular analysis and there were no cystic partially differentiated nephroblastomas.

Table 25: Mean patient age for SIOP histological tumour types

SIOP Classification	Mean age (months)	Standard deviation
Blastemal	54.5	33.3
Diffuse anaplastic	49.5	29.3
Epithelial	35.3	38.7
Mixed	36.8	30.3
Regressive	46.1	31.4
Stromal	35.4	26.2

p=0.221, χ^2

On average, patients who had tumours with blastemal (54.5 months) or anaplastic histology (49.5 months) were older than those with other histological types (p=0.221, χ^2) (Table 25). Patients with mixed, epithelial and stromal tumours had a mean age of 35 to 36 months. In treatment group A high risk neoplasms occurred in 14 male patients and 13 female patients. The mean age of patients with high risk neoplasms (55 months, SD 32.3) was significantly higher than that for intermediate risk neoplasms (39.2 months, SD 30.3) (p=0.018, χ^2). The median age for this group with high risk neoplasms was 48 months. The ages ranged from 10 months to 10 years.

Table 26: Histological classification (SIOP) and clinicopathological stage – treatment group A

Classification	No. of deaths	Stage				Total
		I	II	III	IV	
Blastemal	11	0	1 (1)	3 (2)	12 (8)	16
Diffuse anaplastic	9	0	1	3 (2)	7 (7)	11
Epithelial	1	2	3	0	1 (1)	6
Mixed	14	8	14 (5)	5 (2)	9 (7)	36
Regressive	3	5	9	12 (1)	4 (2)	30
Stromal	1	3	3	3	3 (1)	12
Total	39	18	31 (6)	26 (7)	36 (26)	111

p=0.001 (Pearson χ^2)

Number of deaths in brackets.

All four stage V patients were substaged and included above according to the substage.

Blastemal histology and diffuse anaplasia were more likely than the other histological types to be associated with higher disease stage ($p<0.001$) (Table 26). Fifteen of 16 blastemal tumours and 10 of 11 anaplastic tumours were either stage III or IV. The mixed, regressive and stromal types had a close to equal distribution between low and advanced disease stage. The majority of the epithelial type tumours had low disease stage.

Table 27: Risk group (SIOP) and clinicopathological stage – treatment group A

Risk group	No. of deaths	Stage				Total
		I	II	III	IV	
High	20	0	2	6	19	27
Intermediate	19	18	29	20	17	84
Total	39	18	31	26	36	111

$p<0.001$ (Pearson χ^2)

All four stage V patients were substaged and included above.

Nineteen of the patients with high risk neoplasms had stage IV disease, 6 had stage III disease and 2 had stage II disease (Table 27). Eleven (68.8%) patients with blastemal type histology died and 9 of 11 patients with diffuse anaplasia died. The death rate for diffuse anaplasia was 81.8%. The comparative death rates for the other histological types were 38.9%, 16.7%, 10% and 8.3% for mixed, epithelial, regressive and stromal types respectively ($p<0.0001$, Pearson χ^2). In total 20 of the 27 (74.1%) patients with high risk neoplasms died. Of the 20 patients who died, 15 had stage IV disease, four had stage III disease and one had stage II disease. There were 84 intermediate risk tumours (75.7%) (**Fig**). Of these 84 cases, 19 patients (22.6%) died. Eleven patients had stage IV disease, 3 had stage III disease and 5 had stage II disease. All but 2 deaths were tumour related. There was a significant difference in the death rate in patients who had intermediate risk neoplasms and those who had high risk tumours (22.6% vs 74.1%)($p<0.0001$, Pearson χ^2).

Six of the 27 patients in treatment group B died. Five of these six patients had stage IV disease and one had stage II disease. All six had intermediate risk tumours. The statistical analysis of treatment group B was affected by the small number of cases.

The statistical analysis that follows is based on the analysis of the 111 cases in treatment group A unless otherwise stated.

4.1.2 IMMUNOHISTOCHEMICAL RESULTS

Table 28: Immunohistochemical scores for the 6 proteins studied - treatment group B (n=27)

Case	p53	p21	Bcl-2	pRb	Cyclin A	p-glycoprotein
1	1	0	1	3	2	3
2	0	0	0	4	2	1
3	0	0	1	1	1	0
4	0	0	1	3	2	1
5	0	0	0	3	2	1
6	2	0	1	3	3	0
7	0	0	1	4	3	1
8	0	1	0	3	2	2
10	1	0	0	1	3	3
11	1	1	1	3	2	3
12	0	0	1	3	2	0
13	0	0	0	2	2	0
14	0	0	0	3	4	3
15	0	1	0	2	3	0
16	0	0	0	3	2	2
17	0	0	0	4	4	4
18	1	0	1	2	2	0
19	2	2	2	3	3	2
20	1	0	1	1	2	2
21	0	2	0	3	3	0
22	1	1	1	2	4	3
23	1	2	2	3	2	3
25	1	0	2	3	0	0
35	1	2	0	4	3	1
142	4	1	2	4	3	2
147	0	0	1	2	3	3
148	0	0	0	2	2	3

Table 29: Immunohistochemical scores for the 6 proteins studied – treatment group A (n=111)

Case	p53	p21	Bcl-2	pRb	Cyclin A	p-glycoprotein
24	1	2	0	4	3	0
26	1	1	2	3	2	3
27	1	1	1	3	2	2
28	1	1	1	2	0	1
29	2	1	1	2	3	3
30	1	1	1	3	1	2
31	0	0	1	4	3	2
32	1	0	2	3	3	3
33	1	1	1	1	1	1
34	1	0	1	3	1	3
36	0	1	0	0	0	3
37	1	0	4	2	1	1
38	1	0	1	2	1	1
39	1	1	0	3	3	2
40	0	0	0	1	2	2
41	0	0	0	1	0	0
42	1	1	1	2	2	2
43	4	0	0	2	3	3
44	4	0	2	3	3	4
45	1	0	1	4	2	3
46	1	0	2	2	2	1
47	4	0	4	3	2	4
48	1	0	4	3	2	1
49	2	0	4	3	2	3
50	1	1	0	2	4	3
51	3	0	3	3	2	1
52	1	2	0	4	3	0
53	1	0	1	3	2	4
54	1	0	3	3	1	0
55	1	0	4	3	1	0
56	1	0	1	2	2	1
57	1	0	1	1	1	1
58	0	0	1	1	1	1
59	1	0	1	3	1	0
60	0	0	1	1	1	1
61	1	1	1	2	3	1
62	0	0	0	2	1	3

Case	p53	p21	Bcl-2	pRb	Cyclin A	p-glycoprotein
63	1	1	0	1	2	2
64	1	0	0	2	1	0
65	1	0	1	1	1	0
66	1	0	1	3	2	2
67	1	1	0	1	1	1
68	1	1	3	2	1	1
69	0	0	1	1	1	0
70	0	0	1	1	1	3
71	0	0	1	3	1	2
72	0	0	1	1	1	0
73	1	0	0	3	1	2
74	1	0	2	2	2	2
75	0	0	1	0	1	3
76	0	0	1	2	1	1
77	0	0	1	2	1	4
78	1	0	1	3	1	0
79	0	0	1	2	1	2
80	1	0	1	1	1	2
81	4	0	2	3	2	4
82	1	0	1	1	1	2
83	1	0	1	0	1	2
84	1	1	1	2	3	1
85	1	0	1	2	2	2
86	2	2	1	4	3	1
87	1	2	1	2	1	3
88	1	0	2	2	2	3
89	1	1	1	1	1	0
90	3	0	1	2	2	3
91	3	0	2	2	1	0
92	3	2	1	2	3	3
93	1	0	1	3	2	2
94	0	1	0	2	2	4
95	0	0	0	1	1	0
96	4	0	0	2	3	3
97	1	1	1	1	2	2
98	1	2	1	1	2	2
99	1	1	2	1	2	2
100	1	0	4	3	3	4
101	1	0	3	1	2	3

Case	p53	p21	Bcl-2	pRb	Cyclin A	p-glycoprotein
102	1	0	3	2	2	3
103	2	0	1	1	2	1
104	0	0	1	3	2	3
105	1	0	4	1	2	4
106	1	0	1	3	4	2
107	0	0	1	3	2	2
108	1	0	3	1	0	4
109	0	0	1	3	1	1
110	0	0	1	1	2	4
111	1	1	2	2	1	2
112	0	0	1	0	2	1
113	1	1	3	4	3	2
114	1	0	1	2	1	2
115	0	0	3	2	3	4
116	0	0	1	1	1	0
118	1	1	0	2	2	3
137	2	0	0	3	1	2
138	1	1	1	1	0	0
139	1	0	1	1	0	0
140	1	1	1	2	3	2
141	1	1	0	3	1	0
143	0	0	3	2	1	1
144	1	0	0	2	1	1
145	0	0	0	2	1	4
146	0	0	0	0	1	0
149	0	1	1	3	3	1
150	0	0	1	3	1	2
151	1	1	1	1	2	2
152	1	0	1	2	3	3
153	1	0	2	2	1	1
154	1	2	1	4	2	0
155	0	0	4	3	1	4
156	0	0	1	1	1	0
157	1	0	3	3	1	2
158	1	0	1	3	2	2

The immunoexpression results for the six proteins are shown in Tables 28, 29 & 30.

Table 30: Immunoexpression status for the six antibodies

Antibody	Score	# with preop chemotherapy	# no preop chemotherapy	Total
p53	0	31	15	46
	1	66	9	75
	2	5	2	7
	3	4	0	4
	4	5	1	6
p21	0	77	18	59
	1	27	5	32
	2	7	4	11
	3	0	0	0
	4	0	0	0
Bcl-2	0	22	12	34
	1	60	11	71
	2	11	4	15
	3	10	0	10
	4	8	0	8
pRb	0	5	0	5
	1	29	3	32
	2	37	6	43
	3	33	13	46
	4	7	5	12
Cyclin A	0	6	1	7
	1	48	1	49
	2	36	13	49
	3	19	9	28
	4	2	3	5
p-glycoprotein	0	21	8	29
	1	24	5	29
	2	31	5	36
	3	22	8	30
	4	13	1	14

4.1.2.1 p53 RESULTS

In total, 121 (87.7%) tumours showed low p53 expression (Plate 2b) and 17 (12.3%) showed high p53 expression (Plate 2c). Normal kidney tissue, when present, did not show immunoreactivity for p53.

Staining was seen in blastemal, epithelial and stromal cells (Plate 2c,e,f). Overall, 61 cases showed p53 immunoreactivity in blastemal cells, 53 in epithelial cells and 23 in stromal cells. In treatment group A, 47 tumours showed p53 expression in epithelial cells, 50 in blastemal cells and 22 in stromal cells.

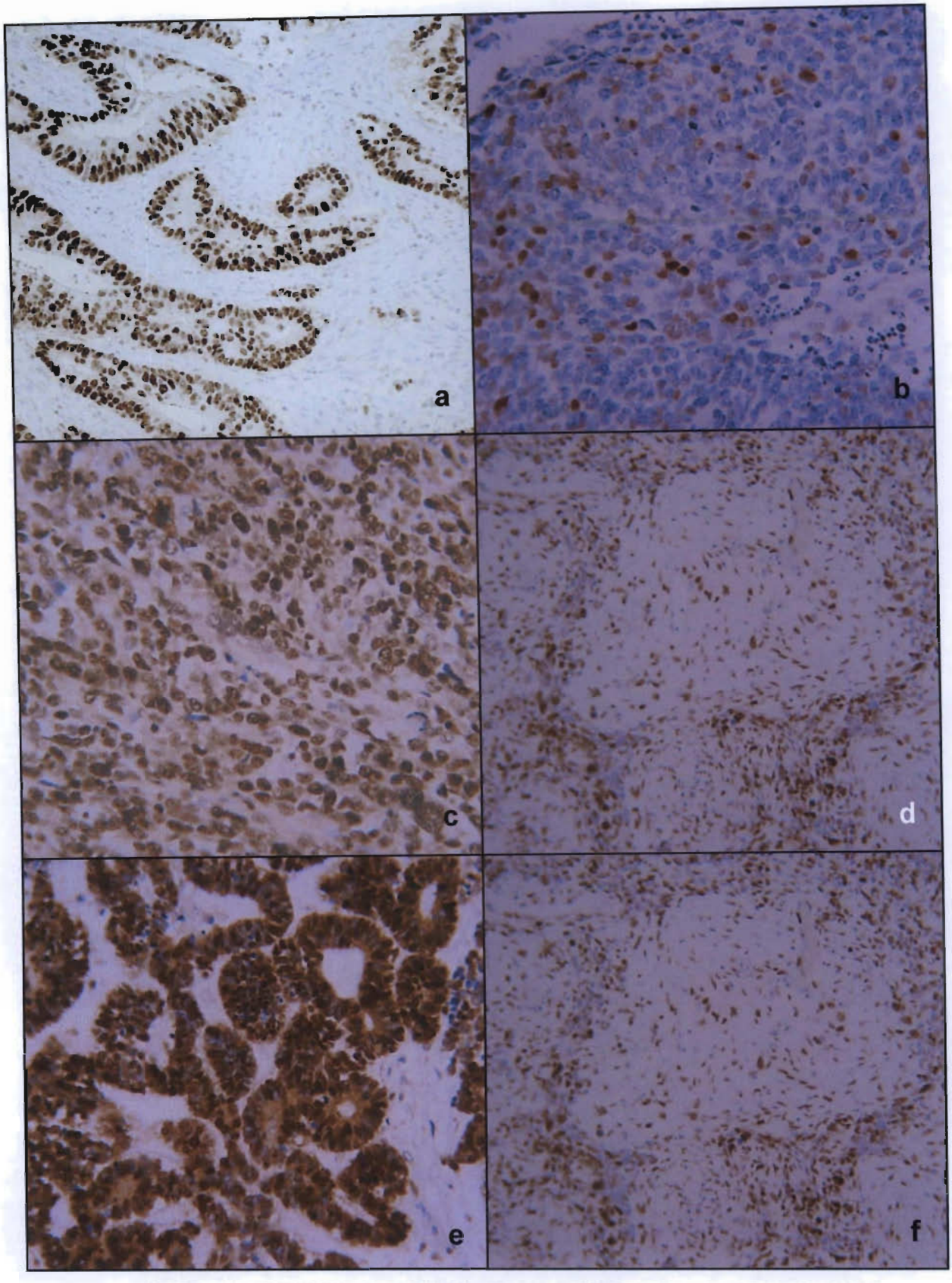


Plate 2: p53 immunostaining, **a**. Control slide – colonic adenocarcinoma showing diffuse nuclear staining, **b**. low staining – less than 25% of nuclei are positive, **c**. high staining – more than 25% of nuclei are positive, **d**. anaplastic nephroblastoma showing high staining including anaplastic nuclei, **e**. non-anaplastic tubules in an anaplastic tumour showing high staining, **f**. staining in stromal cells (anti-p53, DO-7)

In comparison, in treatment group B, 6 showed p53 expression in epithelial cells, 11 in blastemal cells and 1 in stromal cells. There was no difference in the cellular location of p53 immunoexpression between the two treatment groups. Of the 92 cases that showed p53 immunoreactivity, 90 showed staining in either epithelial or blastemal cells or both. Anaplastic nuclei were positive (Plate 2d). p53 expression in anaplastic tumours was present in both anaplastic and non-anaplastic cells. Non-anaplastic tubular and/or glomeruloid structures present in anaplastic tumours, showed intense nuclear staining (Plate 2e).

Tumours from 14 of the 111 (12.6%) patients in treatment group A showed high p53 expression. In comparison, only 3 tumours of the 27 (11.1%) in treatment group B showed similar expression. No statistical difference in p53 staining was found between these two groups (p=0.831, χ^2).

Table 31: Correlation of p53 expression with histological classification – treatment group A

SIOP Classification	p53 immunoexpression					Total
	0	1	2	3	4	
Blastemal	1	12	2	1	0	16
Diffuse anaplastic	1	3	0	3	4	11
Epithelial	3	2	1	0	0	6
Mixed	14	20	1	0	1	36
Regressive	8	22	0	0	0	30
Stromal	4	7	1	0	0	12
Total	31	66	5	4	5	111

p<0.001 (Pearson χ^2)

High risk neoplasms especially the anaplastic type was more frequently associated with high p53 expression (>25%) than intermediate risk tumours (p<0.001, Pearson χ^2) (Tables 31 & 32). When p53 expression was compared with histological classification, seven of the 11 tumours (63.6%) with anaplastic histology and 3 of the 16 tumours with blastemal histology showed high p53 expression while only 4 of remaining 84 (intermediate risk) tumours showed similar expression.

Table 32: Correlation of p53 expression with risk group – treatment group A

SIOP Risk group	p53 immunoexpression					Total
	0	1	2	3	4	
High	2	15	2	4	4	27
Intermediate	29	51	3	0	1	84
Total	31	66	5	4	5	111

$p<0.001$ (Pearson χ^2)

Of these latter 4 cases, 3 patients died and 1 was lost to follow-up. Two of the 3 patients who died had stage IV disease and one had stage II disease. The one patient lost to follow up also had stage II disease. Two had mixed type tumours and there was one case each with epithelial and stromal histology.

All three patients with blastemal tumours which expressed high p53 had stage IV disease and two patients died while one patient was alive with residual disease. In the group with diffuse anaplastic tumours and high p53 expression, there were 3 patients with stage IV disease, 3 with stage III disease and 1 with stage 2 disease. All 3 patients with stage IV disease and 2 with stage III disease died. One patient with stage III disease and the patient with stage II disease were lost to follow up.

Similar to the finding with histological classification, p53 immunoexpression was significantly association with SIOP risk group ($p<0.001$, Pearson χ^2) (Table 32). High p53 expression was seen in a greater percentage of high risk tumours compared to intermediate risk tumours.

The results of the correlation of p53 immunoexpression with clinicopathological stage are shown in Table 33. All stage I tumours showed low p53 staining. In fact, 10 of the 18 cases in this stage were negative for p53 protein immunostaining. Three of the 49 (6.1%) low stage neoplasms and 11 of the 62 (17.7%) high stage neoplasms expressed high p53. Although there was a tendency for more high stage neoplasms to express high p53 this did not reach statistical significance ($p=0.233$, Pearson χ^2).

Table 33: Correlation of p53 expression with clinicopathological stage – treatment group A

Clinicopathological stage	p53 immunoexpression					Total
	0	1	2	3	4	
I	10	8	0	0	0	18
II	10	18	2	0	1	31
III	4	19	0	2	1	26
IV	7	21	3	2	3	36
Total	31	66	5	4	5	111

p=0.233

There were 47 cases that had a combination of low disease stage (stage I and II) and intermediate risk. Forty five of these showed low p53 expression. Similarly, 35 of 37 high stage (stage III and IV) intermediate risk tumours expressed low p53.

Table 34: Correlation of p53 expression with patient outcome – treatment group A

Outcome	p53 immunoexpression					Total
	0	1	2	3	4	
Disease free	12	15	0	0	0	27
Residual/recurrent	3	6	1	0	0	10
Dead	6	23	3	3	4	39
Lost	10	22	1	1	1	35
Total	31	66	5	4	5	111

p=0.125, Pearson χ^2

Although patients who died had a higher frequency of neoplasms with high p53 expression, this was not statistically significant (p=0.125, Pearson χ^2) (Table 34). Tumours from all of the 27 patients who were disease free showed low p53 immunoexpression. Only one of the 10 who had residual/recurrent disease but 10 of the 39 who died showed high p53 expression.

There was no significant association between p53 immunoexpression and age (p=0.055, Spearman correlation coefficient), and gender (p=0.236, Pearson χ^2).

4.1.2.2 p21 RESULTS

Overall, 43 tumours showed nuclear immunoreactivity for p21. Of these 43 cases, 32 showed 1+ and 11 showed 2+ staining (Plate 3b,d). Ninety five cases were negative.

Staining was seen in blastemal, epithelial and stromal cells (Plate 3b,c,d). Overall, 30 cases showed p21 immunoreactivity in blastemal cells, 16 in epithelial cells and 13 in stromal cells. Foci of squamous differentiation showed strong nuclear staining (Plate 3e,f). In treatment group A, 21 cases showed p21 staining in blastemal cells, 13 in epithelial cells and 12 in stromal cells. In treatment group B, 9 cases demonstrated p21 expression in blastemal cells, 3 in epithelial cells and 1 in stromal cells. The cellular location of p21 staining was not significantly different in the two treatment groups.

Three of the 7 cases that showed high p21 expression died. All three of these patients had stage IV disease and all three had high risk tumours (1 anaplastic and 2 blastemal). In comparison, the remaining four cases that showed high p21 expression had intermediate risk tumours. Two of these patients were disease free and two were lost to follow-up.

There was no significant association between p21 immunoexpression and patient age ($p=0.910$, Spearman correlation coefficients), preoperative chemotherapy status ($p=0.143$, χ^2), histological classification ($p=0.344$, Pearson χ^2), risk group ($p=0.494$, Pearson χ^2), clinicopathological stage ($p=0.986$, Pearson χ^2) and outcome ($p=0.923$, Pearson χ^2).

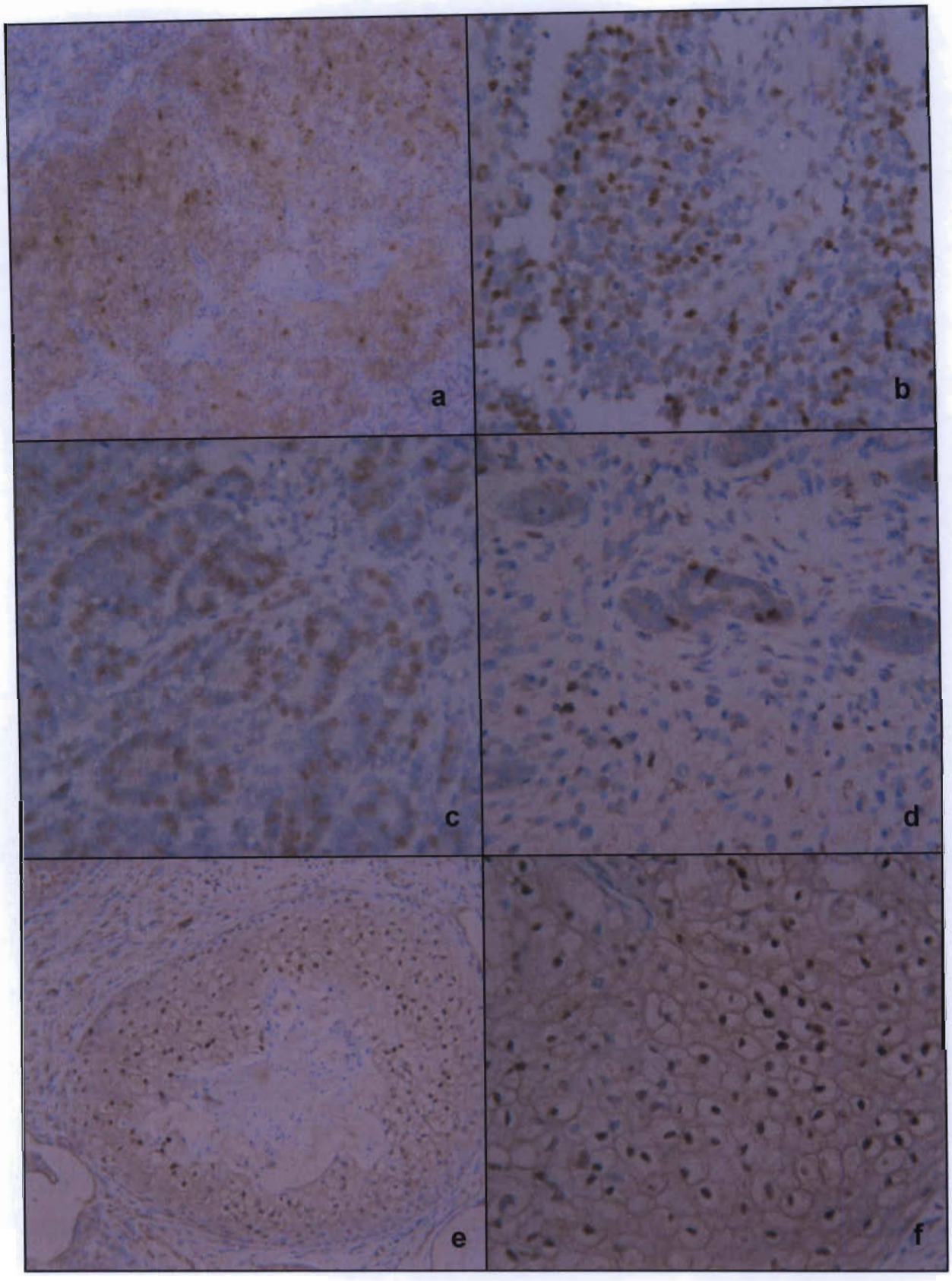


Plate 3: p21 immunostaining, **a**. Control slide – normal tonsil, **b**. high staining – more than 25% of nuclei are positive, **c**. tubular epithelium showing nuclear staining, **d**. low staining – less than 25% of nuclei are positive, **e**. focus of squamous differentiation showing diffuse nuclear staining, **f**. higher power of squamous cells showing diffuse nuclear staining (anti-p21, SX118)

4.1.2.3 bcl-2 RESULTS

In total, 104 tumours (75.4%) showed immunoreactivity for bcl-2 protein. There was marked heterogeneity in bcl-2 staining within each case. Bcl-2 immunoexpression was seen predominantly in blastemal cells (Plate 4b) and glandular epithelial cells (Plate 4c). In some cases, striated muscle cells and mesenchymal cells showed bcl-2 immunoreactivity (Plate 4d). Anaplastic cells were usually negative (Plate 4e). The tufts of immature glomeruloid structures were always positive, while the surrounding immature Bowman's epithelium showed variable staining (Plate 4f).

Table 35: Correlation of bcl-2 expression with administration of preoperative chemotherapy

Preoperative chemotherapy	bcl-2 immunoexpression					Total
	0	1	2	3	4	
No	12	11	4	0	0	27
Yes	22	60	11	10	8	111
Total	34	71	15	10	8	138

p=0.027, χ^2

A significant difference in bcl-2 immunoexpression was found between these two treatment groups (p=0.027, χ^2) (Table 35). Eighty nine tumours from the 111 (80.2%) tumours in treatment group A showed staining for bcl-2 protein. In contrast, only 15 tumours of the 27 (55.6%) in treatment group B showed bcl-2 staining.

Table 36: bcl-2 immunoexpression correlated with clinicopathological stage in treatment group A

Clinicopathological stage	bcl-2 immunoexpression					Total
	0	1	2	3	4	
I	2	12	0	3	1	18
II	7	17	2	2	3	31
III	6	12	6	2	0	26
IV	7	19	3	3	4	36
Total	22	60	11	10	8	111

p=0.663, Pearson χ^2

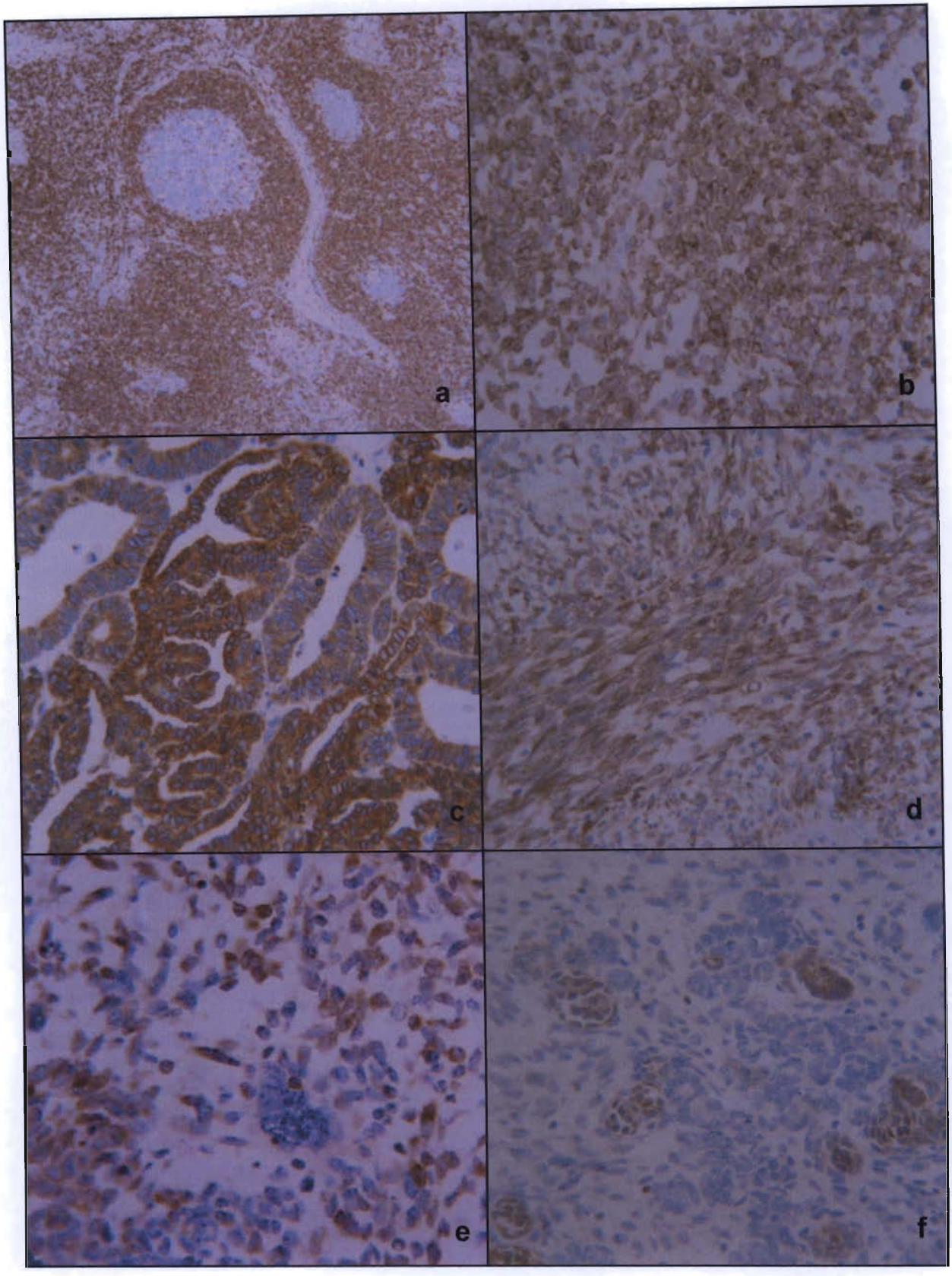


Plate 4: bcl-2 immunostaining, **a**. Control slide – normal tonsil showing lack of staining in a reactive lymphoid follicle and staining in the surrounding lymphoid cells, **b**. diffuse cytoplasmic staining in blastemal cells, **c**. diffuse cytoplasmic staining in mature tubules. Note reduced staining in immature tubules, **d**. cytoplasmic staining in spindle shaped stromal cells, **e**. lack of immunoexpression in anaplastic nuclei, **f**. staining in the tufts of immature glomeruli (anti-bcl-2, 124)

Bcl-2 staining was seen in tumours of all clinicopathological stages and no statistical difference was found between different stages (p=0.663, Pearson χ^2) (Table 36). Only 22 cases from this cohort did not display bcl-2 protein expression. The majority of tumours (60/111) in all four stages showed 1+ positivity (that is, up to 25% of tumour cells being immunopositive).

Table 37: Correlation of bcl-2 expression with risk group – treatment group A

SIOP Risk group	bcl-2 immunoexpression					Total
	0	1	2	3	4	
High	3	11	7	3	3	27
Intermediate	19	49	4	7	5	84
Total	22	60	11	10	8	111

p=0.013, Pearson χ^2

Although no association was found between bcl-2 expression and histological classification (p=0.146, Pearson χ^2), there was a significant association with risk group (p=0.013, Pearson χ^2) (Table 37). In both risk groups bcl-2 expressions of all grades were encountered. Thirteen of the 27 (48.1%) high risk tumours showed high bcl-2 expression, in contrast to 16 of 84 (19.0%) intermediate risk tumours.

There was no significant association between bcl-2 immunoexpression and patient age (p=0.069, Spearman correlation coefficient), gender (p=0.761, Pearson χ^2) and patient outcome (p=0.702, χ^2).

4.1.2.4 pRb RESULTS

In total, 133 tumours showed nuclear immunoreactivity for pRb. Of these 133 cases, 32 showed 1+, 43 showed 2+, 46 showed 3+ and 12 showed 4+ staining. Five cases were negative. Normal kidney tissue, whenever present, showed immunoreactivity for pRb.

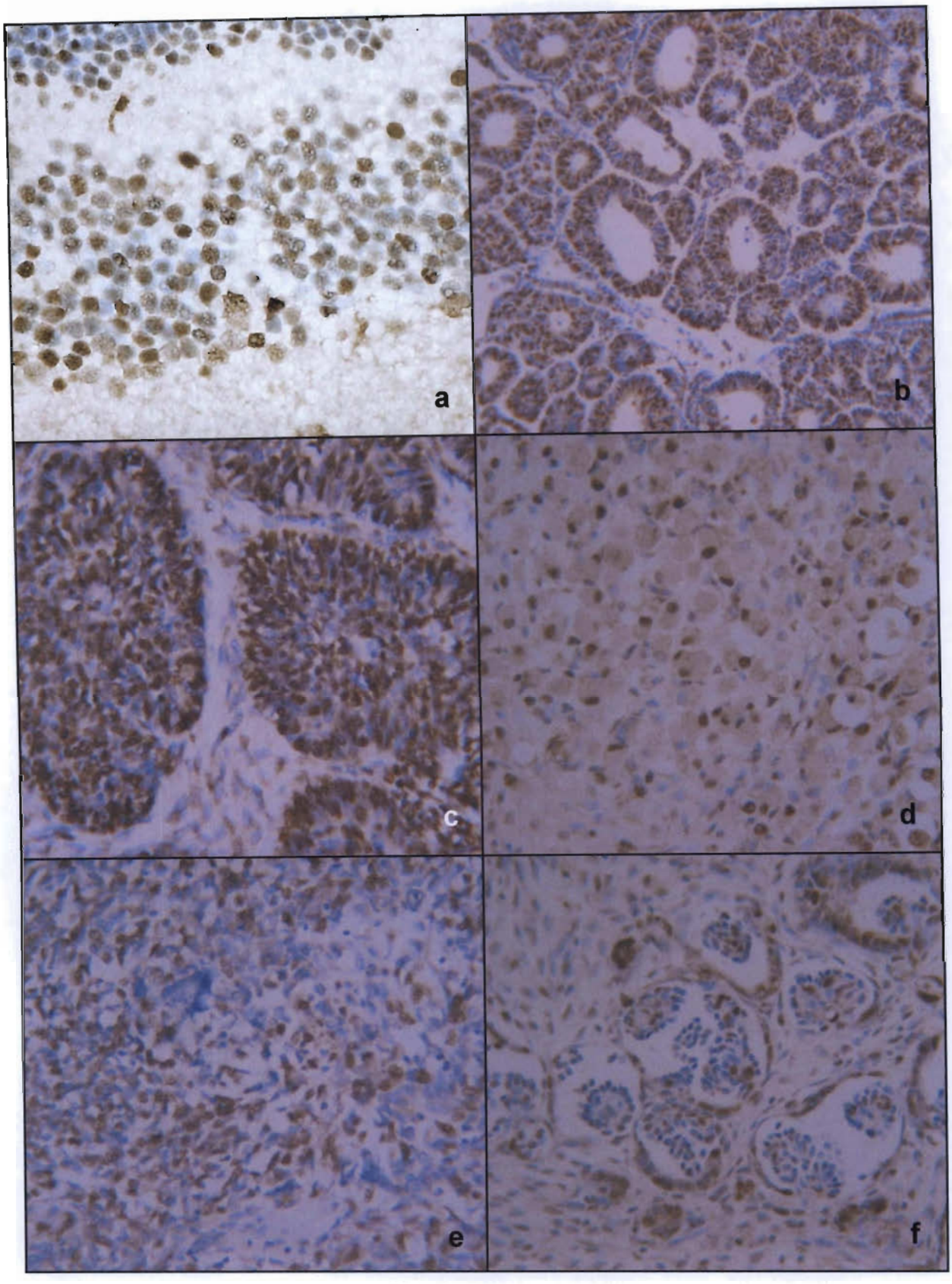


Plate 5: pRb immunostaining, **a**. Control slide – retinal cells showing nuclear staining, **b**. high staining in tubules – more than 25% of nuclei are positive, **c**. high staining in blastema – more than 25% of nuclei are positive, **d**. nuclear staining in striated muscle cells, **e**. lack of staining in anaplastic cells, **f**. focal staining in immature glomerular tuft but diffuse staining in immature Bowman's capsular epithelium (anti-retinoblastoma, Rb1)

Staining was seen predominantly in blastemal and tubular epithelial cells (Plate 5b). Furthermore, the most intense staining was seen in the blastemal and epithelial cells (Plate 5b,c). Of the 133 cases that expressed pRb, 124 showed staining in either epithelial or blastemal cells or both. In thirty two cases, mesenchymal cells including striated muscle cells showed pRb immunoreactivity (Plate 5d). Anaplastic cells were negative (Plate 5e). Immature Bowman's epithelium showed more intense staining than the tuft of immature glomeruli (Plate 5f). Of the 111 cases in treatment group A, 65 showed pRb expression in epithelial cells, 66 in blastemal cells and 21 in stromal cells. In comparison, in treatment group B pRb staining was detected in epithelial cells in 16 cases, in blastemal cells in 23 cases and in stromal cells in 11 cases.

Table 38: Correlation of pRb expression with administration of preoperative chemotherapy

Preoperative chemotherapy	**pRb immunoexpression**					**Total**
	0	**1**	**2**	**3**	**4**	
No	0	3	6	13	5	27
Yes	5	29	37	33	7	111
Total	5	32	43	46	12	138

$p=0.040$, χ^2

A greater proportion of tumours in treatment group B expressed high pRb when compared to preoperatively treated tumours (88.9% vs 69.4%). There appears to be a marginal association between pRb immunoexpression and treatment group ($p=0.040$, χ^2) (Table 38).

All 11 anaplastic tumours showed high pRb immunoexpression. In the mixed, regressive and blastemal types more than half of the tumour showed high expression. In comparison, more than two thirds of the epithelial and stromal types showed low expression. There was a difference in pRb immunoexpression in the various histological types of nephroblastoma, with all anaplastic tumours expressing high pRb ($p=0.003$, Pearson χ^2) (Table 39).

Table 39: Correlation of pRb expression with histological classification – treatment group A

SIOP Classification	pRb immunoexpression					Total
	0	1	2	3	4	
Blastemal	0	7	2	6	1	16
Diffuse anaplastic	0	0	5	6	0	11
Epithelial	0	4	0	2	0	6
Mixed	1	10	12	10	3	36
Regressive	1	3	16	7	3	30
Stromal	3	5	2	2	0	12
Total	5	29	37	33	7	111

p=0.003, Pearson χ^2

Table 40: Correlation of pRb expression with risk group – treatment group A

SIOP Risk group	pRb immunoexpression					Total
	0	1	2	3	4	
High	0	7	7	12	1	27
Intermediate	5	22	30	21	6	84
Total	5	29	37	33	7	111

p=0.270, Pearson χ^2

Despite the association between pRb expression and histological classification, no association was found with risk group. A smaller percentage of high risk tumours (25.9%) showed low pRb expression compared to intermediate risk tumours (32.1%) (p=0.270, Pearson χ^2) (Table 40).

Table 41: Correlation of pRb expression with patient outcome – treatment group A

Outcome	pRb immunoexpression					Total
	0	1	2	3	4	
Disease free	1	7	11	7	1	27
Residual/recurrent	0	6	2	2	0	10
Dead	0	9	13	15	2	39
Lost	4	7	11	9	4	35
Total	5	29	37	33	7	111

p=0.286, Pearson χ^2

pRb immunoexpression was not significantly associated with patient outcome (p=0.286, Pearson χ^2) (Table 41). Four of the 5 negative cases were lost to follow-up and 1 case was disease free after 107 months follow-up. The four cases lost to follow-up consisted of two stage I and two stage III cases. All 5 cases were intermediate risk tumours.

There was no association between pRb immunoexpression and patient age (p=0.982, Spearman correlation coefficient), gender (p=0.441, Pearson χ^2) and clinicopathological stage (p=0.085, Pearson χ^2).

4.1.2.5 Cyclin A RESULTS

In total, 131 tumours showed nuclear immunoreactivity for cyclin A. Of these 131 cases, 49 showed 1+, 49 showed 2+, 28 showed 3+ and 5 showed 4+ staining. Seven cases were negative.

Staining was seen predominantly in blastemal cells (Plate 6b). Furthermore, the most intense staining was seen in the blastemal cells and immature epithelium (Plate 6d). The distribution of staining within blastemal tissue varied. In most cases there was a uniform distribution of positive cells within blastemal tissue (Plate 6b,e). In some cases staining was confined to the periphery of the blastemal tissue, at the junction with stromal tissue (Plate 6c). Of the 131 cases that showed cyclin A immunoreactivity, 119 showed staining in either epithelial or blastemal cells or both. In twenty eight cases, mesenchymal cells including striated muscle cells showed cyclin A immunoreactivity. Anaplastic cells were negative (Plate 6f).

Only 6 of the 111 cases in treatment group A did not express cyclin A. Of the remaining 105 cases that expressed cyclin A, 49 showed expression in epithelium, 61 in blastema and 22 in stroma. Eleven of the 27 cases in treatment group B expressed cyclin A in epithelial cells, 19 in blastemal cells and 6 in stromal cells.

There was no correlation between cyclin A expression and patient age (p=0.445, Spearman correlation coefficient) and gender (p=0.966, Pearson χ^2).

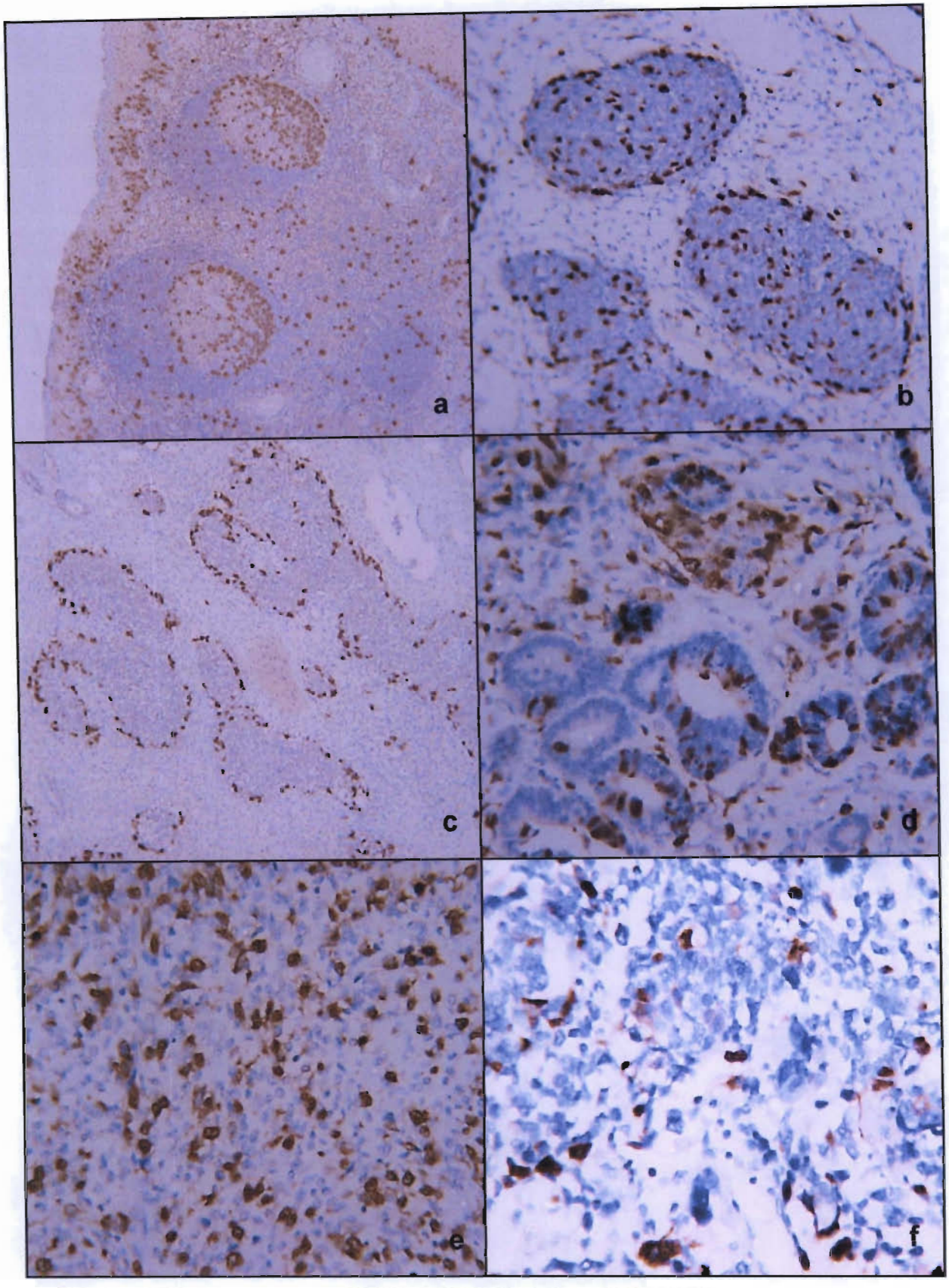


Plate 6: Cyclin A immunostaining, **a**. Control slide – normal tonsil showing nuclear staining in lymphoid follicles and tonsillar epithelium, **b**. randomly distributed positive cells in blastemal islands, **c**. positive blastemal cells at the periphery of blastemal islands, **d**. variable staining in neoplastic tubules, **e**. equal distribution of positive and negative cells, **f**. lack of staining in most anaplastic nuclei (anti-cyclin A, 6E6)

Table 42: Correlation of cyclin A expression with administration of preoperative chemotherapy

Preoperative chemotherapy	Cyclin A immunoexpression					Total
	0	1	2	3	4	
No	1	1	13	9	3	27
Yes	6	48	36	19	2	111
Total	7	49	49	28	5	138

$p<0.001$, χ^2

High cyclin A immunoexpression was significantly more frequent in treatment group B compared to group A (92.6% vs 51.4%) ($p<0.001$, Pearson χ^2) (Table 42).

Table 43: Correlation of cyclin A expression with histological classification – treatment group A

SIOP Classification	Cyclin A immunoexpression					Total
	0	1	2	3	4	
Blastemal	0	0	11	3	2	16
Diffuse anaplastic	0	2	5	4	0	11
Epithelial	0	4	2	0	0	6
Mixed	1	21	8	6	0	36
Regressive	1	15	9	5	0	30
Stromal	4	6	1	1	0	12
Total	6	48	36	19	2	111

$p<0.001$, Pearson χ^2

The majority of blastemal (100%) and diffuse anaplastic (81.8%) tumours expressed high cyclin A compared to the other types which ranged from 16.7% to 46.7% (Table 43) ($p<0.001$, Pearson χ^2).

Table 44: Correlation of cyclin A expression with risk group – treatment group A

SIOP Risk group	Cyclin A immunoexpression					Total
	0	1	2	3	4	
High	0	2	16	7	2	27
Intermediate	6	46	20	12	0	84
Total	6	48	36	19	2	111

$p<0.001$, Pearson χ^2

Not surprisingly, a similar association was found cyclin A expression and risk group. A larger proportion of high risk tumours (92.5%) showed high levels of cyclin A expression compared to intermediate risk tumours (38.1%) ($p<0.001$, Pearson χ^2) (Table 44).

Twenty six of the 36 (72.2%) stage IV tumours showed high cyclin A expression, in contrast to 5 of 18 (27.8%) stage I tumours, 15 of 31 (48.4%) stage II tumours and 11 of 26 (42.3%) stage III tumours. Although there appears to be a direct association between higher stage and higher levels of cyclin A expression, this finding did not reach statistical significance ($p=0.258$, Pearson χ^2). Twenty eight of the 39 (71.8%) patients who died had tumours that showed high cyclin A immunoexpression. Eight of 27 (29.6%) disease free cases and 6 of 10 (60%) cases with residual/recurrent disease also expressed high cyclin A. Cyclin A immunoexpression was not significantly associated with patient outcome ($p=0.394$, Pearson χ^2).

4.1.2.6 p-GLYCOPROTEIN RESULTS

In total, 109 tumours showed immunoreactivity for p-glycoprotein. Of these 109 cases, 29 showed 1+, 36 showed 2+, 30 showed 3+ and 14 showed 4+ staining. Twenty nine cases were negative.

Staining was seen predominantly in tubular epithelial cells and blastemal cells (Plate 7b,c). Furthermore, the most intense staining was seen in the epithelial cells (Plate 7b). Of the 109 cases that showed p-glycoprotein immunoreactivity, 104 showed staining in either epithelial or blastemal cells or both. In nine cases, mesenchymal cells showed p-glycoprotein immunoreactivity (Plate 7d).

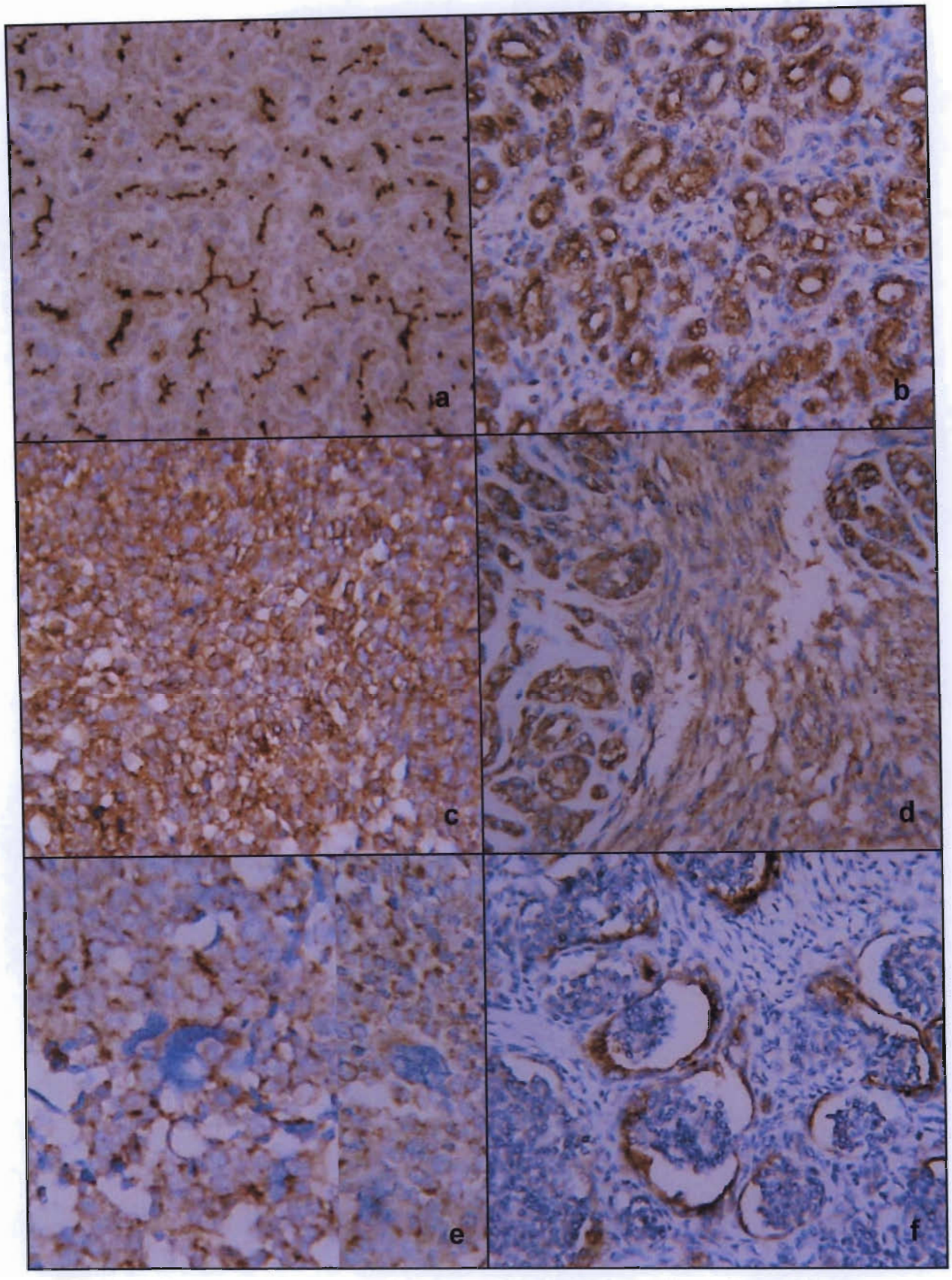


Plate 7: p-glycoprotein immunostaining, **a**. Control slide – liver showing intense membrane staining of the bile canaliculi, **b**. membrane staining of tubular epithelium – more than 25% of cells are positive, **c**. high staining – more than 25% of blastemal cells are positive, **d**. spindle shaped stromal cells showing high staining, **e**. expression in anaplastic cells from two different tumours, **f**. more staining in Bowman's capsule compared to the glomerular tuft in immature neoplastic glomeruloid structures (anti-p-glycoprotein, C219)

In the anaplastic tumours, most of the anaplastic cells were immunoreactive for p-glycoprotein (Plate 7e). Immature Bowman's epithelium showed more intense staining than the tuft of immature glomeruli (Plate 7f). Sixty one of the cases in treatment group A showed p-glycoprotein expression in epithelial cells, 43 in blastemal cells and 8 in stromal cells. In treatment group B, 6 cases expressed p-glycoprotein in epithelial cells, 14 in blastemal cells and 1 in stromal cells.

Table 45: Correlation of p-glycoprotein expression with histological classification – treatment group A

SIOP Classification	p-glycoprotein immunoexpression					Total
	0	1	2	3	4	
Blastemal	0	3	7	5	1	16
Diffuse anaplastic	2	1	1	3	4	11
Epithelial	0	1	3	2	0	6
Mixed	8	10	10	6	2	36
Regressive	6	8	7	4	5	30
Stromal	5	1	3	2	1	12
Total	21	24	31	22	13	111

p=0.165, Pearson χ^2

In each of the mixed, regressive and stromal tumours approximately half the number of cases showed high p-glycoprotein expression. The blastemal, diffuse anaplastic and epithelial tumours showed predominantly high expression (81.3%, 72.7% and 83.3%, respectively). There appears to be a tendency for both blastemal and diffuse anaplastic tumours to show high p-glycoprotein expression, although not significant (Table 45).

Table 46: Correlation of p-glycoprotein expression with risk group – treatment group A

SIOP Risk group	p-glycoprotein immunoexpression					Total
	0	1	2	3	4	
High	2	4	8	8	5	27
Intermediate	19	20	23	14	8	84
Total	21	24	31	22	13	111

p=0.168, Pearson χ^2

Similarly, there was no association between p-glycoprotein expression and risk group (p=0.168, Pearson χ^2), but the majority of high risk tumours showed high expression compared to intermediate risk tumours (77.8% vs 65.5%) (Table 46).

Table 47: Correlation of p-glycoprotein expression with patient outcome – treatment group A

Outcome	p-glycoprotein immunoexpression					Total
	0	1	2	3	4	
Disease free	6	6	4	5	6	27
Residual/recurrent	2	3	3	1	1	10
Dead	4	8	12	11	4	39
Lost	9	7	12	5	2	35
Total	21	24	31	22	13	111

p=0.338, Pearson χ^2

p-glycoprotein expression was not associated with patient outcome (p=0.338, Pearson χ^2) (Table 47). Twelve of the 21 negative cases with adequate follow up consisted of 2 high risk tumours and 10 intermediate risk tumours. Furthermore, this group consisted of two stage I, four stage II, two stage III and four stage IV tumours.

There was no association between p-glycoprotein expression and patient age (p=0.058, Spearman correlation coefficients), administration of preoperative chemotherapy (p=0.473, χ^2) and clinicopathological stage (p=0.926, Pearson χ^2).

4.1.3 INTER-RELATIONS BETWEEN MARKERS

Because of the known inter-relations between the markers Spearman correlation co-efficients were calculated to evaluate these inter-relations between markers. Significant correlations were found for p53 and the following markers; bcl-2 (p=0.0002), p21 (p=0.0087) and cyclin A (p=0.0184). For cyclin A significant correlations were found with p53 (p=0.0184), p21 (p=0.0014), p-glycoprotein (p=0.0003) and pRb (p<0.0001).

Seventy seven of the 97 (79.3%) tumours that showed low p53 also showed low bcl-2 expression and 74 of the 82 (90.2%) tumours that showed low bcl-2 also showed low p53. When comparing high expression, only 6 of the 14 tumours that expressed high p53 showed high bcl-2 and 6 of the 29 tumours that expressed high bcl-2 also expressed high p53. From this comparison it appears that nephroblastoma with low bcl-2 expression is likely to express low levels of p53 and vice versa ($p=0.0002$, Spearman correlation coefficient).

Six of the 57 (10.5%) tumours that expressed high cyclin A also expressed p21 in a similar proportion of cells, in contrast to only 1 of the 54 (1.9%) tumours that showed low cyclin A expression. Conversely, high cyclin A expression was seen in 6 of the 7 (85.7%) tumours that showed high p21 expression and 51 of the 104 (49.0%) tumours that showed low p21 expression. Nephroblastomas expressing high p21 tended to also express high cyclin A ($p=0.0014$, Spearman correlation coefficient).

Fifty two of the 54 (96.3%) tumours that expressed low cyclin A also showed low p53 expression, while 12 of the 14 (85.7%) tumours that showed high p53 expression also showed high cyclin A expression. Fifty two of the 97 (53.6%) tumours that expressed low p53 showed low cyclin A expression. The tendency was for tumours that expressed low cyclin A to be associated with low p53 expression and tumours that expressed high p53 to be associated with high cyclin A expression ($p=0.0184$, Spearman correlation coefficient).

Forty six of the 77 (59.7%) tumours that expressed high pRb cells also showed high cyclin A expression, in contrast, 23 of the 34 (67.6%) tumours that expressed low pRb showed high cyclin A expression. Conversely, 46 of the 57 (80.7%) tumours that showed high cyclin A staining expressed high pRb and 23 of the 54 (42.6%) tumours that showed low cyclin A expression displayed low pRb expression. The majority of tumours that expressed high cyclin A had a tendency to also express high pRb ($p<0.0001$, Spearman correlation coefficient).

Forty four of the 57 (77.2%) tumours that expressed high cyclin A also showed high p-glycoprotein expression, while only 22 of the 54 (40.7%) tumours that showed low cyclin A expression displayed high p-glycoprotein. Conversely, 44 of the 66 (66,7%)

tumours that expressed high p-glycoprotein also expressed high cyclin A and 32 of the 45 (71.1%) that showed low p-glycoprotein expression also displayed low cyclin A. The majority of tumours with high cyclin A had coexpression of high p-glycoprotein and the majority of tumours with low p-glycoprotein had low cyclin A (p=0.0003, Spearman correlation coefficient).

4.1.4 SURVIVAL ANALYSIS

The regression model included covariates such as age, gender, stage and preoperative chemotherapy status to adjust for possible confounders. Hazard ratios and 95% confidence intervals were determined. The marker score was used as a continuous variable. In the joint model where all markers were included cyclin A was the only marker associated with survival (hr=1.7; 95%CI 1.1-2.7; p=0.032). When markers were included in the model one at a time p53 (hr=1.4; 95%CI 1.1-1.8; p=0.019), cyclin A (hr=1.7; 95%CI 1.2-2.4; p=0.002) and pRb (hr=1.5; 95%CI 1.0-2.1; p=0.033) were positively associated with survival.

Graphical representation of the significant markers was estimated by Kaplan-Meier graphs (Figures 8,9,10).

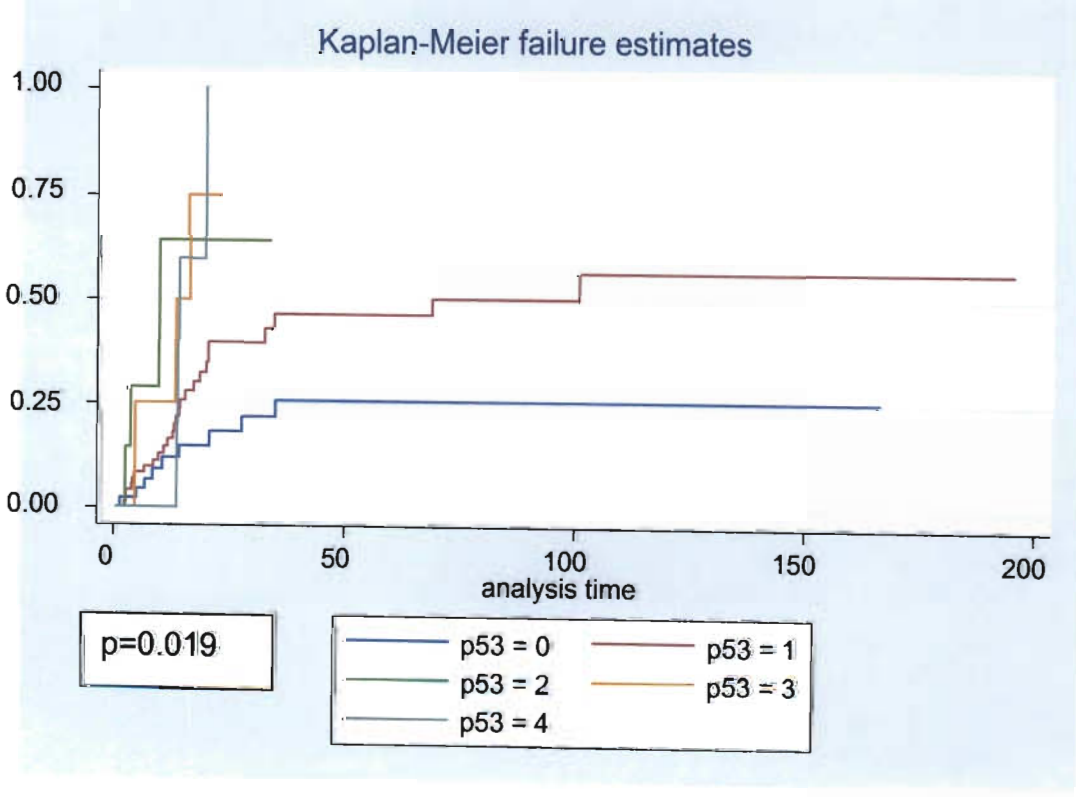


Figure 8: Kaplan-Meier estimates for p53 expression

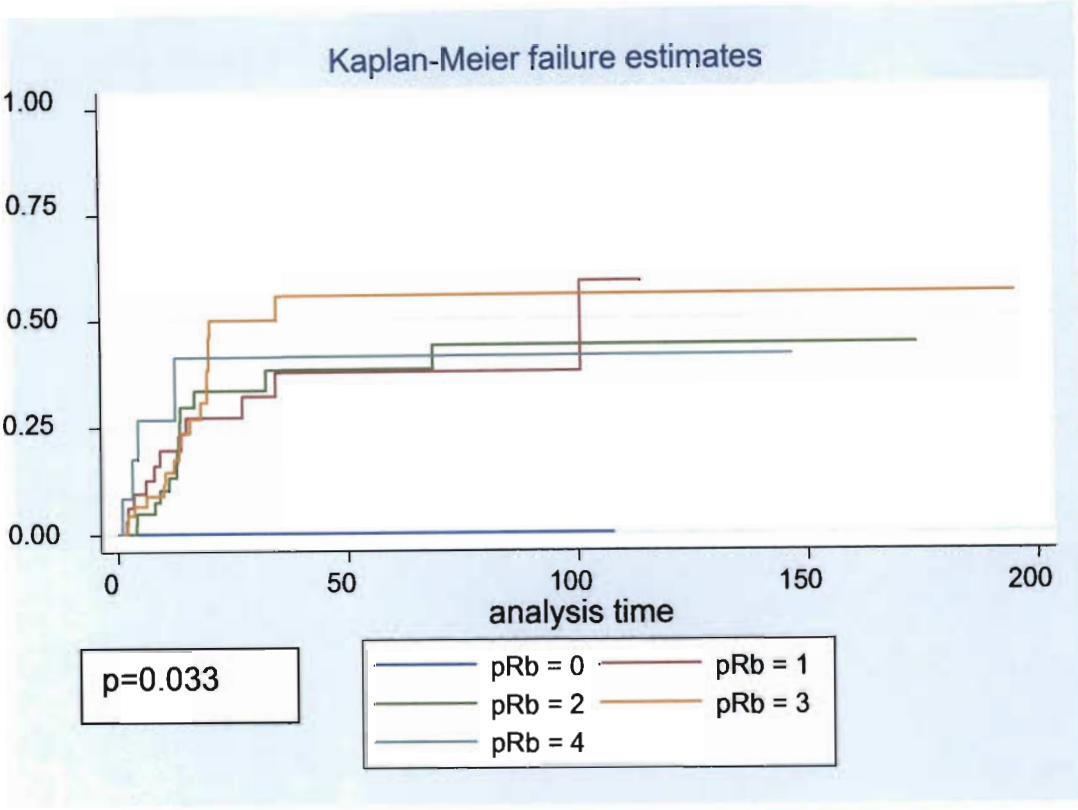


Figure 9: Kaplan-Meier estimates for pRb expression

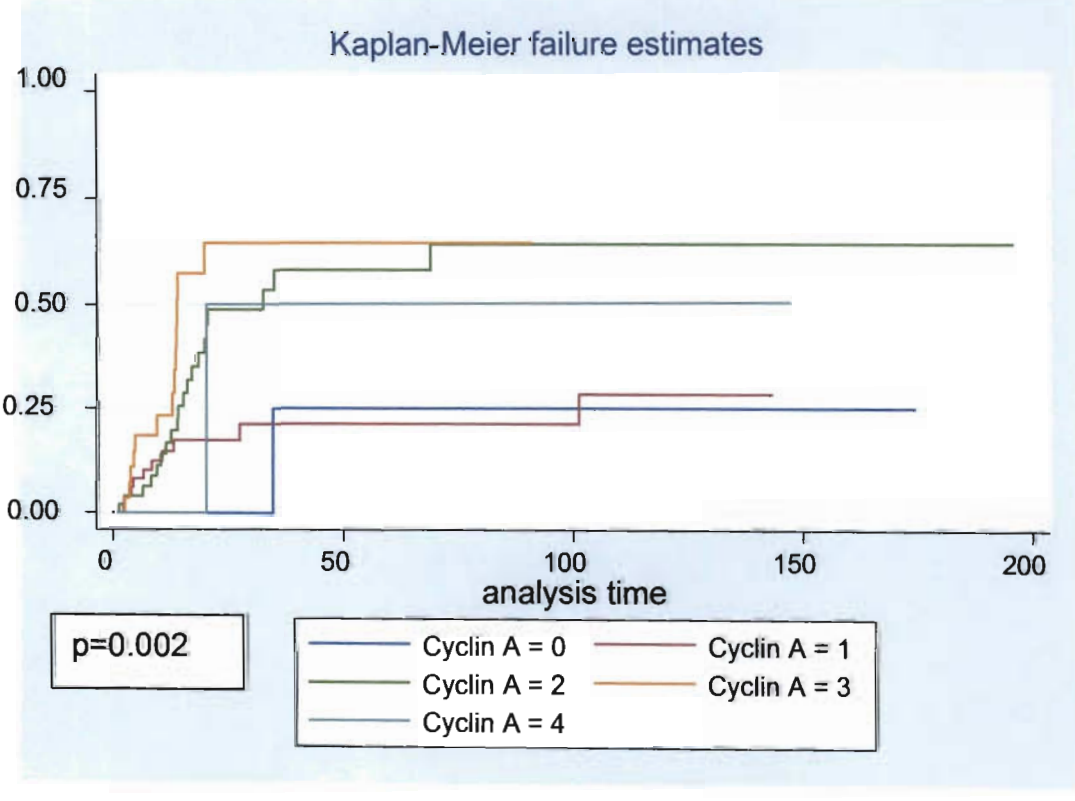


Figure 10: Kaplan-Meier estimates for cyclin A expression

In addition to the survival analysis, crude death rates (CDR) were determined in relation to marker expression. For most markers there was an increase in crude deaths rates with an increase in marker scores. Immunoexpression scores for p53 produced the highest crude death rates. The crude death rate for p53 immunoexpression score of 3+ was 5.4 deaths/100 months. The second highest crude death rate was for a p53 score of 4+ which was 4.7 deaths/100 months.

4.2 MICROSATELLITE STUDY

Table 48: Clinicopathological data for 22 patients in the microsatellite study that did not receive preoperative chemotherapy – treatment group B1

Case No	Age (months)	Gender	Race/ Ethnic	SIOP Classific	Risk Group	Stage	Outcome	Follow up (months)
1	22	F	African	Mixed	Intermediate	1	A	195
2	60	F	African	Mixed	Intermediate	4	C	0.8
3	24	F	African	Mixed	Intermediate	1	L	53.9
4	40	M	African	Mixed	Intermediate	3	L	15.6
5	48	F	African	Blastemal	Intermediate	4	C	9.8
7	12	F	African	Stromal	Intermediate	3	L	35.1
8	9	F	African	Blastemal	Intermediate	1	A	152.9
9	48	F	African	Mixed	Intermediate	2	L	5.5
10	10	M	African	Mixed	Intermediate	2	C	3.3
11	48	F	African	Mixed	Intermediate	3	L	7.7
12	36	F	African	Mixed	Intermediate	4	C	34.6
13	16	M	African	Blastemal	Intermediate	3	A	165.9
14	36	F	African	Mixed	Intermediate	2	L	7.7
15	18	F	African	Mixed	Intermediate	3	A	72.2
16	20	M	African	Mixed	Intermediate	1	L	13.1
17	24	M	Indian	Mixed	Intermediate	3	A	146.5
18	36	M	African	Mixed	Intermediate	3	L	4.5
19	24	M	African	Mixed	Intermediate	3*	L	4.5
21	4	F	Indian	Mixed	Intermediate	1	A	90.5
22	6	F	African	Mixed	Intermediate	1	L	1.8
23	14	F	African	Mixed	Intermediate	1	L	4
35	72	F	African	Blastemal	Intermediate	4	C	12.4

Key:

* one case with stage V disease was substaged as listed above

A – Alive and disease free

B – Residual/recurrent disease

C – Dead

L – Lost to follow-up

M – Male

F – Female

N – No

Y – Yes

Table 49: Clinicopathological data for 48 cases in the microsatellite study that received preoperative chemotherapy – treatment group A1

Case No	Age (months)	Gender	Race/ Ethnic	SIOP Classific	Risk Group	Stage	Outcome	Follow up (months)
24	48	F	African	Mixed	Intermediate	3	L	8
27	19	F	African	Regressive	Intermediate	4	C	19.8
28	23	F	African	Regressive	Intermediate	2	A	173.7
29	60	F	African	Stromal	Intermediate	4	C	9
33	48	M	African	Mixed	Intermediate	4	C	100.5
34	27	F	African	Mixed	Intermediate	3	C	3.4
36	108	F	African	Stromal	Intermediate	3	L	15.4
37	120	F	African	Regressive	Intermediate	4	C	7.9
38	36	M	African	Regressive	Intermediate	1	A	142.5
41	36	F	African	Stromal	Intermediate	4	L	69.7
42	24	F	African	Mixed	Intermediate	2	C	11
44	120	M	African	D. Anaplastic	High	3	C	13.2
47	66	M	African	D. Anaplastic	High	4	C	19.4
48	36	F	African	D. Anaplastic	High	4	C	13.5
52	84	M	African	Regressive	Intermediate	2	L	2.5
53	36	M	African	Regressive	Intermediate	4	B	8.4
54	16	M	African	Mixed	Intermediate	2	L	13.7
55	9	F	African	Regressive	Intermediate	1	L	21.6
57	72	M	African	Mixed	Intermediate	3	C	1.7
58	28	M	African	Regressive	Intermediate	3	L	31.4
60	26	M	African	Mixed	Intermediate	4	B	19
63	36	F	African	Regressive	Intermediate	3	L	22.2
65	24	M	African	Mixed	Intermediate	2	L	8.4
66	84	F	African	Mixed	Intermediate	4	B	25.4
67	22	F	African	Epithelial	Intermediate	2	A	106.9
68	60	F	African	Regressive	Intermediate	2	L	14.1
69	17	M	African	Stromal	Intermediate	2	L	6.9
70	9	F	African	Epithelial	Intermediate	1	A	114
71	11	F	African	Epithelial	Intermediate	1	L	8.6
72	13	M	African	Mixed	Intermediate	1*	B	65.7
73	168	M	African	Mixed	Intermediate	3	L	26.5
76	19	F	African	Mixed	Intermediate	2	A	102.2
77	14	M	African	Mixed	Intermediate	1	L	7.4
78	10	M	African	D. Anaplastic	High	4	C	10.1

Case No	Age (months)	Gender	Race/ Ethnic	SIOP Classific	Risk Group	Stage	Outcome	Follow up (months)
80	12	M	African	Epithelial	Intermediate	2	L	12.4
81	42	M	African	D. Anaplastic	High	2	L	11.7
82	16	F	African	Mixed	Intermediate	1	A	72
83	48	F	African	Stromal	Intermediate	3	L	25.7
84	12	M	African	Regressive	Intermediate	3	L	8.2
88	24	M	African	Mixed	Intermediate	4	C	16.6
89	12	M	African	Stromal	Intermediate	1	L	18.8
90	21	M	African	D. Anaplastic	High	3	L	22.6
91	46	M	African	D. Anaplastic	High	3	C	12.6
92	48	F	African	D. Anaplastic	High	4	C	4
96	48	M	African	D. Anaplastic	High	4	C	13
99	72	M	African	Blastemal	High	4	C	2
102	48	F	African	Regressive	Intermediate	1	A	74
115	72	F	African	D. Anaplastic	High	4	C	13.5

Key:

* one case with stage V disease was substaged as listed above

A – Alive and disease free B – Residual/recurrent disease

C – Dead L – Lost to follow-up

M – Male F – Female

N – No Y – Yes

4.2.1 CLINICAL DATA

4.2.1.1 Patient age

Figure 11 demonstrates the age distribution of the 70 patients in the microsatellite study. The ages ranged from 4 months to 14 years. The mean age was 38.2 months and the median age was 27.5 months. Thirty six (51.4%) patients were between 12 and 36 months of age. Sixty four (91.4%) patients were 6 years or younger.

The mean and median ages for the 22 patients in treatment group B1 were 28.5 months (range 4 months to 6 years) and 24 months, respectively. For the 48 patients in treatment group A1 the mean and median ages were 42.7 months (range 9 months to 14 years) and 36 months, respectively.

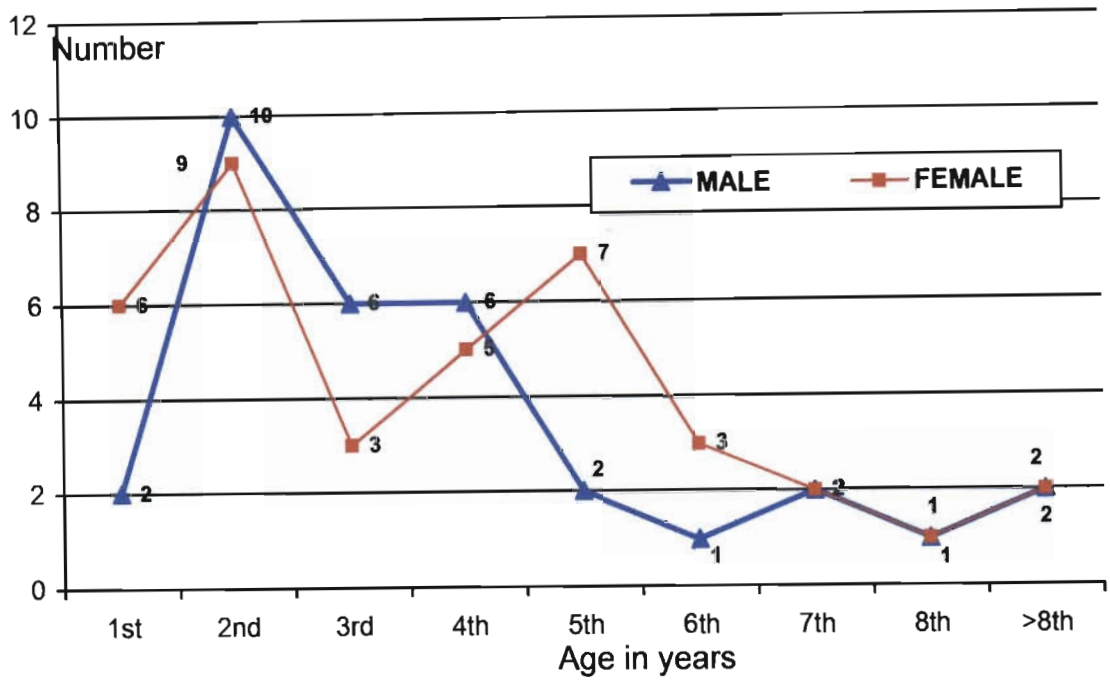


Figure 11: Age and gender distribution (n=70)

4.2.1.2 Gender

The gender distribution and its relationship to age are shown in Figure 11. The female:male ratio was 1,19:1.0 (38:32). The mean age and median age for male patients was 38.7 months and 25 months, respectively. For female patients the mean age and median age was 37.9 months and 36 months, respectively.

For treatment group A1 the mean and median ages for male patients were 42.7 months and 28 months respectively and for female patients these were 42.7 months and 36 months respectively. For the smaller treatment group B1 the mean and median ages for male patients were 24.3 months and 24 months respectively and for females these were 30.5 months and 24 months respectively.

4.2.1.3 Race/Ethnic Group

Overall there were 68 (97.1%) African patients and 2 (2.9%) Asian patients of Indian descent. Treatment group B1 was composed of 20 (90.9%) African patients and the 2 (9.1%) Asian patients. All 48 patients in treatment group A1 were African. The number of Asian patients was too small for any comparison between race groups.

4.2.1.4 Clinicopathological Stage

The stages are shown in Table 50. Forty patients (57.0%) had either stage III or IV disease (high stage disease), while 30 patients (43,0%) had stage I or II disease (low stage disease). Two patients had stage V disease. These were substaged, according to SIOP guidelines, as one stage I/I and one stage III and included in the analysis.

Table 50: Clinicopathological stage (n=70)

Stage	Without preop chemo	With preop chemo	Total	%
I	7	9	16	23.0
II	3	11	14	20.0
III	8	12	20	28.5
IV	4	16	20	28.5
Total	22	48	70	100

Two stage V cases were substaged and included above

4.2.1.5 Preoperative Chemotherapy

Preoperative chemotherapy was administered to 48 (68.6%) patients (treatment group A1). Since this was a retrospective study patients were recruited over many years and the chemotherapeutic regimens varied over this period.

The two treatment groups were analysed separately, however, treatment group B1 (n=22) was too small for any significant statistical analysis.

4.2.1.6 SIOP histological classification and risk group

Mixed histology was the commonest type seen in both treatment groups. The four blastemal histology tumours seen in treatment group B1 were classified as intermediate risk tumours according to SIOP classification system. There were 10 diffuse anaplastic tumours and one with blastemal histology in treatment group A1 (Table 51).

Table 51: SIOP Histological classification

Classification	With preop chemo	No preop chemo	Total
Blastemal	1	4	5
Diffuse anaplastic	10	0	10
Epithelial	4	0	4
Mixed	15	17	32
Regressive	12	0	12
Stromal	6	1	7
Total	48	22	70

Treatment group B1 did not have any tumours with high risk histology (Tables 52). This limited any further analysis based on histological classification and risk group in this patient cohort. Therefore, the analysis that follows is focused on the 48 patients in treatment group A1 unless otherwise stated.

Table 52: SIOP Risk group

Classification	With preop chemo	No preop chemo	Total
High	11	0	11
Intermediate	37	22	59
Total	48	22	70

Nine of the 10 patients with diffuse anaplastic tumours had high stage disease and the only patient with blastemal histology also had high stage disease (Table 53). Eight of the ten (80%) patients with diffuse anaplastic and the one patient with blastemal histology died of disease. All patients with epithelial histology tumours had low stage disease and there were no deaths with this subtype. The remaining tumour types were distributed among the stages. There were five deaths (33.3%) in the group with mixed histology, 2 stage IV, 2 stage III and 1 stage I. There were only 2 deaths in patients with regressive histology and 1 with stromal histology. These 3 patients had stage IV disease.

Table 53: Mortality: histological subtypes and clinicopathological stage – treatment group A1

Classification	No. of deaths	Stage				Total
		I	II	III	IV	
Blastemal	1	-	-	-	1 (1)	1
Diffuse anaplastic	8	-	1	3 (2)	6 (6)	10
Epithelial	0	2	2	-	-	4
Mixed	5	3	4 (1)	4 (2)	4 (2)	15
Regressive	2	3	3	3	3 (2)	12
Stromal	1	1	1	2	2 (1)	6
Total	17	9	11	12	16	48

p=0.551 (Pearson χ^2) Number of deaths in brackets.

Table 54: Mortality: histological risk group and clinicopathological stage – treatment group A1

Classification	No. of deaths	Stage				Total
		I	II	III	IV	
High	9	0	1	3 (2)	7 (7)	11 (9)
Intermediate	8	9	10 (1)	9 (2)	9 (5)	37 (8)
Total	17	9	11	12	16	48

Number of deaths in brackets. p=0.025 (Pearson χ^2)

There were 11 high risk tumours (22.9%) consisting of 1 (2.1%) blastemal and 10 (20.8%) anaplastic tumours (Tables 53 & 54). These occurred in eight male patients (72.7%) and three female patients (27.3%%). The average age for this category was 52.8 months and the median age was 48 months. The ages ranged from 10 months to 10 years. Seven patients had stage IV disease, 3 had stage III disease and 1 had stage II disease. Nine of the 11 (81.8%) patients who had high risk tumours and sufficient follow up died of disease. Two patients were lost to follow-up. Of the 9 patients that died, 2 had stage III disease and 7 had stage IV disease.

There were 37 intermediate risk tumours, 20 occurred in female patients and 17 in male patients. The age range was 9 months to 14 years. The mean and median age for this subtype was 39.7 months and 26 months respectively. There were 9 patients with stage I disease, 10 with stage II disease, 9 with stage III disease and 9

with stage IV disease. The two stage V tumours showed a mixed histological type, intermediate risk.

Eight (21.6%) patients with intermediate risk nephroblastomas died. Five of these 8 patients had stage IV disease, two had stage III disease and one had stage II disease.

4.2.1.7 Patient Outcome

Of the 70 patients studied, thirteen patients were alive and disease free, 4 had either residual/recurrent disease and 22 died of disease, while 31 patients were lost to follow-up after varying periods of initial follow-up.

In treatment group A1, seven were disease free at last follow up, 4 had residual disease, 17 died and 20 were lost to follow up. Four of the 7 disease free patients had stage I disease and 3 had stage II disease. Three of the four patients with residual/recurrent disease had stage IV disease. Sixteen of the 17 patients who died in this group had high stage disease (4 stage III and 12 stage IV). Furthermore, all patients who were alive (disease free or with residual/recurrent disease) had intermediate risk tumours. Nine of the 17 patients who died had high risk tumours.

In treatment group B1, 6 patients were disease free, 5 had died and 11 were lost to further follow up. Of the 6 patients that were disease free 3 had stage I disease and 3 had stage III disease. Four of the five patients that died had stage IV disease. All patients in this group had intermediate risk tumours. Two of the 5 patients that died had blastemal histology.

4.2.2 DNA extraction

DNA extraction was attempted in the cases which had adequate viable tumour and normal tissue. Sufficient good quality, amplifiable DNA acceptable for performing the microsatellite analysis was obtained in 70 cases.

4.2.3 Insulin PCR results

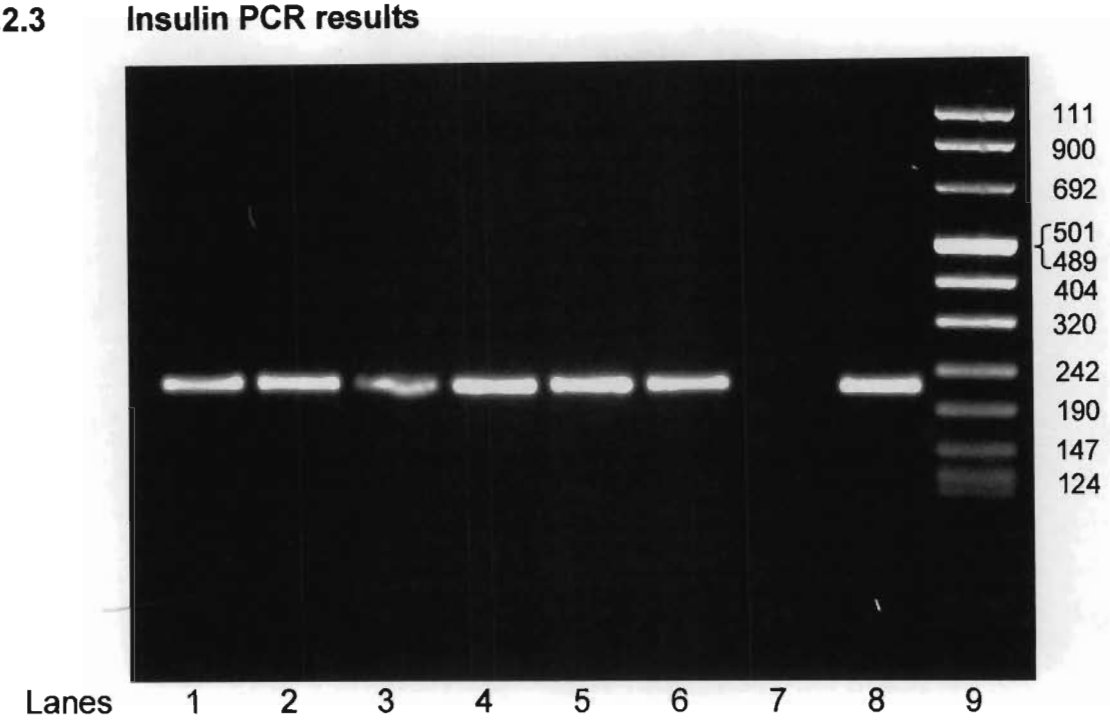


Figure 12: 2% agarose gel electrophoresis of PCR products amplified across the *insulin* exon 2 region (product size 236 base pairs). Lanes 1 to 6 show PCR products of study cases. Lane 7 shows the negative control and Lane 8 the positive control. Lane 9 contains the molecular weight marker.

The magnesium titration optimisations for microsatellite PCR are shown in Appendices 4, 5 and 6.

4.2.4 11p RESULTS

Table 55: Results for 11p microsatellite markers for cases in treatment group B1

Case	D11S902	D11S904	D11S907	D11S922	D11S930	D11S935	LOH MSI status	# of markers
1	NC2	MSI	NC1	NC2	NC2	NC2	MSI	1
2	MSI	NC1	MSI	NC2	MSI	LOH	LOH MSI	1 3
3	LOH	NC1	LOH	NC2	LOH	NC1	LOH	3
4	NC1	NC2	NC1	MSI	NC1	NC1	MSI	1
5	NC1	NC2	LOH	LOH	NC1	NC1	LOH	2
7	LOH	NC1	LOH	NC1	NC2	NC1	LOH	2
8	NC1	NC2	NC1	NC1	NC1	NC1	-	-
9	NC1	MSI	NC2	NC1	NC1	NC1	MSI	1
10	NC2	NC1	NC1	NC2	NC2	NC1	-	-
11	NC1	LOH	NC1	NC1	NC2	LOH	LOH	2
12	NC1	MSI	LOH	NC1	NC2	LOH	LOH MSI	2 1
13	NC1	NC1	NC1	NC1	NC1	MSI	MSI	1
14	NC1	NC1	NC1	NC1	NC1	MSI	MSI	1
15	NC1	LOH	NC1	LOH	LOH	NC1	LOH	3
16	LOH	NC1	LOH	NC1	LOH	LOH	LOH	4
17	NC1	NC2	NC2	NC1	NC1	NC1	-	-
18	NC1	NC1	LOH	NC1	NC1	NC1	LOH	1
19	NC1	MSI	NC2	NC2	NC2	MSI	MSI	2
21	NC2	LOH	NC2	NC1	NC2	LOH	LOH	2
22	NC1	NC1	NC1	LOH	LOH	NC2	LOH	2
23	NC1	NC1	NC1	NC1	LOH	NC2	LOH	1
35	NC1	NC1	NC1	NC1	NC1	NC2	-	-

Key:

LOH - Allelic imbalance/Loss of heterozygosity

MSI - Microsatellite instability

NC1 - No change homozygous, non-informative case

NC2 - No change heterozygous, informative case

Table 56: Results for 11p microsatellite markers for cases in treatment group A1

Case	D11S902	D11S904	D11S907	D11S922	D11S930	D11S935	LOH MSI status	# of markers
24	NC1	NC2	LOH	NC1	NC2	NC1	LOH	1
27	LOH	NC1	NC1	LOH	NC1	NC1	LOH	2
28	NC1	NC1	NC1	NC1	NC2	MSI	MSI	1
29	LOH	LOH	LOH	NC2	NC2	NC2	LOH	3
33	NC2	LOH	LOH	NC1	NC1	NC1	LOH	2
34	NC1	NC2	NC1	NC1	NC1	NC1	-	-
36	NC1	NC1	NC1	LOH	NC1	NC2	LOH	1
37	NC2	NC1	NC1	NC1	NC2	NC1	-	-
38	LOH	LOH	LOH	NC1	NC2	LOH	LOH	4
41	NC1	NC1	NC1	NC2	NC2	NC2	-	-
42	NC1	MSI	NC1	NC1	NC2	NC2	MSI	1
44	LOH	NC2	LOH	NC1	NC1	NC1	LOH	2
47	LOH	MSI	NC1	NC1	NC2	NC1	LOH MSI	1 1
48	NC1	NC1	NC2	LOH	NC1	NC2	LOH	1
52	NC1	NC1	NC1	NC1	LOH	NC1	LOH	1
53	NC1	NC2	NC2	NC2	NC2	NC1	-	-
54	LOH	NC1	LOH	NC1	NC1	NC1	LOH	2
55	NC2	NC1	NC2	LOH	NC1	NC1	LOH	1
57	NC1	LOH	NC1	NC1	LOH	LOH	LOH	3
58	NC1	LOH	NC1	NC2	NC2	NC1	LOH	1
60	NC1	NC2	NC1	NC1	NC1	NC1	-	-
63	NC1	LOH	NC1	NC1	NC2	NC1	LOH	1
65	LOH	NC1	NC1	LOH	NC2	NC2	LOH	2
66	LOH	NC1	LOH	LOH	NC1	MSI	LOH MSI	3 1
67	NC2	NC2	NC1	NC2	NC2	NC1	-	-
68	LOH	LOH	NC1	LOH	NC1	NC1	LOH	3
69	LOH	NC1	LOH	NC1	LOH	NC1	LOH	3
70	NC1	NC1	NC1	NC1	NC1	LOH	LOH	1
71	NC1	NC1	NC1	NC1	NC1	NC1	NI	-
72	NC1	NC1	NC1	NC2	NC2	LOH	LOH	1
73	NC2	NC2	MSI	NC2	LOH	LOH	LOH MSI	2 1

Case	D11S902	D11S904	D11S907	D11S922	D11S930	D11S935	LOH MSI status	# of markers
76	NC1	NC2	MSI	NC1	NC1	NC2	MSI	1
77	LOH	NC1	NC2	LOH	NC1	NC2	LOH	2
78	NC2	NC1	NC1	NC1	NC1	NC1	-	-
80	NC2	NC1	NC1	NC1	NC2	NC1	-	-
81	LOH	NC1	MSI	NC1	NC1	NC1	LOH MSI	1 1
82	NC1	NC1	NC1	LOH	NC1	NC1	LOH	1
83	LOH	LOH	NC1	NC2	LOH	NC1	LOH	3
84	LOH	NC1	NC1	NC1	NC2	LOH	LOH	2
88	NC2	NC1	NC1	NC1	NC2	NC2	-	-
89	NC2	NC1	NC2	NC1	NC1	NC1	-	-
90	NC1	NC2	NC1	LOH	NC1	NC1	LOH	1
91	NC1	NC1	LOH	NC1	NC2	NC1	LOH	1
92	NC1	NC1	NC1	LOH	LOH	NC1	LOH	2
96	NC2	NC1	NC2	LOH	NC1	LOH	LOH	2
99	NC2	NC1	NC1	NC2	NC2	NC1	-	-
102	LOH	LOH	LOH	LOH	LOH	LOH	LOH	6
115	NC1	NC1	NC1	NC1	NC1	NC1	NI	

Key:

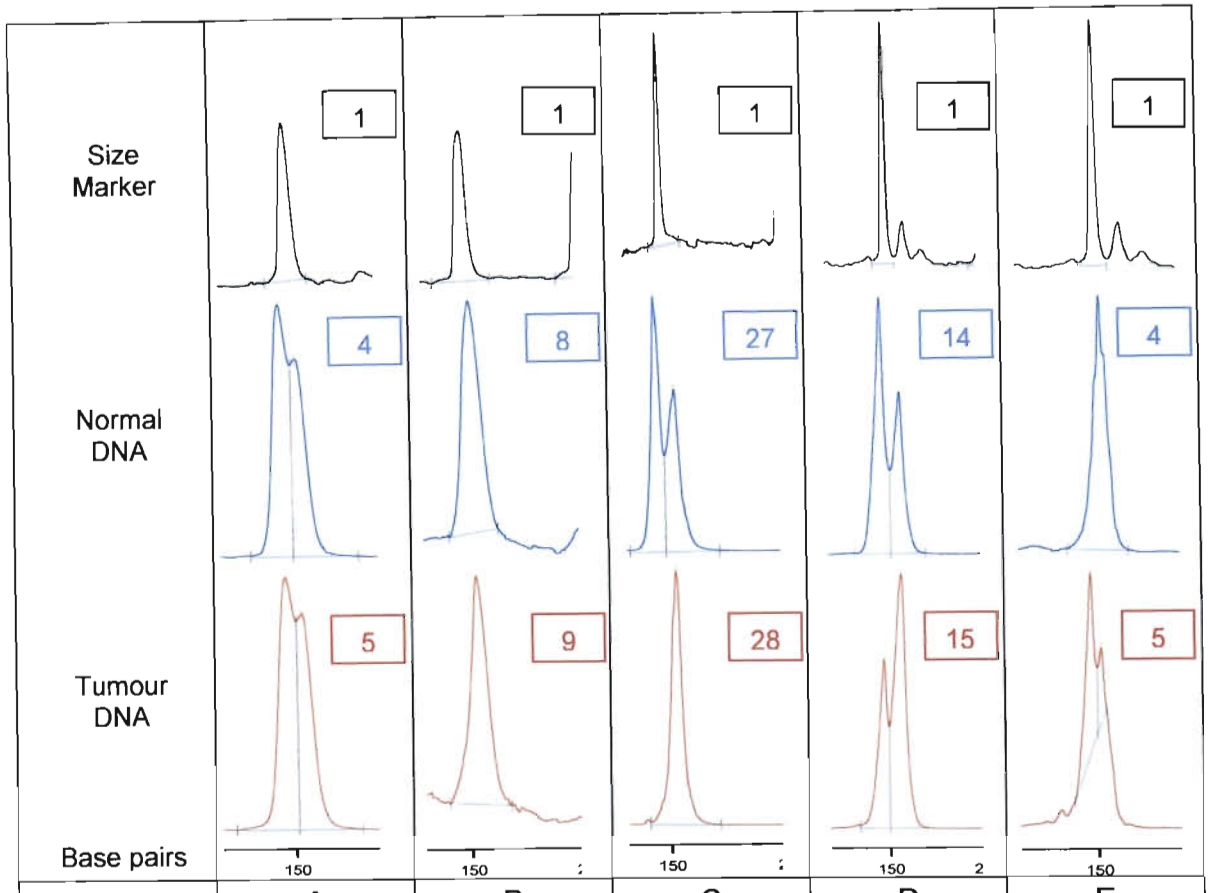
LOH - Allelic imbalance/Loss of heterozygosity

MSI - Microsatellite instability

NC1 - No change homozygous, non-informative case

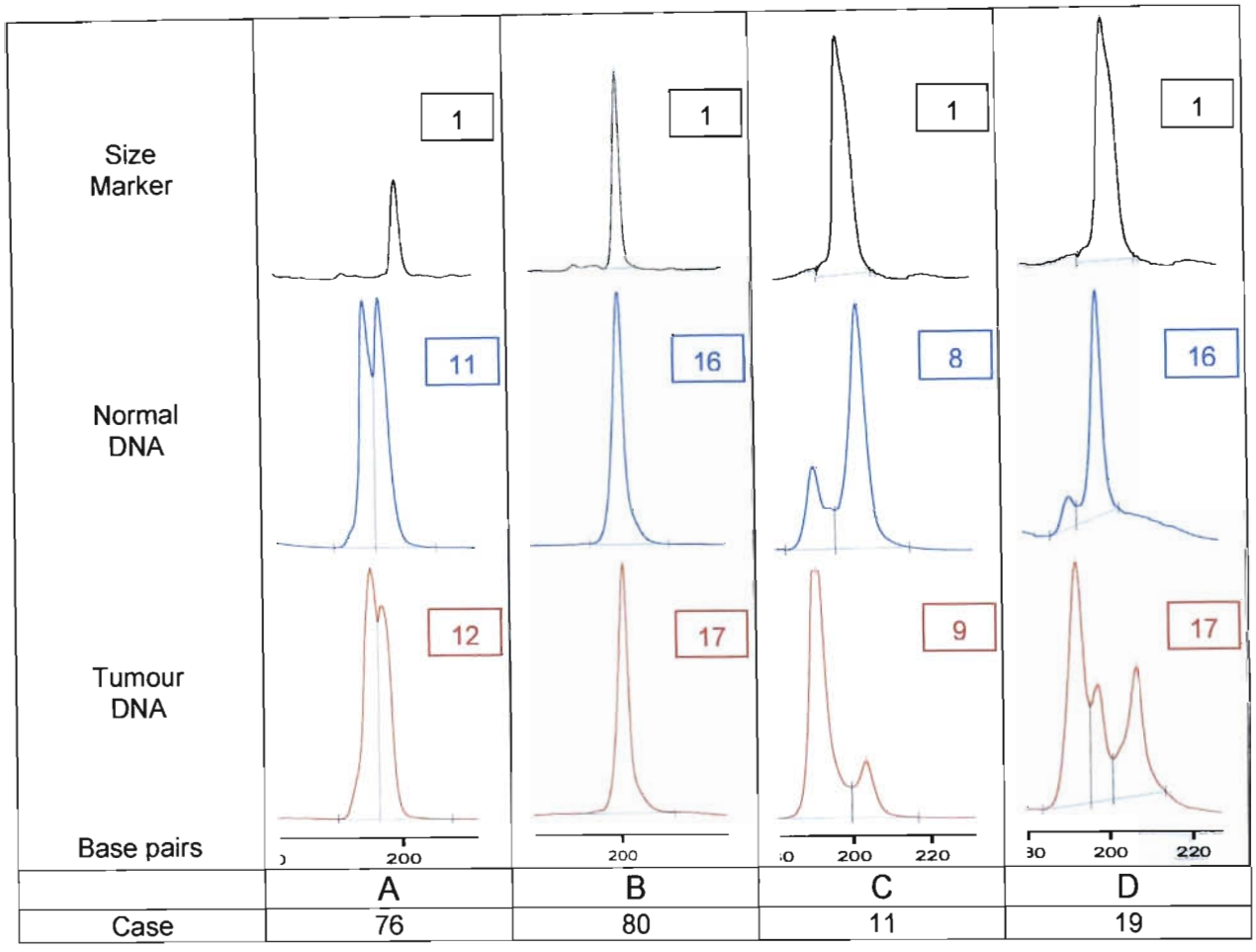
NC2 - No change heterozygous, informative case

NI - Not informative for all 11p markers



	Lane	Peak area	Size (base pairs)
A	4 (67N)	553.4	146.2
		386.7	153.0
	5 (67T)	675.3	146.9
		504.8	153.5
B	8 (12N)	93.9	152.1
	9 (12T)	78.3	153.0
C	27 (16N)	387.3	146.1
		301.7	154.0
	28 (16T)	532.6	153.3
D	14 (65N)	1667.2	147.1
		1097.7	156.7
	15 (65T)	1094.5	147.4
		2148.1	156.7
E	4 (2N)	221.5	151.7
	5 (2T)	103.7	147.3
		26.1	151.1

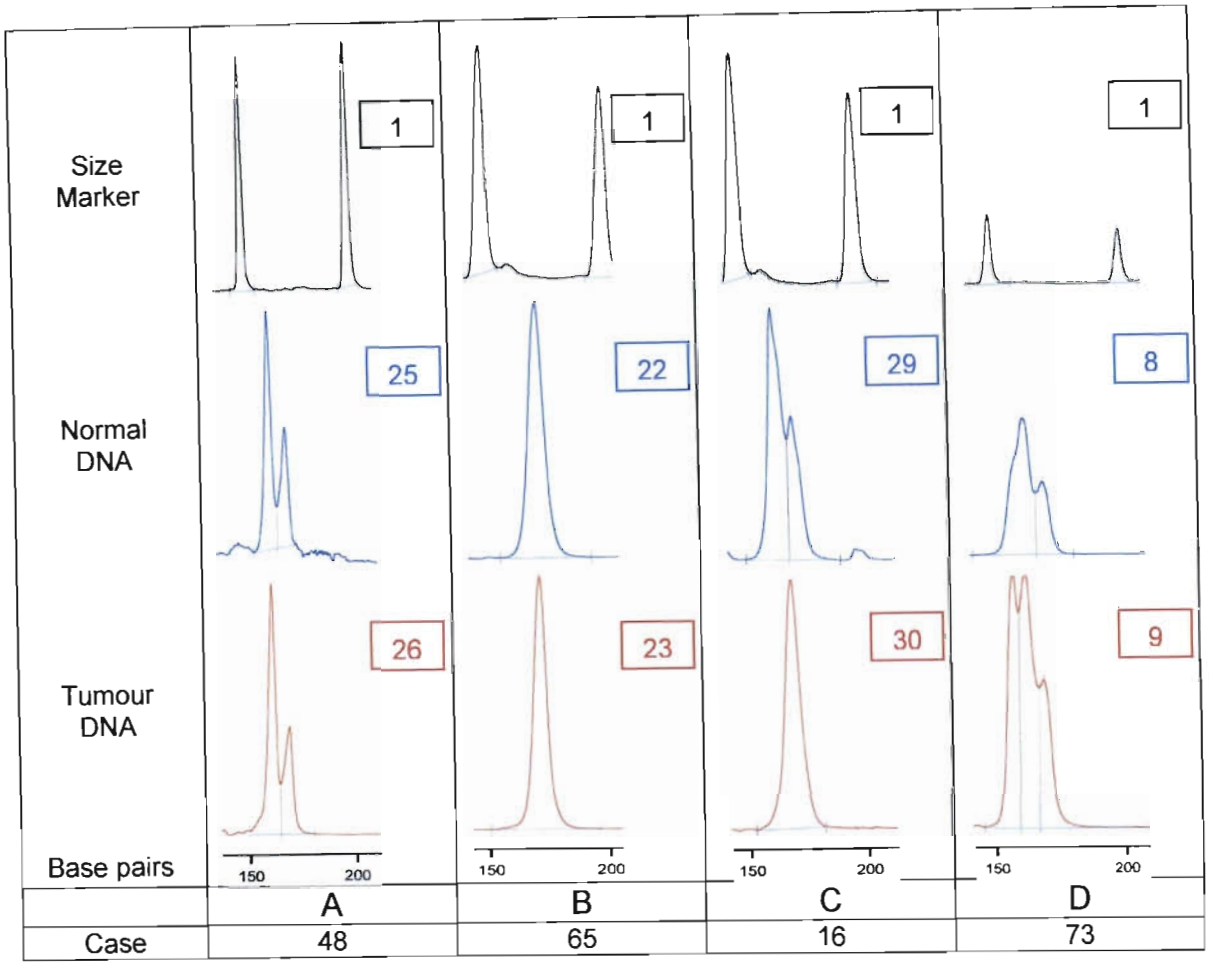
Figure 13: Electrophoretograms for marker **D11S902** showing **(A)** no change heterozygous, **(B)** no change homozygous, **(C)** loss of heterozygosity, **(D)** allelic imbalance (R = 2.98) with loss of the smaller allele, and **(E)** microsatellite instability with a novel allele in the tumour sample



	A	B	C	D
Case	76	80	11	19

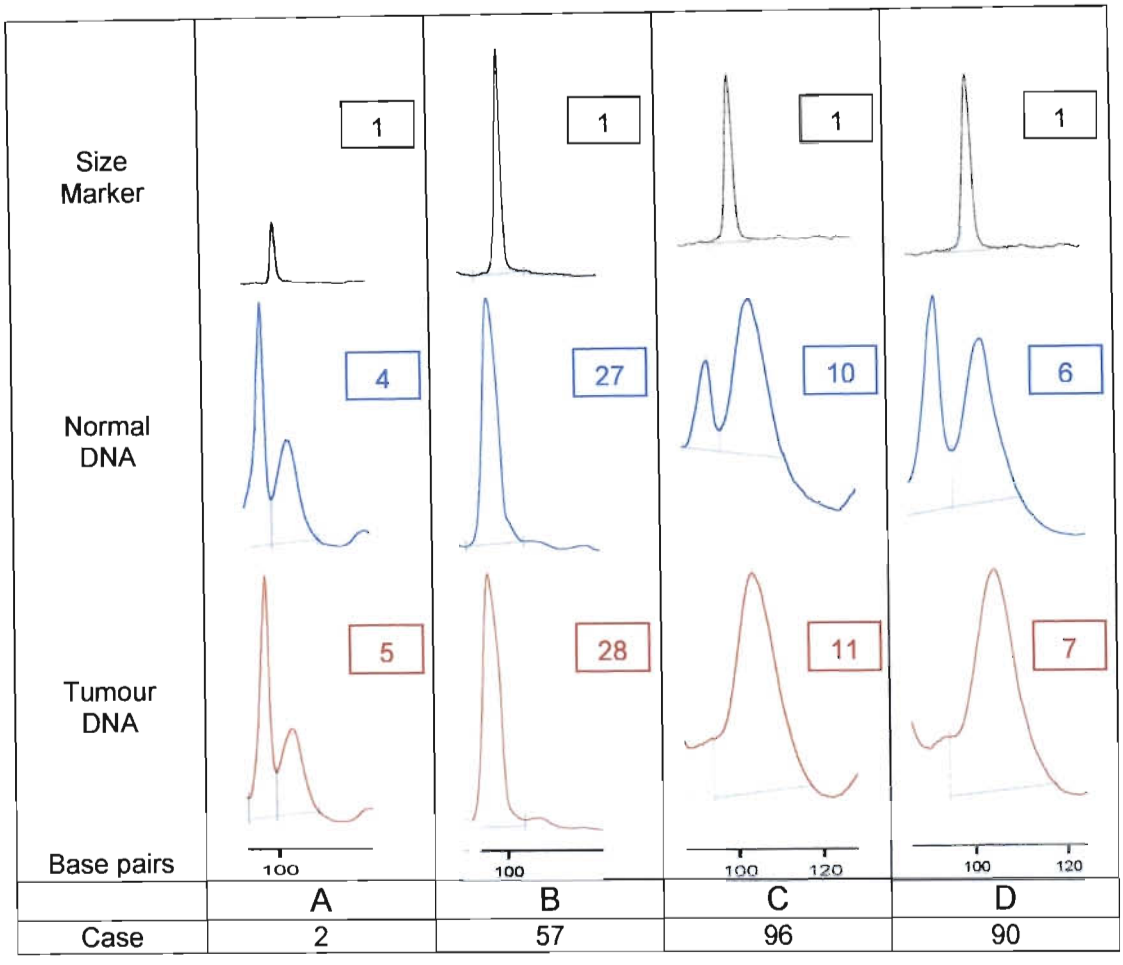
	Lane	Peak area	Size (base pairs)
A	11 (76N)	898.9	187.8
		954.1	193.5
	12 (76T)	3950.6	188.1
		2982.6	192.9
B	16 (80N)	3957.1	200.0
	17 (80T)	692.3	200.9
C	8 (11N)	408.4	191.6
		1185.6	201.4
	9 (11T)	4626.7	191.7
		997.0	201.7
D	16 (19N)	14.7	190.9
		104.3	197.9
	17 (19T)	309.8	191.4
		124.4	197.5
		197.1	206.9

Figure 14: Electrophoretograms for marker **D11S904** showing **(A)** no change heterozygous (R = 0.71, no allelic imbalance), **(B)** no change homozygous, **(C)** allelic imbalance (R = 0.07) with loss of the larger allele, and **(D)** microsatellite instability with a new allele in the tumour sample



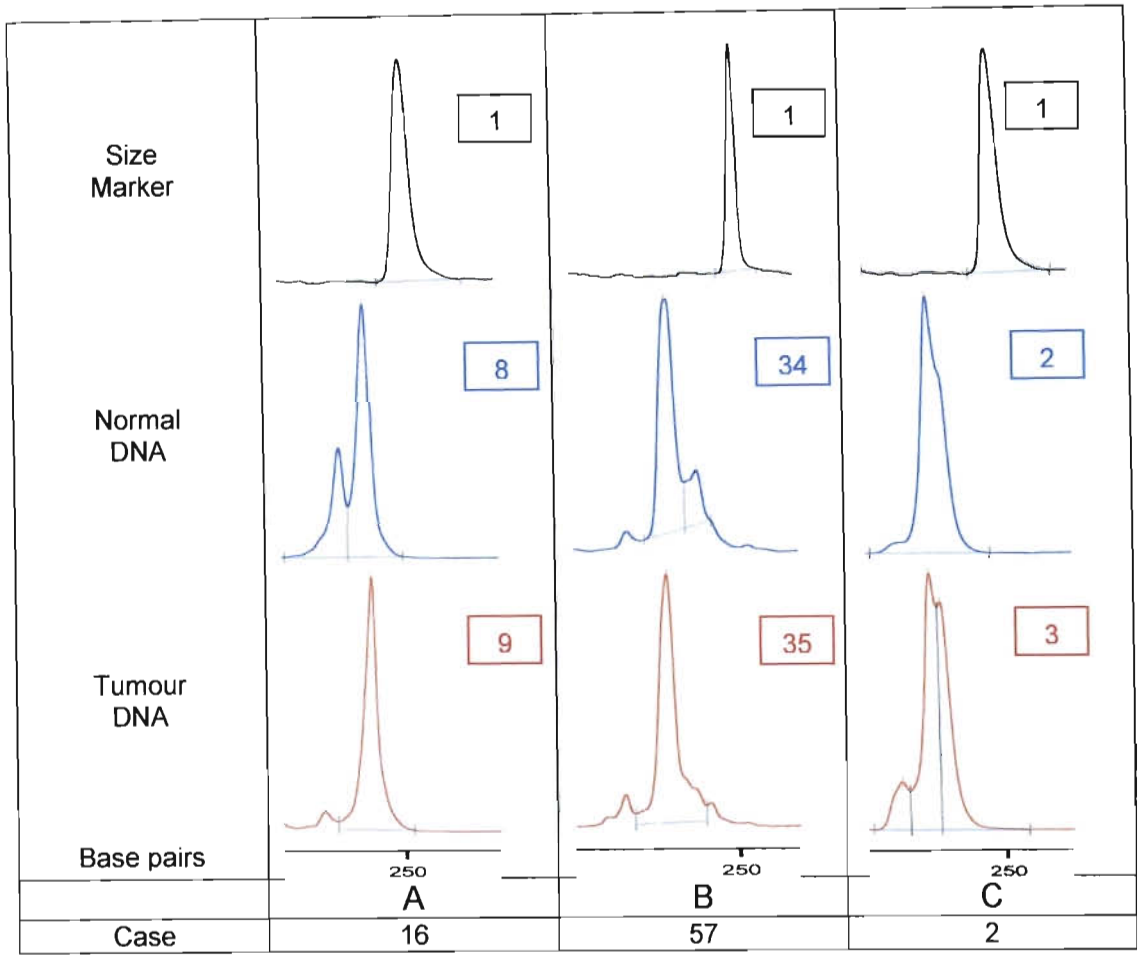
	Lane	Peak area	Size (base pairs)
A	25 (48N)	38.6	161.5
		21.1	169.1
	26 (48T)	744.6	161.5
		360.4	169.1
B	22 (65N)	2470.9	171.4
	23 (65T)	4297.8	171.6
C	29 (16N)	1354.2	163.9
		647.6	171.6
	30 (16T)	516.7	170.9
D	8 (73N)	3927.9	162.4
		1392.1	169.7
	9 (73T)	4190.4	157.7
		5293.6	162.4
		2734.7	169.1

Figure 15: Electrophoretograms for marker **D11S907** showing **(A)** no change heterozygous, **(B)** no change homozygous, **(C)** loss of heterozygosity, and **(D)** microsatellite instability with a novel allele in the tumour sample



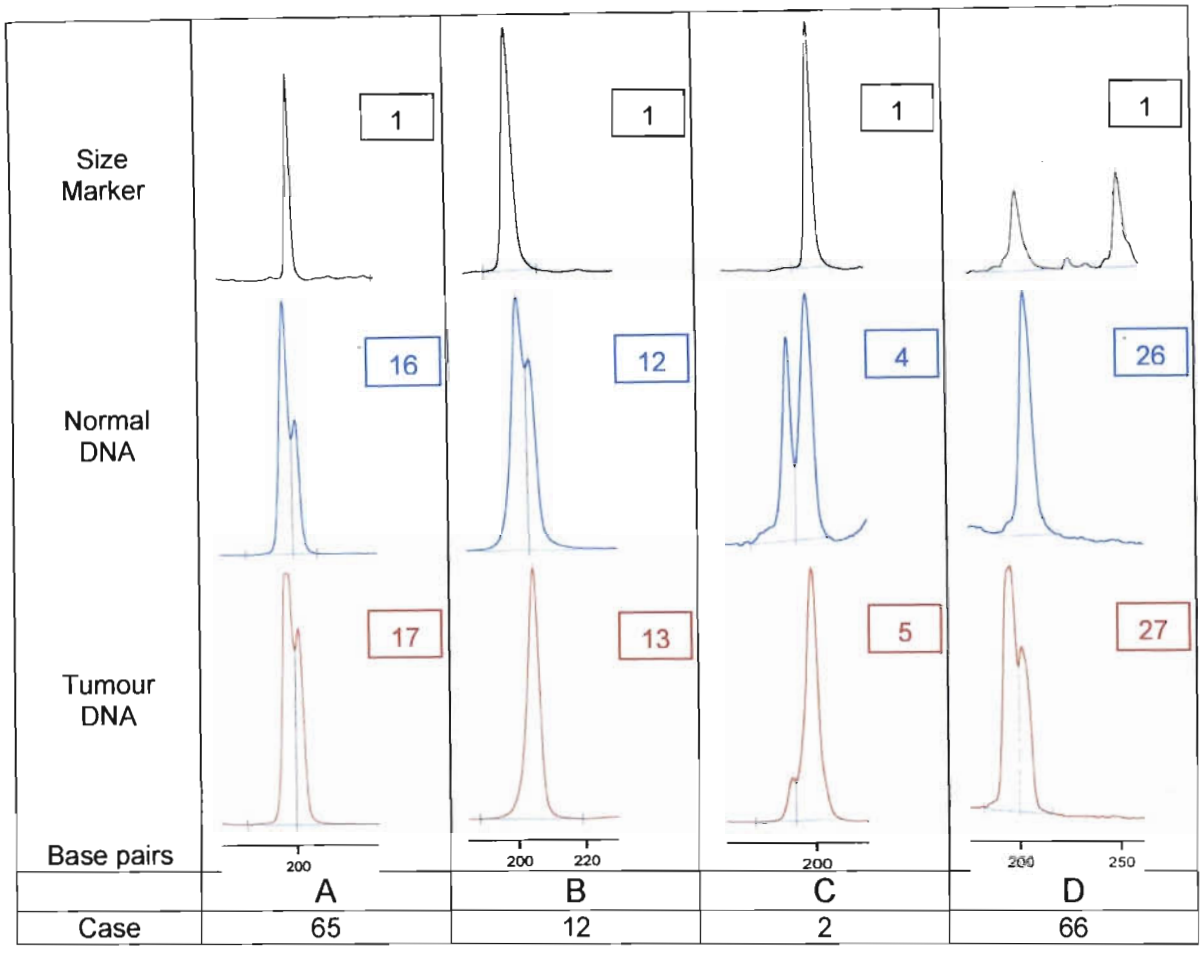
	Lane	Peak area	Size (base pairs)
A	4 (2N)	277.0	94.6
		189.1	104.7
	5 (2T)	258.0	95.1
		154.9	105.3
B	27 (57N)	1443.7	94.1
	28 (57T)	1341.8	93.8
C	10 (96N)	33.9	93.7
		101.2	103.8
	11 (96T)	194.7	103.8
D	6 (90N)	325.9	92.2
		351.3	102.2
	7 (90T)	276.2	102.6

Figure 16: Electrophoretograms for marker **D11S922** showing **(A)** no change heterozygous, **(B)** no change homozygous, and **(C & D)** loss of heterozygosity with loss of the smaller allele



	Lane	Peak area	Size (base pairs)
A	8 (16N)	238.2	230.7
		558.3	239.6
	9 (16T)	794.2	240.0
B	34 (57N)	317.0	232.2
		67.4	239.7
	35 (57T)	260.2	231.9
C	2 (2N)	1204.8	228.9
	3 (2T)	555.7	219.2
		2686.7	228.6
		1911.1	231.6

Figure 17: Electrophoretograms for marker **D11S930** showing **(A)** loss of heterozygosity with loss of the smaller allele, **(C)** loss of heterozygosity with loss of the larger allele, and **(C)** microsatellite instability with new alleles in the tumour sample



	Lane	Peak area	Size (base pairs)
A	16 (65N)	2186.4	195.7
		908.5	201.3
	17 (65T)	4490.1	195.4
		2413.9	201.0
B	12 (12N)	3102.1	201.5
		1690.9	204.9
	13 (12T)	1232.4	204.8
C	4 (2N)	110.2	189.9
		163.2	197.3
	5 (2T)	249.9	190.3
		2471.5	198.0
D	26 (66N)	51.2	200.8
	27 (66T)	83.5	194.9
		46.0	200.5

Figure 18: Electrophoretograms for marker **D11S935** showing **(A)** no change heterozygous, **(B)** loss of heterozygosity, **(C)** allelic imbalance (R = 6.67) with loss of the smaller allele, and **(E)** microsatellite instability with a novel allele in the tumour sample

Of the 70 cases studied, only 1 case (Case #102) showed LOH for all six 11p markers. Two cases showed LOH for 4 markers, 8 cases for 3 markers, 16 cases for 2 markers and 17 cases for 1 marker. Only one case showed MSI for three markers, 1 case for 2 markers and 13 for 1 marker. Six cases showed both MSI and LOH for 11p markers.

In treatment group A1, there was 1 case that showed LOH for all six 11p markers, 1 case for 4 markers, 6 for 3 markers, 10 for 2 markers and 14 for 1 marker (Table 56). Seven cases showed MSI for 1 marker only. Four cases in the group showed both MSI and LOH for 11p markers.

In treatment group B1, there was one case that showed LOH for 4 markers, 2 cases for 3 markers, 6 cases for 2 markers and 3 cases for 1 marker (Table 55). One case (case #2) showed MSI for 3 markers, 1 for 2 markers and 6 for 1 marker. Two cases showed MSI and LOH for 11p markers.

Table 57: Summary of data for the 11p microsatellite markers – treatment group A1

	D11S902 (%)	D11S904 (%)	D11S907 (%)	D11S922 (%)	D11S930 (%)	D11S935 (%)	Overall	
							#	% [a]
LOH	15 (57.6)	9 (45.0)	10 (52.6)	13 (59.1)	7 (26.9)	8 (42.1)	32	69.6*
MSI	0	2 (10.0)	3 (15.8)	0	0	2 (10.5)	7	15.2*
NC2	11	9	6	9	19	9		
NC1	22	28	29	26	22	29		
Total	48	48	48	48	48	48		
Informativity	54.2	41.7	39.6	45.8	54.2	39.6		

* - 2 cases were non informative for all six 11p markers.

a - this refers to the percentage of informative cases (n= 46).

Table 58: Summary of data for the 11p microsatellite markers – treatment group B1

	D11S902 (%)	D11S904 (%)	D11S907 (%)	D11S922 (%)	D11S930 (%)	D11S935 (%)	Overall	
							#	%
LOH	3 (42.9)	3 (27.3)	6 (54.5)	3 (33.3)	5 (38.5)	5 (41.7)	12	54.5
MSI	1 (14.3)	4 (36.4)	1 (9.1)	1 (11.1)	1 (7.7)	3 (25.0)	8	36.4
NC2	3	4	4	5	7	4		
NC1	15	11	11	13	9	10		
Total	22	22	22	22	22	22		
Informativity	31.8	50.0	50.0	41.0	59.1	54.5		

Of the 70 cases studied 68 were informative and 44 (64.7%) showed LOH for at least one 11p marker and 15 cases (22.1%) showed MSI for at least one 11p marker. In treatment group A1, 32 (69.6%) cases showed LOH for at least one 11p marker and 7 (15.2%) showed MSI for at least one marker (Table 57). In treatment group B1, 12 (54.5%) showed LOH for at least one marker and 8 (36.4%) showed MSI for at least one marker (Table 58). There was no significant difference in 11p alterations between the two treatment groups.

The most number of cases of LOH (18) was seen with D11S902 and of MSI (6) with D11S904. In treatment group A1 the frequency of LOH varied from 26.9% for D11S930 to 59.1% for D11S922. In treatment group B1 the frequency varied from 27.3 for D11S904 to 54.5 for D11S907.

The number of patients lost to follow limited the statistical analysis. For example, for marker D11S902 eight of the 15 (53.3%) cases with LOH in treatment group A1 were lost to follow up.

4.2.4.1 Correlation of 11p markers with clinicopathological features

Case #102 which demonstrated LOH for all six 11p markers, had stage I disease, was disease free at 74 months follow up and had a regressive, intermediate risk tumour. Case #38 which demonstrated LOH for four markers had stage I disease, was disease free at 142.5 months follow up and had a regressive, intermediate risk

tumour. Case #16 which demonstrated LOH for 4 markers also had stage I disease, was disease free at 13.1 months and then lost to follow up and had a mixed, intermediate risk tumour.

Tumours with LOH for four or more 11p markers tended to have low disease stage, intermediate risk tumours and disease free follow up, however this was not statistically significant.

There was no statistically significant association between LOH for individual 11p markers and patient age, gender, preoperative chemotherapy status, histological classification, risk group, stage and outcome. For D11S907 the ANOVA was marginal for age (p=0.056) but the non-parametric comparison was not significant (p=0.165, Kruskal Wallis test - χ^2).

4.2.4.2 Analysis of 11p by region

Table 59: Frequency of LOH on chromosome 11p by region

11p regions	Preoperative chemotherapy (n=48)		No preoperative chemotherapy (n=22)	
	Number	%	Number	%
11p13 only	7	14.6	5	22.7
11p15 only	5	10.4	0	0
11p13-11p15	20	41.6	7	31.8
None	14	29.2	10	45.5
Not informative for all 11p markers	2	4.2	0	0

LOH for 11p13 only was seen in twelve tumours, seven of which were treated with preoperative chemotherapy. In treatment group A1 five tumours showed LOH for the 11p15 locus only (Table 59). Although 13 tumours showed LOH for D11S922 (11p15 locus), eight of these tumours also demonstrated LOH for 11p13 markers. An additional 20 tumours in treatment group A1 showed LOH for markers that included both the 11p13 and 11p15 loci. Overall 27 of the 46 informative cases showed LOH for 11p13 and 25 of the 46 informative cases showed LOH for 11p15. In treatment group B1 five cases showed LOH for 11p13 and none for 11p15 only,

but seven showed LOH for both 11p13 and 11p15 (Table 59). In both groups LOH was more frequent at the 11p13 locus compared to the 11p15 locus.

There was no statistically significant association between LOH for the 11p regions and patient age, gender, preoperative chemotherapy status, histological classification, risk group, stage and outcome.

4.2.5 16q RESULTS

Table 60: Results for 16q microsatellite markers – treatment group B1

Case	D16S411	D16S413	D16S496	D16S503	D16S520	LOH MSI status	# of markers
1	NC2	NC1	NC1	MSI	LOH	LOH MSI	1 1
2	LOH	NC2	NC1	NC1	NC1	LOH	1
3	LOH	NC1	NC1	LOH	MSI	LOH MSI	2 1
4	NC2	NC1	NC1	NC2	NC1		
5	NC2	NC2	NC2	NC1	LOH	LOH	1
7	NC2	NC1	NC2	NC1	NC2		
8	LOH	NC1	LOH	LOH	NC2	LOH	3
9	NC1	LOH	NC2	NC1	NC1	LOH	1
10	NC1	NC2	LOH	NC1	NC2	LOH	1
11	LOH	NC1	NC2	NC1	NC1	LOH	1
12	NC1	NC1	MSI	NC1	NC1	MSI	1
13	LOH	NC1	NC2	NC1	NC2	LOH	1
14	LOH	NC1	NC1	NC1	NC2	LOH	1
15	LOH	NC1	NC2	NC1	NC2	LOH	1
16	NC2	NC1	NC2	NC2	NC2		
17	NC1	NC1	NC2	NC1	NC1		
18	NC1	NC1	NC2	LOH	NC2	LOH	1
19	NC2	NC1	NC2	NC1	NC1		
21	NC1	NC1	NC1	NC1	LOH	LOH	1
22	NC2	NC1	NC1	MSI	NC2	MSI	1
23	NC2	NC2	NC2	LOH	LOH	LOH	2
35	NC1	NC1	LOH	LOH	LOH	LOH	3

Table 61: Results for 16q microsatellite markers – treatment group A1

Case	D16S411	D16S413	D16S496	D16S503	D16S520	LOH MSI status	# of markers
24	NC2	NC1	LOH	NC1	LOH	LOH	2
27	NC1	NC1	NC2	NC1	NC2		
28	NC1	NC1	NC1	NC2	NC1		
29	LOH	NC2	LOH	NC2	NC2	LOH	2
33	NC2	NC2	NC1	LOH	NC2	LOH	1
34	LOH	NC1	NC1	NC1	NC2	LOH	1
36	NC1	NC1	NC1	NC2	NC2		
37	NC1	NC2	MSI	NC1	NC2	MSI	1
38	NC2	NC1	NC1	NC2	NC2		
41	NC2	NC1	NC2	LOH	LOH	LOH	2
42	LOH	NC2	NC1	NC2	NC2	LOH	1
44	NC1	NC2	NC1	NC1	NC1		
47	NC2	LOH	LOH	NC1	NC1	LOH	2
48	NC2	NC2	LOH	NC2	NC1	LOH	1
52	NC2	NC1	NC1	NC1	LOH	LOH	1
53	NC1	LOH	LOH	NC1	NC2	LOH	2
54	NC1	NC1	NC1	NC1	NC1	NI	
55	NC2	NC2	NC1	NC1	NC2		
57	NC1	NC1	NC2	NC2	NC2		
58	NC1	NC2	NC1	NC2	NC2		
60	NC2	NC2	NC2	NC1	NC2		
63	NC1	NC1	NC1	LOH	NC2	LOH	1
65	NC1	NC1	LOH	NC1	NC1	LOH	1
66	NC1	NC1	NC1	NC1	NC1	NI	
67	LOH	LOH	NC1	NC1	NC2	LOH	2
68	NC2	NC1	NC1	NC1	NC1		
69	NC2	MSI	NC2	NC1	NC1	MSI	1
70	NC2	NC1	NC2	LOH	NC1	LOH	1
71	NC2	LOH	NC1	NC2	NC1	LOH	1
72	NC1	NC1	LOH	NC1	NC1	LOH	1
73	MSI	NC1	NC1	LOH	NC2	LOH MSI	1 1
76	NC1	NC2	NC2	NC1	LOH	LOH	1
77	NC1	NC1	NC1	NC1	NC1	NI	

Case	D16S411	D16S413	D16S496	D16S503	D16S520	LOH MSI status	# of markers
78	NC1	LOH	MSI	NC1	LOH	LOH MSI	2 1
80	NC1	NC1	NC1	NC1	NC1	NI	
81	NC1	NC1	LOH	NC1	NC1	LOH	1
82	LOH	NC2	NC2	NC1	NC2	LOH	1
83	NC2	LOH	NC2	NC2	NC1	LOH	1
84	NC1	NC1	NC2	NC1	NC2		
88	NC1	NC2	NC2	NC2	NC2		
89	NC1	NC1	NC1	NC1	LOH	LOH	1
90	LOH	NC1	MSI	LOH	LOH	LOH MSI	3 1
91	MSI	NC2	LOH	NC1	NC1	LOH MSI	1 1
92	LOH	NC2	NC1	NC1	LOH	LOH	2
96	NC1	NC1	NC2	NC1	MSI	MSI	1
99	LOH	NC1	LOH	NC1	LOH	LOH	3
102	NC2	NC1	LOH	NC1	NC2	LOH	1
115	NC1	NC1	NC1	LOH	LOH	LOH	2

Key:

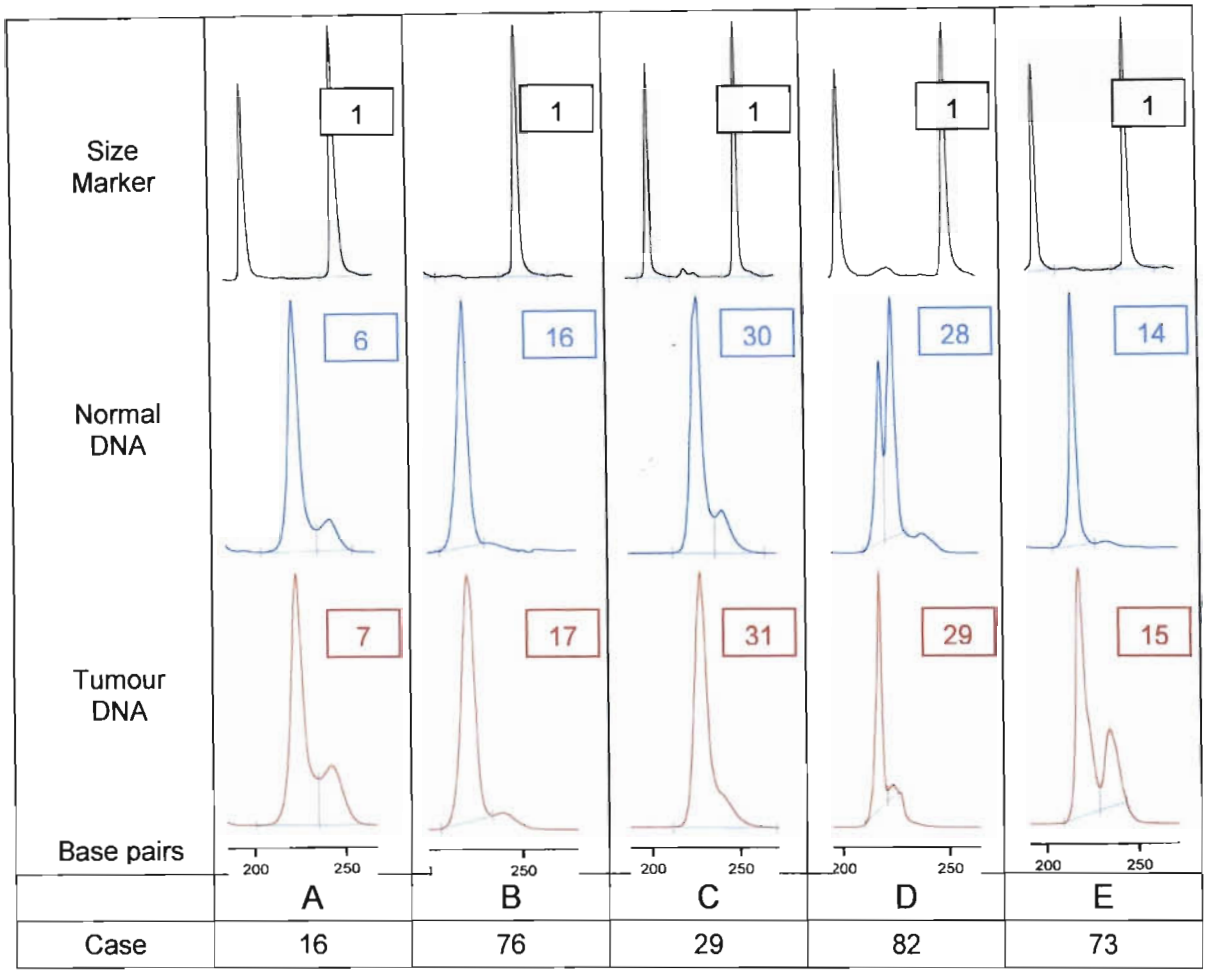
LOH - Allelic imbalance/Loss of heterozygosity

MSI - Microsatellite instability

NC1 - No change homozygous, non-informative case

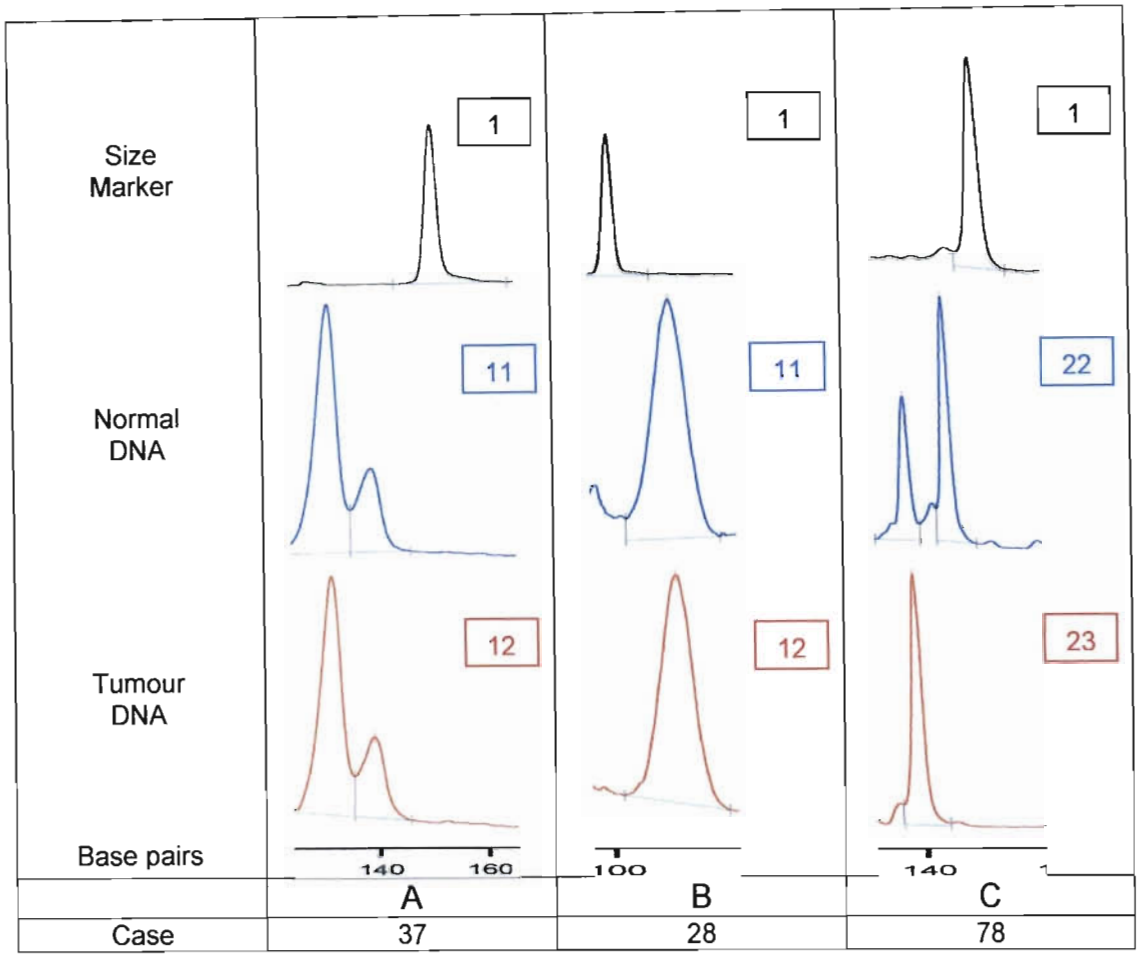
NC2 - No change heterozygous, informative case

NI - Not informative for all 16q markers



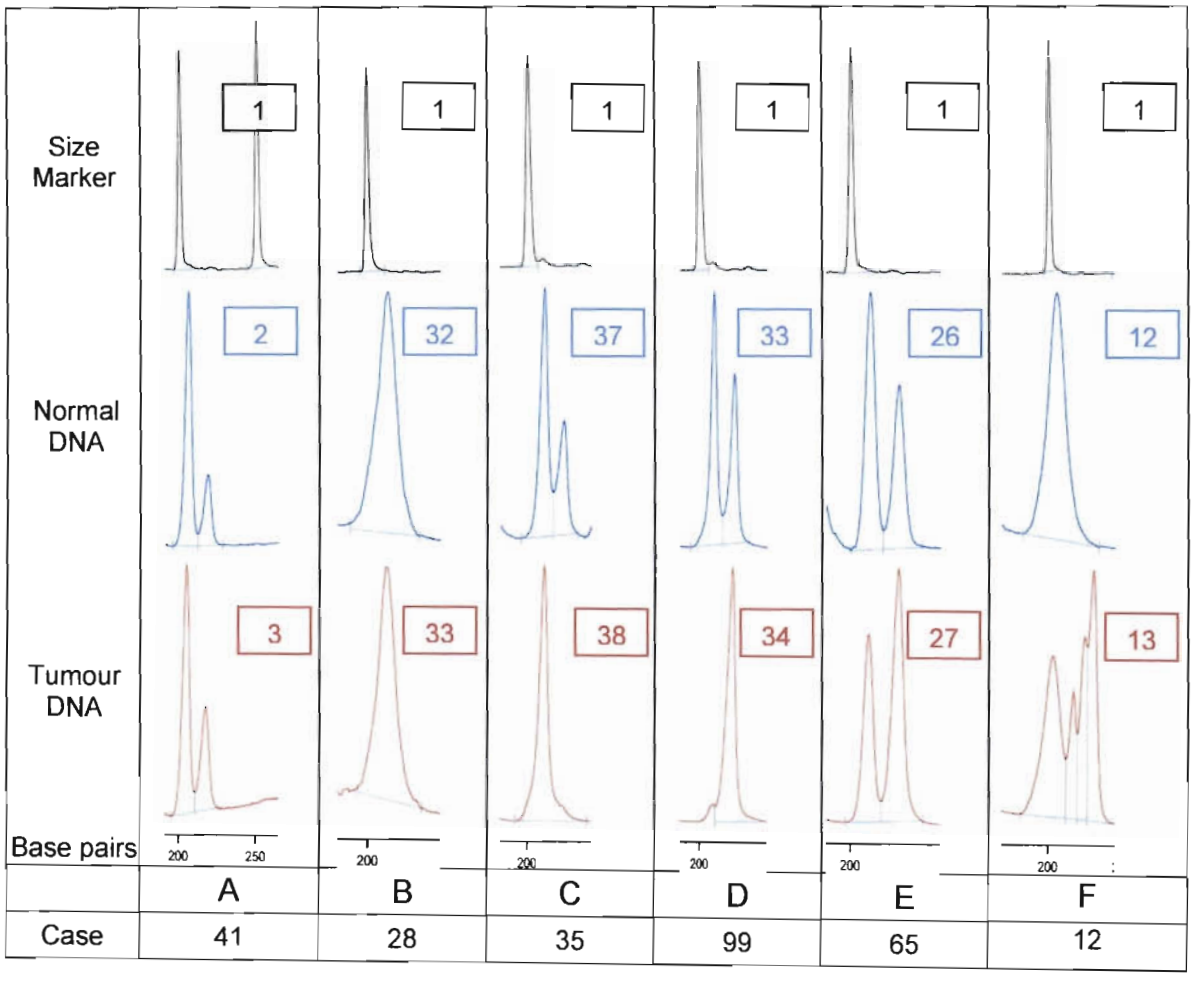
	Lane	Peak area	Size (base pairs)
A	6 (16N)	1221.2	225.8
		235.5	243.4
	7 (16T)	1537.1	225.0
		557.6	242.9
B	16 (76N)	168.6	223.2
	17 (76T)	2527.8	223.8
C	30 (29N)	1590.2	227.0
		349.0	240.2
	31 (29T)	2056.9	227.6
D	28 (82N)	296.7	218.5
		456.3	224.2
	29 (82T)	1279.7	217.9
		83.0	223.9
E	14 (73N)	278.0	218.2
	15 (73T)	3879.3	218.4
		1516.2	235.1

Figure 19: Electrophoretograms for marker **D16S411** showing **(A)** no change heterozygous, **(B)** no change homozygous, **(C)** loss of heterozygosity, **(D)** allelic imbalance (R = 0.04) with loss of the larger allele, and **(E)** microsatellite instability with a novel allele in the tumour sample



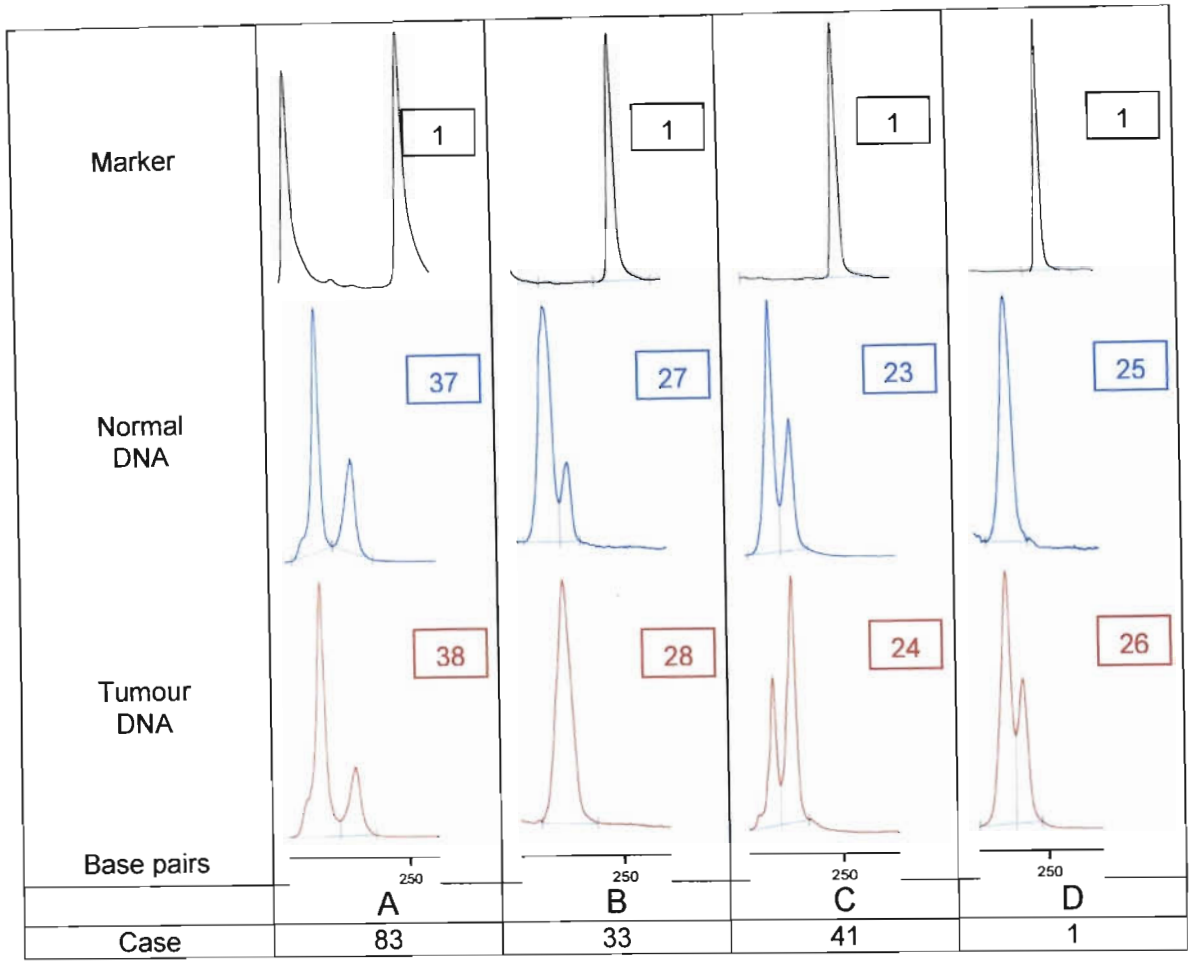
	Lane	Peak area	Size (base pairs)
A	11 (37N)	124.9	130.8
		43.0	138.5
	12 (37T)	82.9	131.2
		29.6	139.0
B	11 (28N)	92.1	112.6
	11 (28T)	106.7	113.0
C	22 (78N)	261.9	138.5
		409.3	144.7
	23 (78T)	1002.3	139.0

Figure 20: Electrophoretograms for marker **D16S413** showing **(A)** no change heterozygous, **(B)** no change homozygous, **(C)** loss of heterozygosity



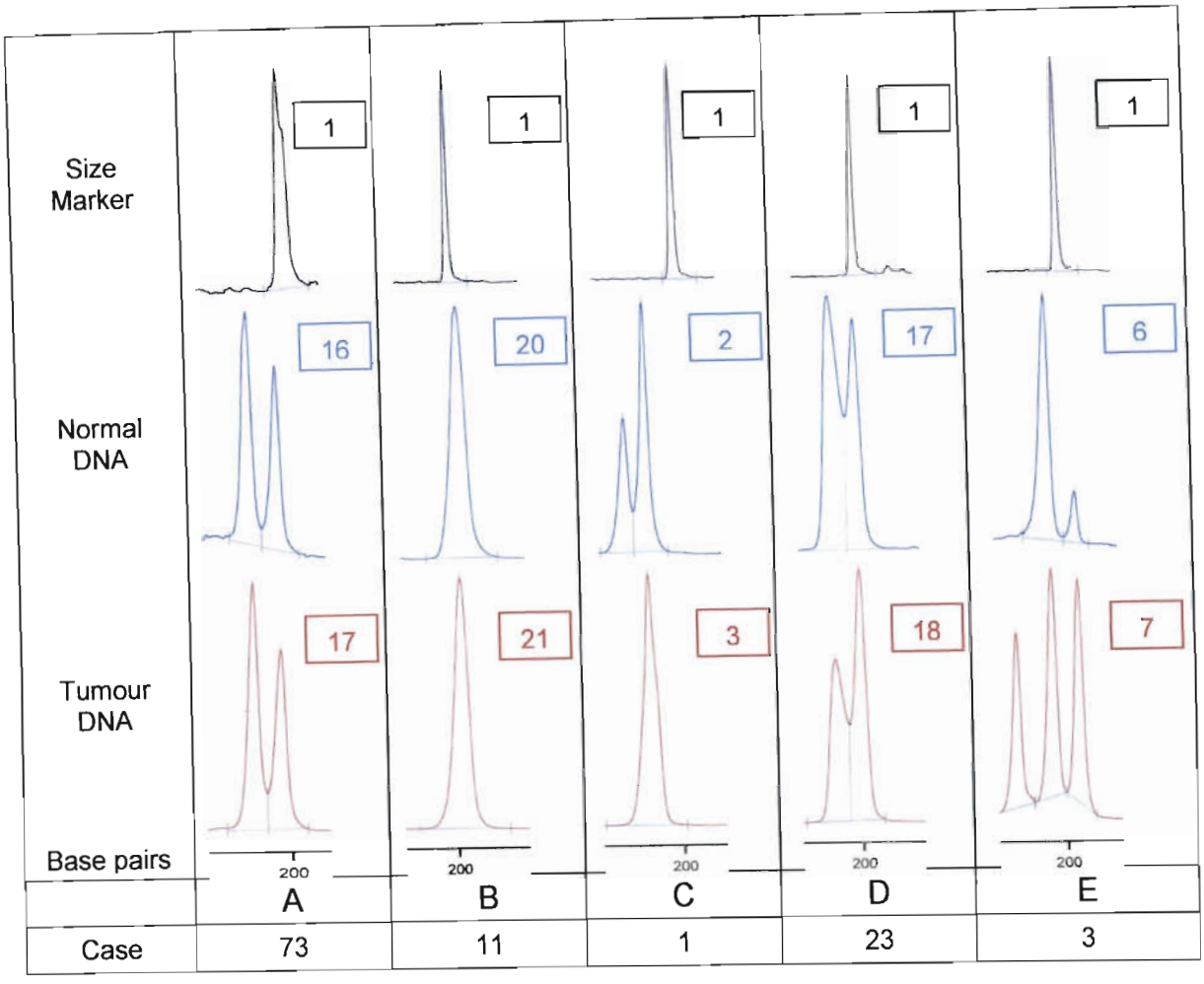
	Lane	Peak area	Size (base pairs)
A	2 (41N)	384.8	205.9
		126.4	218.5
	3 (41T)	165.9	205.4
		82.5	218.1
B	32 (28N)	208.7	211.6
	33 (28T)	255.7	211.4
C	37 (35N)	84.3	208.8
		44.5	219.1
	38 (35T)	748.4	208.7
D	33 (99N)	520.3	208.7
		402.6	220.9
	34 (99T)	686.1	220.3
E	26 (65N)	106.3	209.4
		77.0	223.5
	27 (65T)	1087.2	208.9
		1675.2	223.4
F	12 (12N)	453.2	204.9
	13 (12T)	347.5	204.1
		116.5	216.6
		151.7	224.3
		257.9	230.1

Figure 21: Electrophoretograms for marker **D16S9496** showing **(A)** no change heterozygous, **(B)** no change homozygous, **(C)** loss of heterozygosity with loss of the larger allele, **(D)** loss of heterozygosity with loss of the smaller allele, **(E)** allelic imbalance (R = 2.13) with loss of the smaller allele, and **(E)** microsatellite instability with a novel allele in the tumour sample



	Lane	Peak area	Size (base pairs)
A	37 (83N)	318.7	211.0
		160.5	226.0
	38 (83T)	540.2	211.7
		178.5	226.6
B	27 (33N)	75.9	217.7
		17.5	227.2
	28 (33T)	131.6	226.4
C	23 (41N)	352.6	222.5
		203.6	230.2
	24 (41T)	73.6	222.3
		135.7	230.0
D	25 (1N)	100.3	232.4
	26 (1T)	515.7	231.9
		269.9	238.7

Figure 22: Electrophoretograms for marker **D16S503** showing **(A)** no change heterozygous, **(B)** loss of heterozygosity, **(C)** allelic imbalance (R = 3.19) with loss of the smaller allele, and **(E)** microsatellite instability with a novel allele in the tumour sample



	Lane	Peak area	Size (base pairs)
A	16 (73N)	62.5	184.1
		46.1	196.3
	17 (73T)	434.1	184.5
		338.7	196.3
B	20 (11N)	3311.9	204.0
	21 (11T)	3521.2	203.8
C	2 (1N)	261.3	174.2
		454.4	184.3
	3 (1T)	1714.7	184.1
D	17 (23N)	3360.5	186.4
		2305.7	199.1
	18 (23T)	1329.8	186.7
		1660.8	199.6
E	6 (3N)	138.4	192.3
		19.7	206.5
	7 (3T)	165.0	174.1
		237.2	193.0
		222.5	206.9

Figure 23: Electrophoretograms for marker **D16S520** showing **(A)** no change heterozygous, **(B)** no change homozygous, **(C)** loss of heterozygosity, **(D)** allelic imbalance (R = 1.82) with loss of the smaller allele, and **(E)** microsatellite instability with a novel allele in the tumour sample

In treatment group A1, case #90 showed LOH for three markers and MSI for one marker (Table 61). This case showed the most number of alterations with 16q markers. There was one additional case which showed LOH for 3 markers, 9 cases for 2 markers and 18 cases for one marker. There were 7 cases that demonstrated MSI for at least one marker. Three cases showed MSI for D16S496 (16q22.1), the most for any 16q marker. Three of these 7 cases also demonstrated LOH for 16q markers. None of the cases showed MSI for more than one marker.

In treatment group B1, there were 2 cases that showed LOH for 3 markers, 2 for 2 markers and 11 for 1 marker. Four cases showed MSI for at least one 16q marker.

Table 62: Summary of data for the 16q microsatellite markers – treatment group A1

	D16S411 (%)	D16S413 (%)	D16S496 (%)	D16S503 (%)	D16S520 (%)	Overall	
						#	% [a]
LOH	8	6	11	7	10	29	65.9*
MSI	2	1	3	0	1	7	15.9*
NC2	15	14	12	11	20		
NC1	23	27	22	30	17		
Total	48	48	48	48	48		
Informativity	52.1	43.8	54.2	37.5	64.6		

* 4 cases were non informative for all five 16q markers.

a - this refers to the percentage of informative cases (n= 46).

Table 63: Summary of data for the 16q microsatellite markers – treatment group B1

	D16S411 (%)	D16S413 (%)	D16S496 (%)	D16S503 (%)	D16S520 (%)	Overall	
						#	% [a]
LOH	7	1	3	5	5	15	68.2
MSI	0	0	1	2	1	4	18.2
NC2	8	4	11	2	9		
NC1	7	17	7	13	7		
Total	22	22	22	22	22		
Informativity	68.2	22.7	68.2	40.9	68.2		

Overall 44 cases of the 66 informative cases (66.7%) showed LOH for at least one 16q marker and 11 cases (16.7%) showed MSI for 16q markers. Fourteen cases showed LOH with D16S496, eleven of these were in treatment group A1 (Table 62). Of all 70 cases, 15 showed LOH for each of the markers D16S411 and D16S520. The smallest number of cases had LOH with marker D16S413.

In treatment group A1, 29 of the 44 informative cases (65.9%) showed LOH for at least one 16q marker and 7 cases (15.9%) showed MSI for at least one 16q marker (Table 62). Eleven cases had LOH for D16S496 and 10 for D16S520.

In treatment group B1, 15 cases (68.2%) showed LOH for at least one marker and 4 cases (18.2%) showed MSI for at least one marker (Table 63). LOH was most frequently seen with markers D16S503 (5 cases) and D16S411 (7 cases).

Twenty six of the 29 cases with 16q LOH showed LOH for a locus telomeric to 16q21. Furthermore, 5 of these 26 cases also showed LOH at 16q12.1.

4.2.5.1 Correlation of 16q markers with clinicopathological features

For D16S411 the ANOVA was marginal for age (p=0.056) but the non-parametric comparison was not significant (p=0.443, Kruskal Wallis test - χ^2). There were significantly more female patients with tumours that demonstrated LOH for D16S411 (31.6% vs 9.4%) (p=0.020, Fisher's exact test).

Analysis of D16S496 which maps to 16q22.1 showed a marginal association with histological classification (p=0.092, Fisher's exact test) and a significant association with risk group (p=0.030, Fisher's exact test). In treatment group A1 there was a greater frequency of LOH in the blastemal (100%) and diffuse anaplastic (40%) tumours compared to the other histological types (0 – 20%). Two of the four cases of MSI seen with this marker had diffuse anaplastic histology. Five of the 11 (45.5%) high risk tumours showed LOH for D16S496 compared to 6 of 37 (16.2%) intermediate risk tumours and 18.2% of high risk tumours displayed MSI compared to 2.7% of intermediate tumours.

Another 16q marker D16S520 which maps to 16q24.2-qter showed similar results with a significant association with both histological classification (p=0.019, Fisher's exact test) and risk group (p<0.001, Fisher's exact test). In treatment group A1 the only blastemal tumour and 40% of the diffuse anaplastic tumours showed LOH in comparison to the frequency in the other tumour types which ranged from 0 to 33%. One of the diffuse anaplastic tumours also showed MSI. The analysis for this marker produced similar results to the analysis for D16S496. Five of the 11 (45.5%) high risk tumours showed LOH in contrast to 5 of the 37 (13.5%) intermediate risk tumours. Alterations at this marker locus are more frequent in high risk tumours (54.5% vs 13.5%).

Although there was a significant association of D16S413 with outcome (p=0.011, Fisher's exact test) all outcome analysis in the microsatellite study was viewed with caution because of the small number of patients in this cohort and the large number of patients that were eventually lost to follow up.

4.2.5.2 Analysis of 16q by region

Table 64: Frequency of LOH on chromosome 16q by region

16q regions	Preoperative chemotherapy (n=48)		No preoperative chemotherapy (n=22)	
	Number/informative cases	%	Number/informative cases	%
16q12.1	8/25	32.0	7/15	46.7
16q21-22	18/36	50.0	6/19	31.6
16q24-qter	15/38	39.5	6/17	35.3

LOH for the 16q12.1 region only was found in 37.5% of informative cases (Table 64). LOH for the 16q21-22 region was detected in 24 of 55 cases (43.6%) and for the 16q24-qter region it was 38.2% (21/55). The most frequent region of 16q that showed LOH was the 16q21-22 region, followed by 16q24 and 16q12.1. There was no significant difference in results between the two treatment groups, although 16q21-22 LOH was more frequent in treatment group A1 and 16q12.1 was more frequent in treatment group B1.

In treatment group A1 LOH 16q was more frequent in females than males (69.6% vs 52.0%), a marginal association (p=0.050, Fisher's exact test).

No association was found between the combined 16q regions and clinicopathological features.

4.2.6 Mismatch repair gene results

Table 65: Results of MMR gene microsatellite markers – treatment group B1

Case	BAT25	BAT26	BAT40	D2S123	D3S659	D3S1255	LOH MSI status	# of markers
1	MSI	NC1	NC1	NC1	NC1	NC2	MSI	1
2	NC1	LOH	NC1	NC1	MSI	NC2	LOH MSI	1 1
3	NC1	NC1	NC1	NC2	NC2	LOH	LOH	1
4	NC2	NC2	NC1	NC2	NC2	NC1		
5	NC1	NC1	NC1	NC1	NC1	NC1	NI	
7	NC1	NC1	NC1	NC1	NC1	NC2		
8	NC1	NC1	NC1	NC1	NC2	NC1		
9	NC1	LOH	NC2	NC1	NC2	NC1	LOH	1
10	NC2	NC1	NC1	NC1	NC2	NC1		
11	NC1	NC1	NC1	NC1	NC2	NC1		
12	LOH	NC1	NC1	NC1	MSI	NC1	LOH MSI	1 1
13	MSI	NC1	NC1	NC1	NC2	NC1	MSI	1
14	NC1	NC1	NC1	NC1	NC2	NC1		
15	NC1	NC1	LOH	LOH	NC1	NC1	LOH	2
16	NC2	NC1	LOH	NC1	NC1	NC1	LOH	1
17	NC1	NC1	NC1	NC1	NC2	LOH	LOH	1
18	NC1	NC1	LOH	NC1	NC2	NC1	LOH	1
19	NC1	NC1	NC1	NC1	NC2	LOH	LOH	1
21	NC1	NC1	NC1	LOH	NC2	NC2	LOH	1
22	NC1	NC1	NC2	NC1	NC2	NC2		
23	NC1	NC1	NC1	NC1	NC2	NC1		
35	NC1	NC1	NC1	NC1	NC2	LOH	LOH	1

Table 66: Results of MMR gene microsatellite markers – treatment group A1

Case	BAT25	BAT26	BAT40	D2S123	D3S659	D3S1255	LOH MSI status	# of markers
24	NC1	NC1	NC1	NC1	NC2	NC2		
27	NC1	LOH	NC2	NC1	MSI	NC1	LOH MSI	1 1
28	NC2	NC1	NC1	NC1	NC1	NC1		
29	NC1	NC1	NC1	NC1	MSI	LOH	LOH MSI	1 1
33	NC1	LOH	NC1	NC1	NC2	NC1	LOH	1
34	NC1	NC1	NC1	NC1	NC2	NC1		
36	NC1	NC1	NC1	NC1	NC2	NC2		
37	NC1	NC1	NC1	NC1	LOH	NC1	LOH	1
38	NC1	NC2	NC2	NC1	NC1	NC1		
41	NC1	LOH	NC1	NC1	NC2	MSI	LOH MSI	1 1
42	NC1	NC2	NC1	NC1	NC1	NC1		
44	NC1	LOH	NC1	LOH	NC2	NC1	LOH	2
47	NC1	LOH	NC1	LOH	NC2	NC2	LOH	2
48	NC1	NC2	NC2	NC1	NC1	NC1		
52	NC1	NC1	NC2	NC1	NC2	NC1		
53	NC1	NC1	NC1	NC1	NC2	NC1		
54	NC1	NC1	NC1	NC2	NC1	NC2		
55	NC1	NC1	NC1	NC2	NC2	NC1		
57	NC1	MSI	NC1	NC2	NC1	NC2	MSI	1
58	NC1	NC1	NC1	LOH	NC1	NC2	LOH	1
60	NC1	NC1	NC1	NC1	NC2	NC2		
63	NC1	NC2	NC1	NC1	NC2	NC2		
65	NC1	NC1	NC1	NC1	NC1	NC2		
66	NC1	NC1	NC1	NC2	NC2	NC1		
67	NC2	NC1	NC2	NC1	NC1	NC2		
68	NC1	NC1	NC1	NC1	NC1	NC2		
69	NC1	NC1	NC1	NC2	LOH	LOH	LOH	2
70	NC1	NC1	NC1	NC1	NC2	NC1		
71	NC1	NC1	NC1	NC1	NC1	LOH	LOH	1
72	NC1	NC1	NC1	NC1	NC2	NC2		

Case	BAT25	BAT26	BAT40	D2S123	D3S659	D3S1255	LOH MSI status	# of markers
73	NC1	NC1	NC1	NC2	NC1	NC2		
76	NC1	NC1	NC1	NC1	NC2	NC1		
77	NC1	NC1	LOH	NC1	NC1	NC1	LOH	1
78	NC1	NC1	NC1	NC2	NC2	NC1		
80	NC1	NC1	NC1	NC2	NC2	NC1		
81	NC1	LOH	LOH	LOH	NC2	NC1	LOH	3
82	NC2	NC1	NC1	NC1	NC1	LOH	LOH	1
83	NC1	NC1	NC1	LOH	NC1	NC1	LOH	1
84	NC1	NC1	NC1	NC2	NC2	NC2		
88	NC2	NC2	NC1	NC2	NC2	NC1		
89	NC1	NC1	NC1	NC1	NC1	NC2		
90	NC1	NC1	NC1	NC1	NC2	LOH	LOH	1
91	NC1	NC2	NC2	NC2	NC1	NC1		
92	NC1	NC1	LOH	NC1	NC2	LOH	LOH	2
96	NC2	NC1	NC1	NC2	NC1	NC1		
99	LOH	NC1	NC1	NC1	NC1	NC2	LOH	1
102	NC1	LOH	NC1	LOH	NC1	NC2	LOH	2
115	NC1	NC1	NC1	NC1	NC1	LOH	LOH	1

Key:

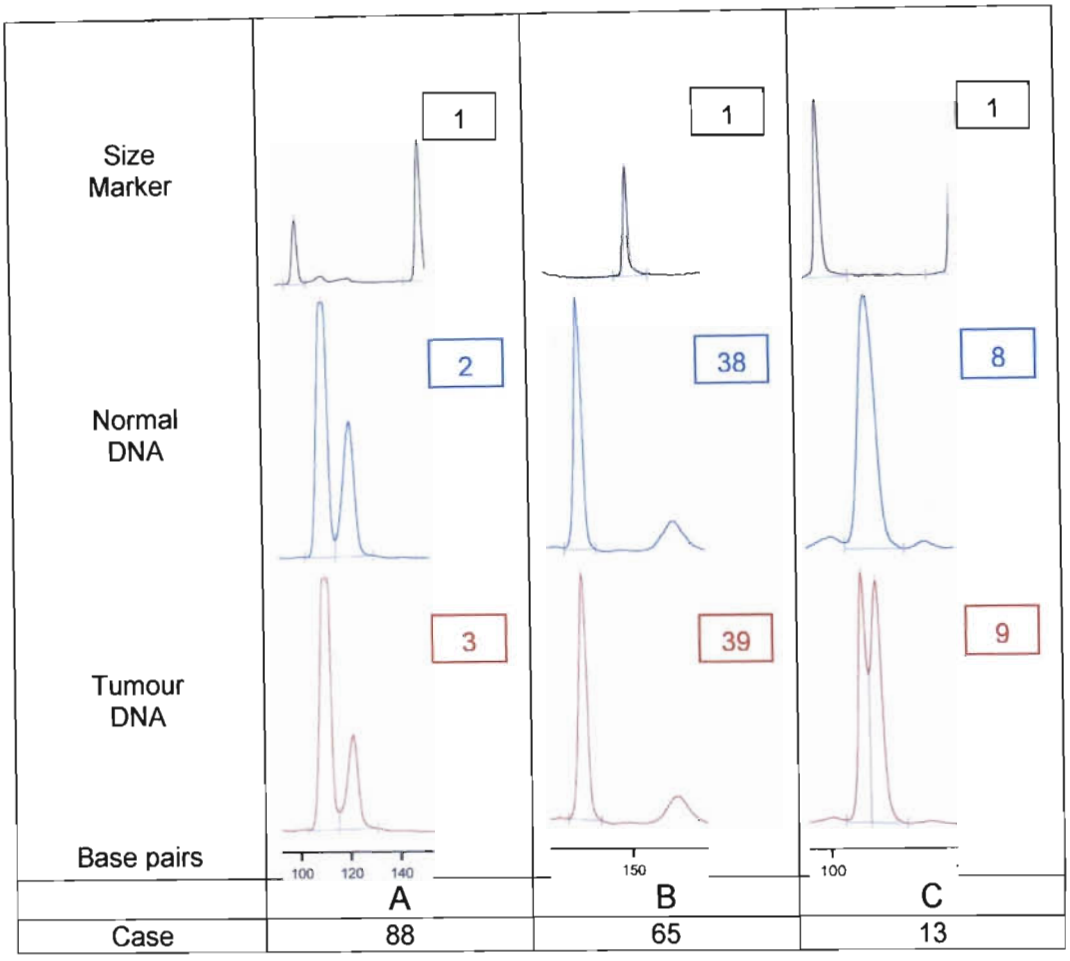
LOH - Allelic imbalance/Loss of heterozygosity

MSI - Microsatellite instability

NC1 - No change homozygous, non-informative case

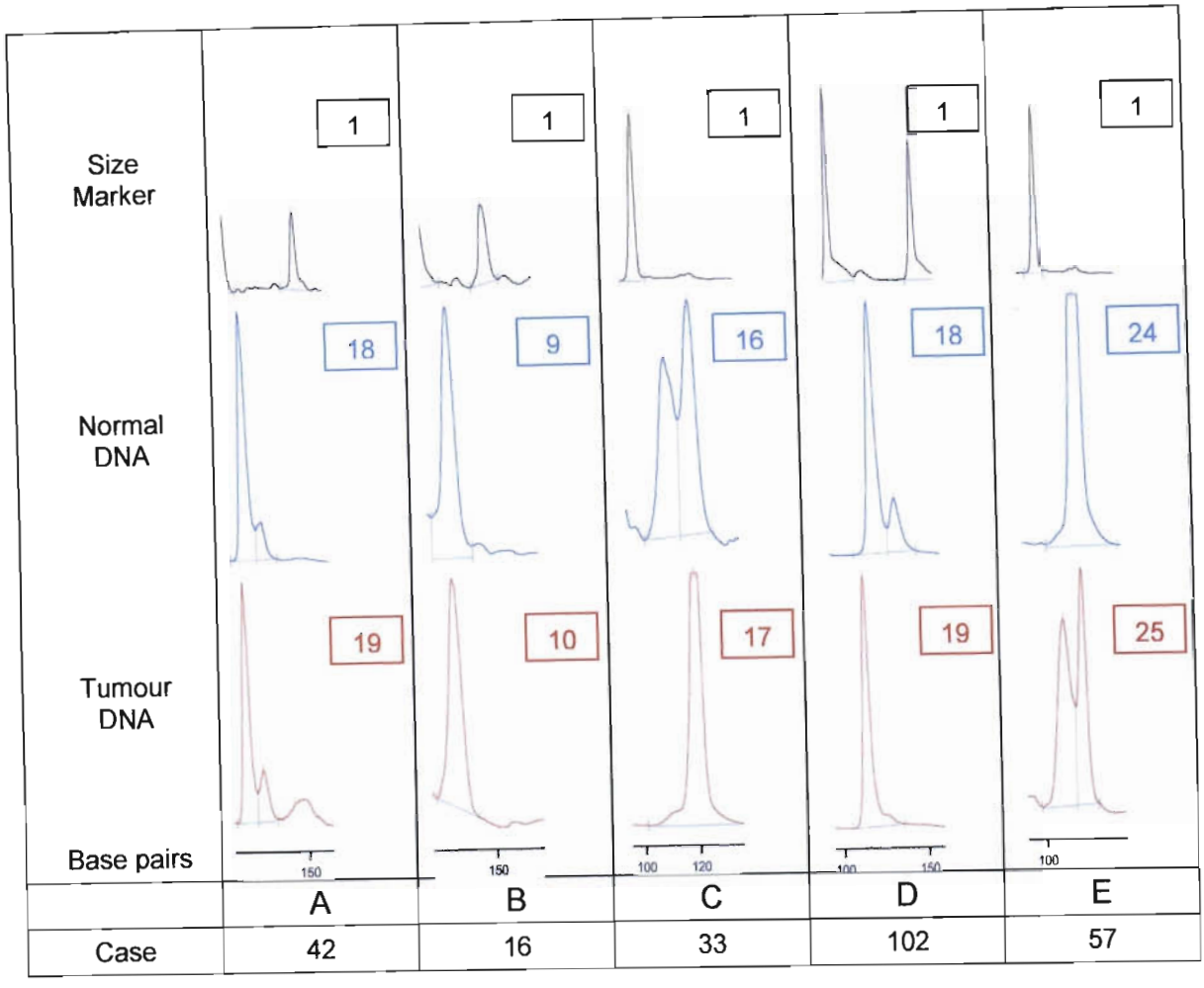
NC2 - No change heterozygous, informative case

NI - Not informative



	Lane	Peak Area	Size (base pairs)
A	2 (88N)	2992.6	110.1
		1657.8	120.4
	3 (88T)	3052.9	110.1
		1127.2	120.9
B	38 (65N)	176.7	122.2
	39 (65T)	147.9	122.7
C	8 (13N)	263.1	116.2
	9 (13T)	292.4	112.6
		350.6	116.9

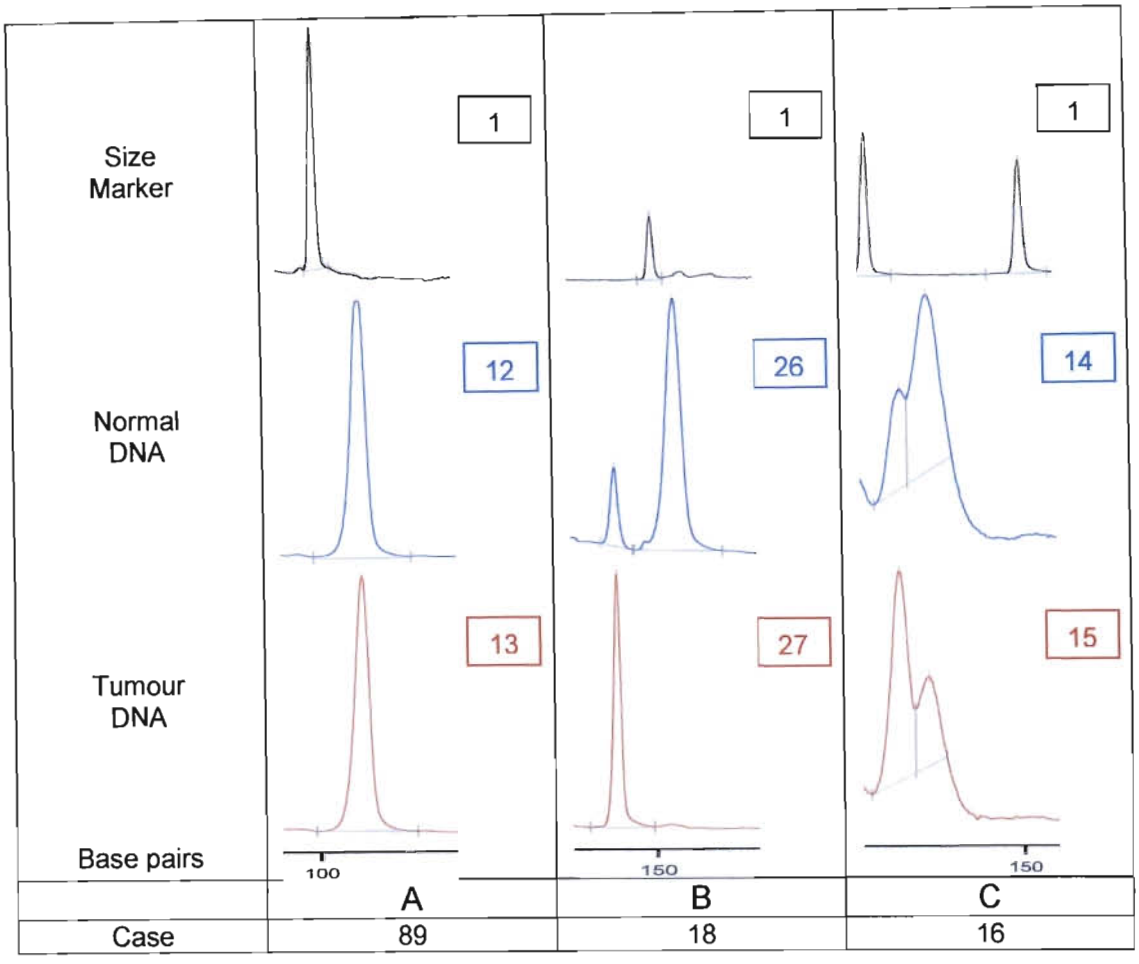
Figure 24: Electrophoretograms for marker **bat25** showing **(A)** no change heterozygous, **(B)** no change homozygous, and **(C)** microsatellite instability in which there is a novel allele in the tumour sample



	A	B	C	D	E
Case	42	16	33	102	57

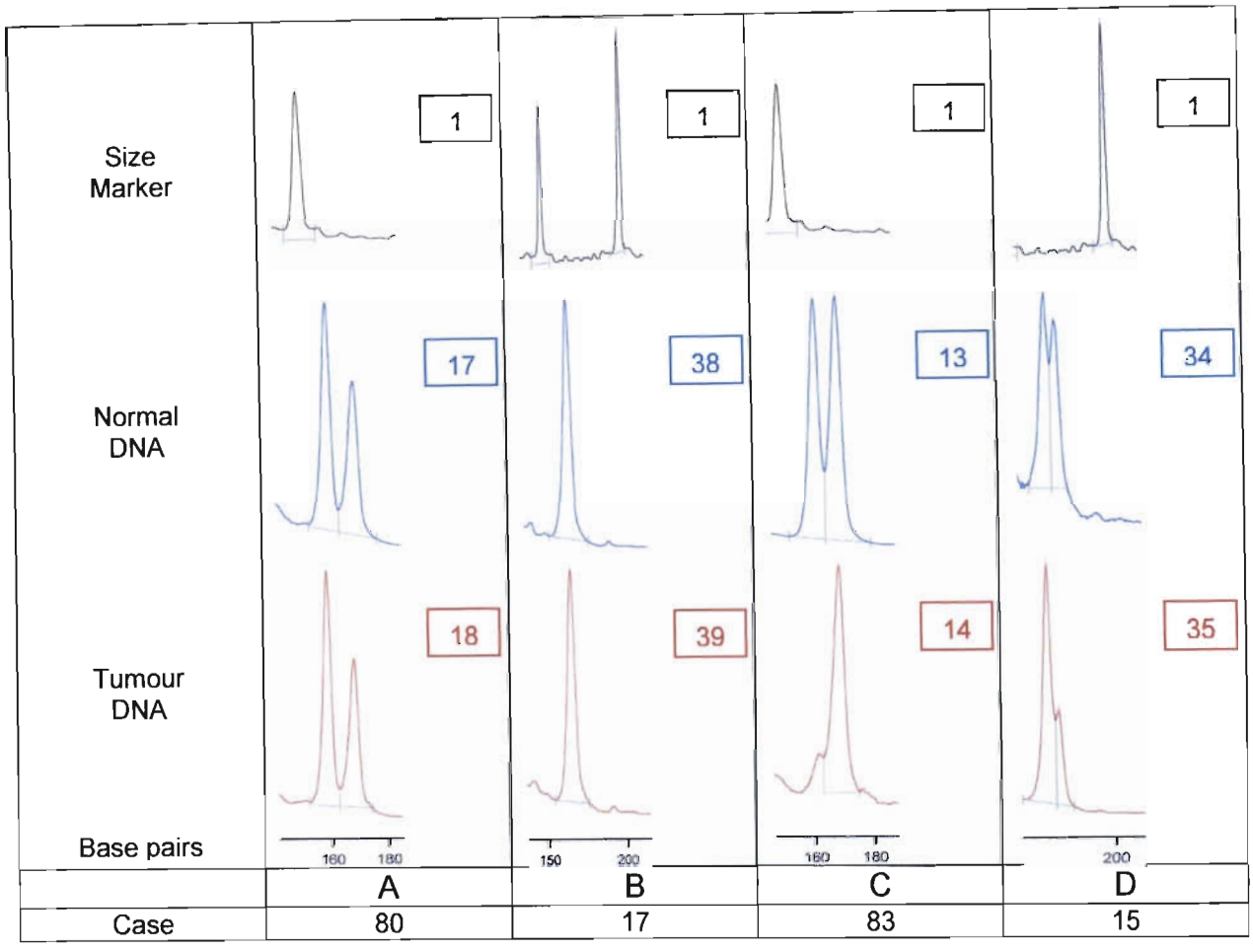
	Lane	Peak area	Size (base pairs)
A	18 (42N)	1246.3	110.2
		215.2	122.1
	19 (42T)	91.5	109.8
		26.1	121.3
B	9 (16N)	7255.4	120.5
	10 (16T)	2771.8	119.9
C	16 (33N)	207.6	110.0
		234.8	119.0
	17 (33T)	3793.1	119.1
D	18 (102N)	1598.5	121.6
		424.2	134.3
	19 (102T)	996.7	120.0
E	24 (57N)	5237.2	117.8
	25 (57T)	204.8	109.7
		154.7	118.0

Figure 25: Electrophoretograms for marker **bat26** showing **(A)** no change heterozygous, **(B)** no change homozygous, **(C)** loss of heterozygosity with loss of the smaller allele, **(D)** loss of heterozygosity with loss of the larger allele, and **(E)** microsatellite instability in which there is a novel allele in the tumour sample



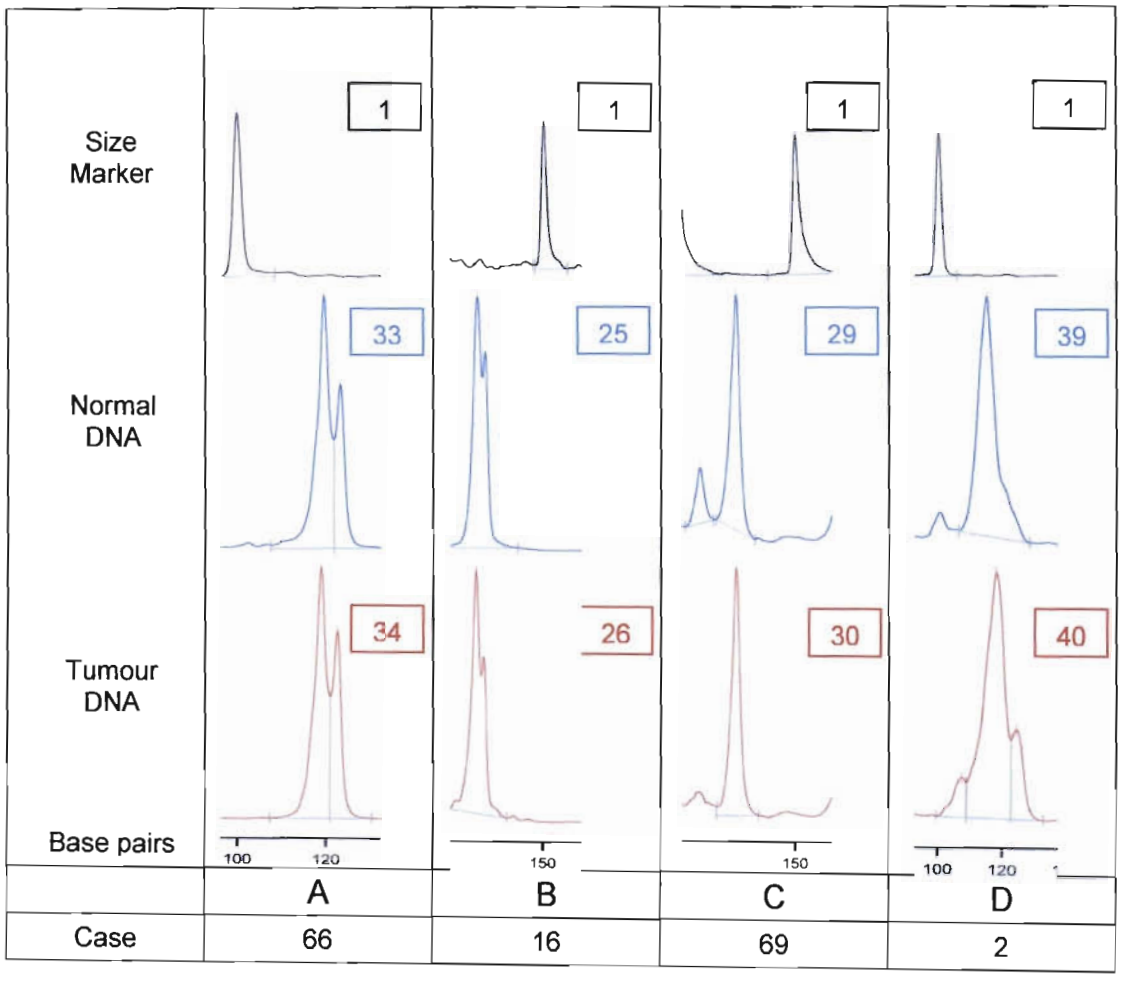
	Lane	Peak Area	Size (base pairs)
A	12 (89N)	3586.3	113.3
	13 (89T)	1475.2	113.6
B	26 (18N)	148.9	136.0
		928.0	157.4
	27 (18T)	967.5	136.3
C	14 (16N)	22.3	110.3
		61.2	119.4
	15 (16T)	86.7	109.7
		39.9	119.1

Figure 26: Electrophoretograms for marker **bat40** showing **(A)** no change homozygous, **(B)** loss of heterozygosity, and **(C)** allelic imbalance (R = 0.17) with loss of the larger allele



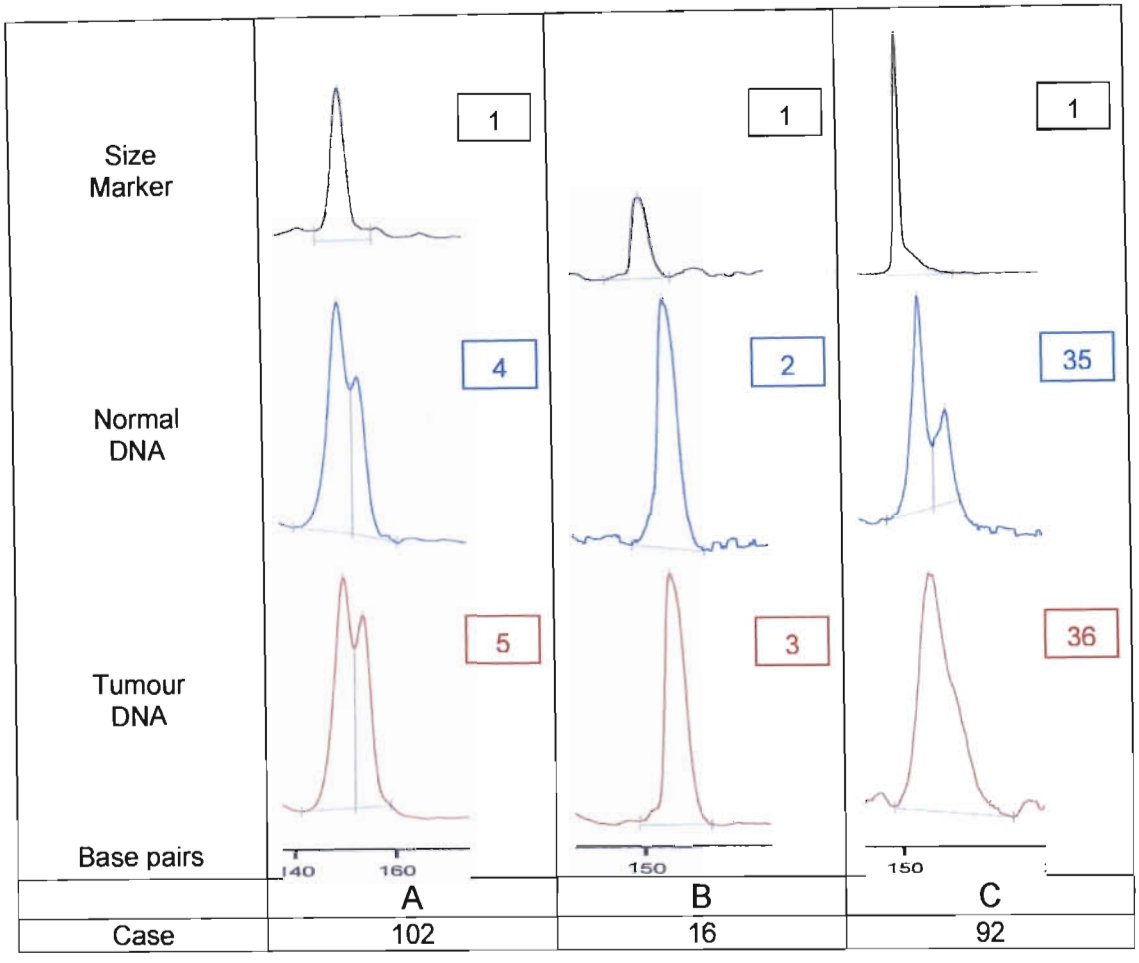
	Lane	Peak area	Size (base pairs)
A	17 (80N)	72.8	159.2
		58.9	168.7
	18 (80T)	79.2	158.6
		59.0	168.0
B	38 (17N)	436.9	164.6
	39 (17T)	239.5	165.3
C	13 (83N)	193.5	161.0
		221.3	168.5
	14 (83T)	36.1	168.5
D	34 (15N)	45.8	168.0
		33.3	173.1
	35 (15T)	307.5	167.8
		88.1	172.7

Figure 27: Electrophoretograms for marker **D2S123** showing **(A)** no change heterozygous, **(B)** no change homozygous, **(C)** loss of heterozygosity, and **(D)** allelic imbalance (R = 0.39) with loss of the larger allele



	Lane	Peak area	Size (base pairs)
A	33 (66N)	1651.6	119.4
		746.8	123.0
	34 (66T)	1313.1	118.8
		727.3	122.4
B	25 (16N)	3531.9	119.2
	26 (16T)	1525.1	118.8
C	29 (69N)	17.9	109.4
		88.7	124.7
	30 (69T)	92.6	124.8
D	39 (2N)	223.8	117.8
	40 (2T)	31.4	107.5
		322.9	118.0
		67.5	124.3

Figure 28: Electrophoretograms for marker **D3S659** showing **(A)** no change heterozygous, **(B)** no change homozygous (note stutter band not detected by computer software), **(C)** loss of heterozygosity, and **(D)** microsatellite instability with new alleles appearing in the tumour sample



	Lane	Peak area	Size (base pairs)
A	4 (102N)	66.7	149.2
		31.2	153.0
	5 (102T)	127.0	149.8
		83.6	153.6
B	2 (16N)	62.3	158.5
	3 (16T)	154.0	158.9
C	35 (92N)	46.9	156.3
		21.9	164.6
	36 (92T)	1042.9	157.3

Figure 29: Electrophoretograms for **D3S1255** showing **(A)** no change heterozygous, **(B)** no change homozygous and **(C)** loss of heterozygosity

Overall, only one case showed LOH for 3 mismatch repair gene markers (case #81). Six cases showed LOH for 2 markers, 5 of these were from treatment group A1. Of the 23 cases that showed LOH for 1 marker, 13 were from treatment group A1. There were four cases in each treatment group that showed MSI for one marker.

Table 67: Data for the mismatch repair gene markers - treatment group A1

	Bat25 (%)	Bat26 (%)	Bat40 (%)	D2S123 (%)	D3S659 (%)	D3S1255 (%)	Overall	
							#	% [a]
LOH	1 (16.7)	7 (50.0)	3 (33.3)	6 (33.3)	2 (7.4)	7 (28.0)	19	39.6
MSI	0	1 (7.1)	0	0	2 (7.4)	1 (4.0)	4	8.3
NC2	5	6	6	12	23	17		
NC1	42	34	39	30	21	23		
Total	48	48	48	48	48	48		
Informativity	12.5	29.2	18.8	37.5	56.3	52.1		

Table 68: Data for the mismatch repair gene markers – treatment group B1

	Bat25 (%)	Bat26 (%)	Bat40 (%)	D2S123 (%)	D3S659 (%)	D3S1255 (%)	Overall	
							#	% [a]
LOH	1 (16.7)	2 (66.7)	3 (60.0)	2 (50.0)	0	4 (44.4)	11	52.4*
MSI	2 (33.3)	0	0	0	2 (11.8)	0	4	19.0*
NC2	3	1	2	2	15	5		
NC1	16	19	17	18	5	13		
Total	22	22	22	22	22	22		
Informativity	27.3	13.6	22.7	18.2	77.3	40.9		

* One case was non informative for all six mismatch repair gene markers.

a - this refers to the percentage of informative cases (n= 21).

In the 70 cases analysed, LOH was seen most frequently with marker D3S1255 followed by bat26 and D2S123. Similar results were seen in treatment group A1.

In all cases, inclusive of both treatment groups, LOH for at least one MMR marker was seen in 30 of 69 informative cases (43.5%) (Tables 67 & 68). Twenty-three of these 30 cases showed LOH for only one marker only, 6 showed LOH for 2 markers and 1 case showed LOH for three markers. Nineteen (39.6%) of the 30 cases were in treatment group A1 and 11 (52.4%) in group B1. Although MMR LOH was more frequent in treatment group B1, this did not reach significance. MSI was seen in 8 of 69 (11.6%) informative cases.

Because these markers map to four different chromosomes only limited grouping of markers was possible. Bat26 and D2S123 were grouped together since both map to 2p16. LOH for the 2p16 region only (bat26 or D2S123) was found in 41.9% (13/31) of informative cases. Four of the thirteen cases showed LOH for both bat26 and D2S123. The remaining cases showed LOH for only one of these two markers and were non informative for other marker. Three of the four cases that showed LOH for both 2p16 markers had anaplastic histology. Two patients died of disease and one was lost to follow up. The one case with regressive histology that had LOH for both these markers was disease free at 74 months of follow up.

D3S659 and D3S1255 were also grouped since both map to the short arm of chromosome 3 but unlike the 2p markers these two markers have different loci on 3p. LOH for the 3p region (either D3S659 or D3S1255) was present in 12 of 59 informative cases (20.3%). Only one tumour (Case #69) showed LOH for both these markers. This case is likely to have loss of a larger region of 3p. This tumour received preoperative chemotherapy, had stromal histology, showed LOH for three 11p markers and was lost to follow up after 6.9 months.

The association between D2S123 and gender ($p=0.016$, Fisher's exact test) was influenced by the higher number of non informative cases among female patients. This result is therefore viewed with caution. There was only one other marginal association between bat25 and outcome. This was also interpreted with caution because of the small number of patients in the study and the large group of patients lost to follow up. There were no other significant associations between mismatch repair markers and clinicopathological features.

4.2.7 Comparative results for all 3 sets of markers

Table 69: LOH and MSI for the three sets of markers – treatment group B1

Case	LOH				MSI					
no.	11p	16q	MMR	Total	11p	16q	MMR	Total	%	Status
1	-	1	-	1	1	1	1	3	17.6	MSI-L
2	1	1	1	1	3	-	1	4	23.5	MSI-L
3	3	2	1	6	-	1	-	1	5.9	MSI-L
4	-	-	-	0	1	-	-	1	5.9	MSI-L
5	2	1	-	3	-	-	-	-	-	MSS
7	2	-	-	2	-	-	-	-	-	MSS
8	-	3	-	3	-	-	-	-	-	MSS
9	-	1	1	2	1	-	-	1	5.9	MSI-L
10	-	1	-	1	-	-	-	-	-	MSS
11	2	1	-	3	-	-	-	-	-	MSS
12	2	-	1	3	1	1	1	3	17.6	MSI-L
13	-	1	-	1	1	-	1	2	11.8	MSI-L
14	-	1	-	1	1	-	-	1	5.9	MSI-L
15	3	1	2	6	-	-	-	-	-	MSS
16	4	-	1	5	-	-	-	-	-	MSS
17	-	-	1	1	-	-	-	-	-	MSS
18	1	1	1	3	-	-	-	-	-	MSS
19	-	-	1	1	2	-	-	2	11.8	MSI-L
21	2	1	1	4	-	-	-	-	-	MSS
22	2	-	-	2	-	1	-	1	5.9	MSI-L
23	1	2	-	3	-	-	-	-	-	MSS
35	-	3	1	4	-	-	-	-	-	MSS

Table 70: LOH and MSI for the three sets of markers – treatment group A1

Case	LOH				MSI					
no.	11p	16q	MMR	Total	11p	16q	MMR	Total	%	Status
24	1	2	-	3	-	-	-	-	-	MSS
27	2	-	1	3	-	-	1	1	5.9	MSI-L
28	-	-	-	0	1	-	-	1	5.9	MSI-L
29	3	2	1	6	-	-	1	1	5.9	MSI-L
33	2	1	1	4	-	-	-	-	-	MSS
34	-	1	-	1	-	-	-	-	-	MSS
36	1	-	-	1	-	-	-	-	-	MSS
37	-	-	1	1	-	1	-	1	5.9	MSI-L
38	4	-	-	4	-	-	-	-	-	MSS

Case no.	LOH				MSI					
	11p	16q	MMR	Total	11p	16q	MMR	Total	%	Status
41	-	2	1	3	-	-	1	1	5.9	MSI-L
42	-	1	-	1	1	-	-	1	5.9	MSI-L
44	2	-	2	4	-	-	-	-	-	MSS
47	1	2	2	5	1	-	-	1	5.9	MSI-L
48	1	1	-	2	-	-	-	-	-	MSS
52	1	1	-	2	-	-	-	-	-	MSS
53	-	2	-	2	-	-	-	-	-	MSS
54	2	-	-	2	-	-	-	-	-	MSS
55	1	-	-	1	-	-	-	-	-	MSS
57	3	-	-	3	-	-	1	1	5.9	MSI-L
58	1	-	1	2	-	-	-	-	-	MSS
60	-	-	-	0	-	-	-	-	-	MSS
63	1	1	-	2	-	-	-	-	-	MSS
65	2	1	-	3	-	-	-	-	-	MSS
66	3	-	-	3	1	-	-	1	5.9	MSI-L
67	-	2	-	2	-	-	-	-	-	MSS
68	3	-	-	3	-	-	-	-	-	MSS
69	3	-	2	5	-	1	-	1	5.9	MSI-L
70	1	1	-	2	-	-	-	-	-	MSS
71	-	1	1	2	-	-	-	-	-	MSS
72	1	1	-	2	-	-	-	-	-	MSS
73	2	1	-	3	1	1	-	2	11.8	MSI-L
76	-	1	-	1	1	-	-	1	5.9	MSI-L
77	2	-	1	3	-	-	-	-	-	MSS
78	-	2	-	2	-	1	-	1	5.9	MSI-L
80	-	-	-	0	-	-	-	-	-	MSS
81	1	1	3	5	1	-	-	1	5.9	MSI-L
82	1	1	1	3	-	-	-	-	-	MSS
83	3	1	1	5	-	-	-	-	-	MSS
84	2	-	-	2	-	-	-	-	-	MSS
88	-	-	-	0	-	-	-	-	-	MSS
89	-	1	-	1	-	-	-	-	-	MSS
90	1	3	1	5	-	1	-	1	5.9	MSI-L
91	1	1	-	2	-	1	-	1	5.9	MSI-L
92	2	2	2	6	-	-	-	-	-	MSS
96	2	-	-	2	-	1	-	1	5.9	MSI-L
99	-	3	1	4	-	-	-	-	-	MSS
102	6	1	2	9	-	-	-	-	-	MSS
115	-	2	1	3	-	-	-	-	-	MSS

Nine cases in treatment group A1 and five in treatment group B1 showed LOH for at least one marker from each of the three sets of markers analysed (Tables 69 & 70). Case #102 showed LOH for 9 different markers. These included all six 11p markers, one 16q marker and two MMR markers. There were two cases from each treatment group (cases #3, #15, #29, #92) which showed LOH for a total of six markers from across all three sets of markers. Cases #15 and #92 also was microsatellite stable but the other two showed MSI for only one marker.

Of the 70 cases studied, there were no cases with high frequency microsatellite instability (MSI-H). A tumour would need to show MSI for more than 5 markers for it to be classified as MSI-H. The closest to the criteria for MSI-H was case #2 in treatment group B1 which showed MSI for four markers, three of which were 11p markers and 1 MMR marker. Twenty seven of the 70 (38.6%) cases showed MSI-L. Seventeen (35.4%) cases were from treatment group A1 and 10 (45.5%) from group B1. Two cases showed MSI for 3 markers. Both of these cases were in treatment group B1 and each showed MSI for one marker from 11p, 16q and MMR. There was no direct association between the combined MSI status and clinicopathological features.

4.2.8 SURVIVAL ANALYSIS

The Kaplan-Meier estimates showed that clinicopathological stage had a significant association with survival (Figure 30). The mortality rate was directly related to increasing stage. Other markers with a significant association with survival were D11S902, D11S930, D16S413, D3S659 and bat26.

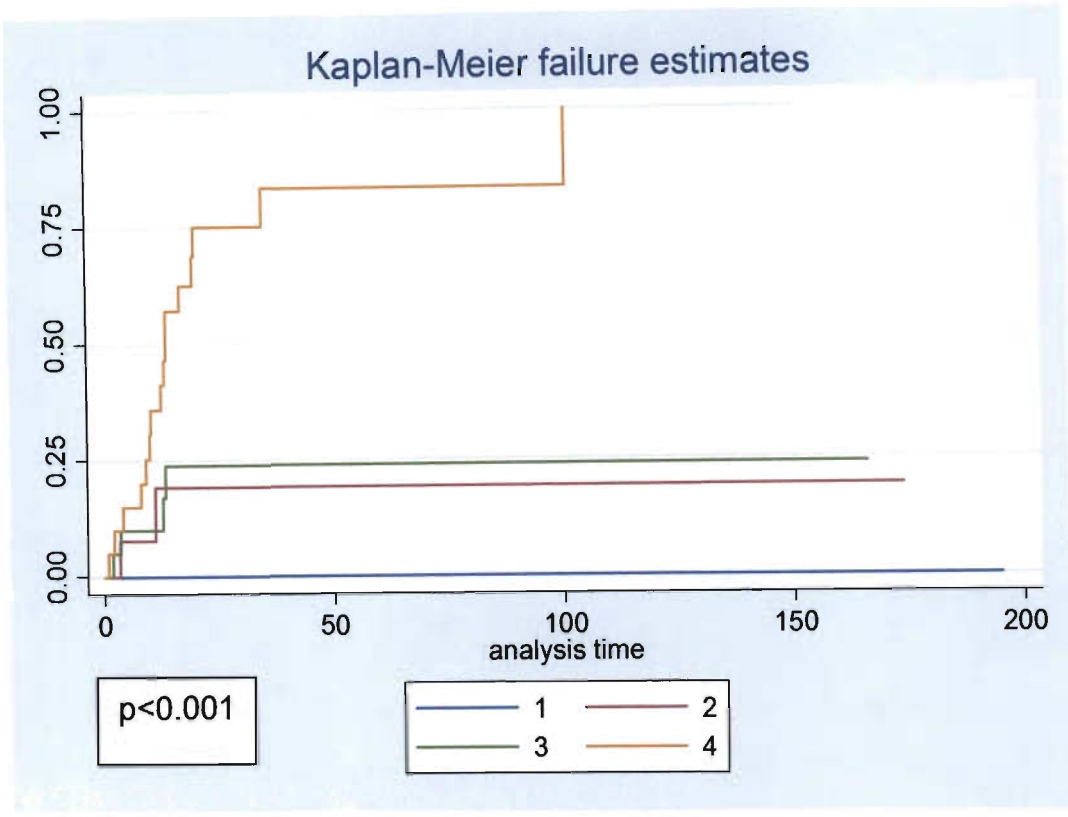


Figure 30: Kaplan-Meier estimates for clinicopathological stage

The five markers (D11S902, D11S930, D16S413, D3S659, bat26) that were significant from the Kaplan-Meier analysis were used in the Cox regression model with adjustment for the following possible confounders. This was done since the study design was mainly observational. From the Cox regression none of the markers were significantly associated with survival after adjustment for preoperative chemotherapy, stage, gender, and age. However, stage was found to be a significant factor ($p<0.001$) (HR=5.6, 95%CI: 2.1-14.9). The small sample number of subjects and especially the small number of events across the covariate patterns will have low power.

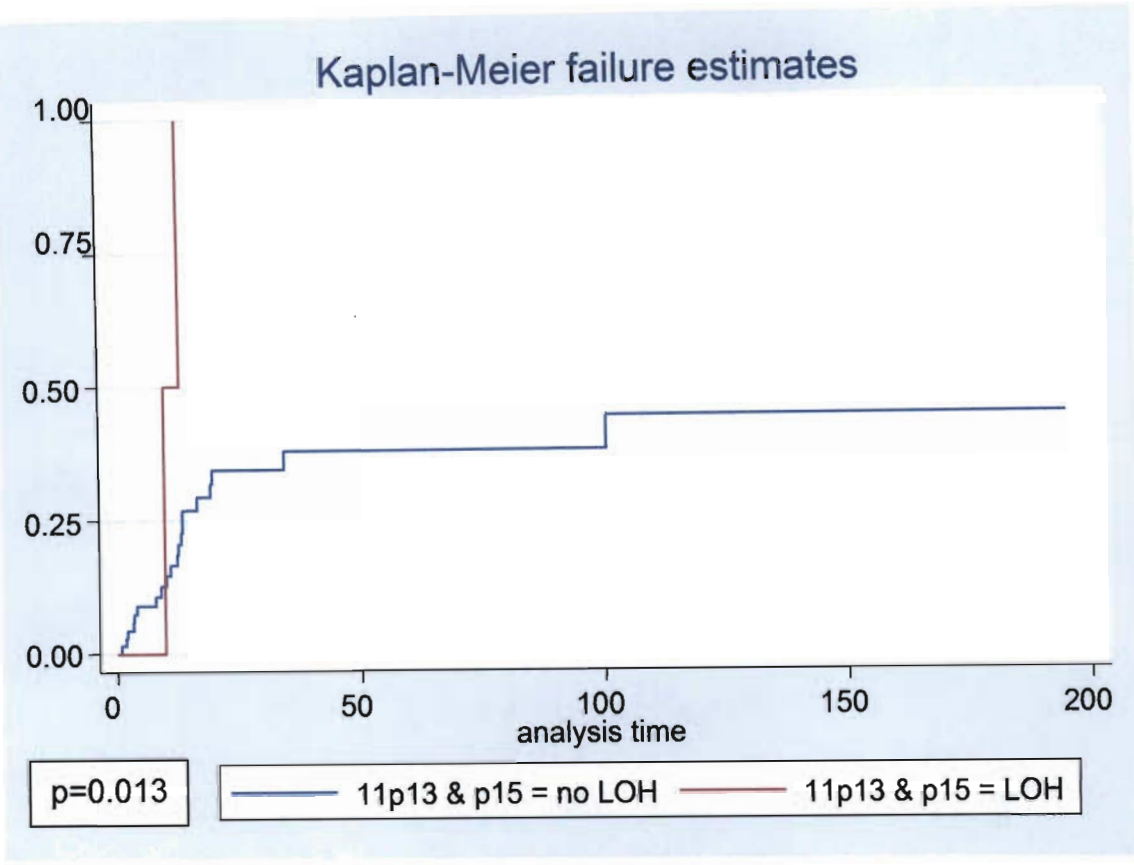


Figure 31: Kaplan-Meier estimates for the group with LOH for both 11p13 and 11p15

The Kaplan-Meier estimates showed that cases with LOH for both 11p13 and 11p15 had a worse survival compared to cases without LOH at these loci (Figure 31). After adjustment for confounding factors this did not retain its significance. The Kaplan-Meier estimate was unreliable because of the very small number of events in the LOH group.

The region 16q12.1 was found to be a highly significant marker for survival by the Cox regression model (p=0.004) (HR=5.7, 95%CI: 1.7-18.6). The wide confidence interval reflects a lack of precision due to a small sample size and the log rank test was not significant (p=0.431).

CHAPTER FIVE

DISCUSSION

5.1 Demographics and Clinical features

King Edward VIII hospital (KEH) is a government hospital serving as an academic referral hospital for the province of KwaZulu-Natal. KwaZulu-Natal is the most populous province with a total population of 9.43 million, which is predominantly African, followed by white and mixed race groups.[510] Asians, made up predominantly of Indians, account for 0.79 million of the population of KwaZulu-Natal. Prior to 1994 KEH catered mainly for Africans and Indians. Most white patients attended other government hospitals reserved for whites or used private health care facilities. This explains the predominance of African patients in the present study. In the United States the age-standardised rate of nephroblastomas is 8.5 cases per million White children per year.[511] The corresponding figure for United States Black children is 10,9.[511] In Britain the relative incidence rate in West Indians is twice the rate in Whites and Whites have a rate twice that of Asians.[24] Although this study was unable to comment on any clinical or pathological differences between racial groups because of the study population, another study from another centre in South Africa reported no significant differences in presentation and outcome for the different racial groups.[512]

It is also significant to note that the political situation in South Africa changed during the study period. This accounts for the peak in admissions in the early 1990s which coincided with changes in patient referral patterns.

Just over 80% of patients received preoperative chemotherapy according to various SIOP protocols. Because of the long period of recruitment in this retrospective study, patients were managed according to different treatment regimens. This introduced some variation into the study population.

The majority of patients were between 12 and 36 months of age. The overall mean age of 42.3 months was slightly higher than published reports.[23] The average age for females was 42.7 months, comparable with the average age in the NWTS (42,5 months).[23] For males the average age was higher when compared to the NWTS, i.e. 41.8 months and 36,5 months, respectively. In comparison to the NWTS findings of over 90% of patients being younger than 6 years, the present study found that 87% of patients were 6 years or younger. Two patients were older than 10 years and there were no adult patients. Patients who had immediate surgery were younger than those

that had preoperative chemotherapy. A similar finding was reported in a study that analysed a larger group of patients.[513] These authors suggested that this was directly related to the smaller tumours seen in this group. We were unable to analyse this because tumour masses were not available for all cases, however, an earlier study conducted at the same institution reported the mean mass as 947 grams (range 80 – 5000 grams).[1]

The slight female predominance in the present study (F:M=1.12:1) is similar to that described by others.[165,514-517] Overall, there does not appear to be a consistent gender preponderance.

The majority of patients in this cohort presented with advanced disease (either stage III or IV). Most of these patients (31.2%) had stage IV disease. There were 65% more patients with stage IV disease than stage I disease. Stages II and III constituted half of all the cases. Patients at this institution usually present late with advanced disease. The majority of children seen at this institution live in rural areas. In an earlier 5 year study conducted at the same institution 77% of nephroblastoma admissions were from rural areas.[29] These authors reported that irregular discharge, death before investigation and refusal of Western medical treatment were some of the problems experienced with children from rural areas. The mean tumour mass in rural children was higher than urban children. The authors accounted for the high frequency of advanced disease by the late presentation of patients which in turn was multifactorial and included socio-economic factors, educational factors and inadequacy of primary and secondary health facilities. Another major problem was the high incidence of associated infectious diseases in these patients. The authors outlined some of the factors influencing the management of nephroblastomas in developing countries which explains some of the limitations of the present study. Other reports from South Africa and the rest of Africa also highlighted these problems including the inadequate follow up of nephroblastoma patients.[512,518,519]

We also noted a significant association between stage and patient age. We feel that this is also directly related to the delay in seeking medical attention. For varying

reasons patients do not seek medical attention at the time of first symptoms. The reasons for this delay were discussed earlier.

As reported previously clinicopathological stage was a significant predictor of outcome. There was an increasing death rate with increasing stage.

5.2 SIOP histopathological classification and SIOP risk group

The most frequently diagnosed histological types were the mixed and regressive types. In preoperatively treated cases regressive histology and mixed histology have been reported as the predominant types[513,520], not unlike the findings of the present study. A significantly higher frequency of blastemal and epithelial histology tumours in the primary surgery group was reported previously.[513] These authors also reported that stromal histology was rare in the group treated with primary surgery. The present study found that mixed histology tumours were the most frequent histological type seen in patients who had primary surgery and blastemal histology constituted 18.5% of tumours in this treatment group. Furthermore, there was no difference in the frequency of stromal histology between the two treatment groups, however one has to view these results with caution because of the small number of cases that underwent primary surgery.

Diffuse anaplasia was seen in 8% of tumours, all of which received preoperative chemotherapy. The rate of anaplasia was slightly higher than that reported in the literature, which varies between 4,5% and 7,5%.[181,183,520-522] The higher rate may be due to the exclusion of cases that had complete necrosis or insufficient material for analysis. In the present study only 18.2% (2/11) of anaplastic nephroblastomas occurred in patients under 2 years, consistent with most studies in which anaplastic histology was rare in children under 12 months.[182,521] In contrast, 43.3% (55/127) of non-anaplastic nephroblastomas occurred in patients under 2 years.

Significant ethnic differences in the frequency of anaplasia have been described. Bonadio *et al.*[521] found that a larger proportion of anaplastic tumours occurred in "non-white" patients, including "Black and Latin American" patients. We were unable to comment on ethnic differences in the frequency of anaplasia since our hospital served a selective population comprising mainly Africans.

Patients who were diagnosed with anaplastic tumours were older than the other patients (mean age 49.5 months). Anaplasia tends to occur in older patients. A mean age of 62-63 months has been reported and another found that more than half of the cases occurred in patients older than 5 years.[181,182,521] Others have also confirmed a significant association between anaplasia and patient age.[520] These are interesting findings which provide some insight into the question whether anaplasia arises *de novo* or is the result of dedifferentation following as yet unknown genetic alterations. Blastemal tumours in treatment group A were also diagnosed in older patients. In combination, high risk histology tumours occurred in older patients. This suggests that the resistance of blastemal tumours to chemotherapy is acquired over time and may have a similar mechanism to anaplastic tumours.

In the present study the majority of patients with anaplasia were male (72.7%). Although other studies[181,182,521] have reported a female predominance, a definite gender predilection for anaplasia has not been confirmed. In our study the small number of anaplastic tumours and the absence of focal anaplastic tumours do limit the significance of the finding.

Most anaplastic tumours present with advanced disease stage. The tumour related mortality rate for anaplastic tumours (81.8%) was different to those reported in the NWTS, which were 47% and 41.7%.[183,521] This may be due to the fact that the majority of patients with anaplastic tumours had advanced stage disease. The significant interaction between anaplasia and clinicopathological stage found in the present study is supported by Weirich *et al.*[520], however, others have not found such an association.[181,182] Stage I disease with anaplasia has a relatively good prognosis and falls into the focal anaplasia category.[23,162,182] There were no stage I tumours with anaplasia in the present study. The overall higher disease stage seen with the high risk group is consistent with the poorer prognosis associated with this group.

Not unexpectedly, advanced disease stage, anaplasia and blastemal histology individually and high risk histology were associated with a higher death rate.

5.3 IMMUNOHISTOCHEMICAL STUDY

5.3.1 p53 immunoexpression

The *p53* gene has also been suggested to play a role in the pathogenesis of nephroblastomas.[338,339] The present study looked at the prognostic significance of the immunohistochemical detection of p53 protein in nephroblastomas. It should be noted that the immunohistochemical detection of p53 protein is neither a perfect nor an accurate reflection of underlying *p53* mutations because mechanisms other than mutations may also result in p53 protein accumulation. Nevertheless, there seems to be good agreement between the frequency of positive immunohistochemical staining and mutations detected by DNA sequencing and this is generally accepted in practice.[523] Lahoti *et al.*[335] however, found that most nephroblastomas with immunodetectable p53 protein did not have *p53* mutations in the hot-spot regions. They postulated that this may be due to the presence of mutations in regions not examined or that the tumour overexpressed or retained wild-type p53. It has been stated by Lahoti *et al.*[335] that the phenomenon of tumour heterogeneity may cause variation in the results of immunohistochemical and molecular analyses of tumour. It was therefore recommended that multiple blocks be examined to ascertain whether immunostaining varies from area to area. In the present study up to three blocks of non-necrotic tumour were examined without finding a marked variation in staining patterns. The overall scoring of different blocks from the same tumour remained the same.

The majority of cases (87.7%) showed low expression of p53. Expression was most frequently seen in blastemal cells and least in stromal cells. No significant difference in p53 expression was found between the two treatment groups in the present study, but there have been conflicting results from studies investigating the influence of chemotherapy on p53 expression. One study found that the level of p53 immunopositivity was not altered by preoperative chemotherapy,[335] in contrast to the observation by Moll *et al.*[524] that chemotherapy may influence p53 protein expression.

Although some previous studies have suggested that increased p53 expression is associated with advanced stage and biologically aggressive Wilms tumours,[184,337,338,343,345] others have not.[339] In the present study there was a

significant difference in p53 expression between non anaplastic and anaplastic histology tumours and between high risk and intermediate risk tumours. p53 immunoexpression was a frequent finding in anaplastic tumours. The finding of high p53 expression in all cell types, including overtly anaplastic and non-anaplastic cells, in anaplastic histology tumours is potentially of practical value. Detection of anaplasia is dependent on the thorough examination and adequate sampling of the tumour specimen. It is possible that if the tumour is poorly sampled anaplasia may not be detected in some cases. If a tumour shows high p53 expression and no anaplasia is found in the sections examined, additional sections of the tumour should be examined carefully for anaplasia. Generous sectioning of the tumour is recommended.[183] One section for each centimetre of tumour diameter is the minimum guideline. Proper and careful specimen handling together with tissue sectioning is essential in order to avoid artefacts that may mimic anaplasia. It may well be that tumours with high p53 expression may require more aggressive chemotherapy. The high p53 expression seen in anaplastic tumours was not present in the other high risk histology tumour, the blastemal type. This suggests that *p53* mutations are not frequent events in blastemal type tumours.

Apart from its role in growth arrest, DNA repair, and apoptosis, p53 also affects the expression of drug resistance genes. Wild-type p53 down-regulates the expression of drug resistance genes, e.g. *MDR1*, *MRP1* and *DNA topoisomerase IIα*.[416-418] but mutant p53 may activate *MDR1* (multidrug resistant gene).[418,525] Hence, the worse prognosis seen with tumours with high p53 expression may be related to *MDR1* activation by the lack of wild type p53. This is based on the assumption that the high p53 expression detected immunohistochemically is due to *p53* mutations. In support of this possible mechanism one would expect to find that all tumours expressing high p53 would also express high levels of p-glycoprotein. However, in the present study a significant proportion of tumours with low p53 expressed high levels of p-glycoprotein. This suggests that p-glycoprotein is activated not only by mutant p53 but also by some other mechanism. Although a direct correlation between up-regulation of p53 protein and chemotherapy resistance in anaplastic Wilms tumours is generally accepted, the significance of p53 expression in non-anaplastic Wilms tumour is currently unresolved.[184,335,341,343,344]

Previous studies of patients with favourable histology Wilms tumour have revealed correlation of p53 immunoreactivity to tumour stage and prognosis[340,341,343,345] In the present study there was no direct association with stage and outcome nevertheless all stage I tumours showed low expression with 10 of the 18 stage I tumours actually failing to express any p53 protein. This finding strongly favours p53 alterations as late events in natural history of nephroblastomas. p53 mutations are acquired long after development of the tumour. Furthermore, patients who died were more likely to have tumours that expressed high p53, however, this did not reach statistical significance.

Lahoti *et al.*[335] found that most of the nephroblastomas (94%) that are immunopositive for p53 carry a higher risk of metastasis or recurrence in contrast to the majority of p53 negative primary tumours, a finding which was not reproduced in the present study.

A subset of patients who died despite having relatively low disease stage and favourable histology had tumours that showed low p53 staining. It is possible that in these cases *MDM-2* overexpression may be responsible for the inactivation of p53 in an autoregulatory fashion[297] since *MDM-2* overexpression may overcome p53 regulated growth control in the absence of *p53* mutations.[526,527] It is also possible that there are interactions between p53 and other cellular proteins. Evidence is accruing that the cellular environment plays a crucial role in p53 stability and ultimately, immunodetection. Therefore, p53 protein expression may be a reflection of not only *p53* gene mutations but also other cellular and/or genetic events. It has been shown that p53 interacts with various viral gene products, such as human papilloma virus E6, simian virus 40 T-antigen and adenovirus E1B.[204,261,528] Cellular proteins, heat shock protein 70 and transcriptional factor WT1, are also associated with p53.[11,252,260]

Whatever the mechanism, it is clear that p53 immunoexpression is of importance to both those diagnosing and treating nephroblastomas. The findings of the present study are in agreement with the previous studies of p53 analysis in nephroblastomas, where anaplasia is associated with significantly high levels of p53 expression.[335,341] However, there are additional features that emanate from the

current study. p53 immunoexpression is low in the majority of nephroblastomas. Low p53 expression occurs in all stages i.e. there is no correlation with disease stage.

5.3.2 p21 immunoexpression

Expression of p21 was seen in less than half of tumour cells in all cases. Only 11 cases showed high p21 immunoexpression. Staining was seen in all three histological components. An interesting finding was consistent staining in foci of squamous cell differentiation, similar to that reported in laryngeal carcinomas. The expression of p21 in these cells is consistent with their terminally differentiated state and the relative reduction in cell proliferation. In laryngeal tumours, expression of $p21^{cip1/WAF1/sdi1}$ protein correlates with differentiation.[529] Low levels of p21 staining were seen in grade 4 poorly differentiated laryngeal tumours, while grade 1-3 tumours expressed normal to high levels of $p21^{cip1/WAF1/sdi1}$ at both mRNA and protein level. The association of increased $p21^{cip1/WAF1/sdi1}$ protein levels with differentiation has significant exceptions.

Based on our current understanding, it would be reasonable to expect that tumours which were treated preoperatively would show elevated levels of p21, since DNA damage induces p21 expression via a p53 dependent pathway.[262] However, p21 induction was not found to be a consistent feature in treated nephroblastomas. One explanation would be the absence of wild-type p53, a situation in which p53 dependent p21 induction cannot occur.[355] A complete absence of wild-type p53 would require loss of both functional copies of *p53*.[351] However, this mechanism cannot be supported in the present study because correlation between p53 immunoexpression and p21 expression was not significant. Although the majority of cases with high p53 expression showed low p21 expression, this finding was not statistically significant. p21 did not show any significant correlation with any of the clinicopathological parameters. Hence p21 does not seem to have any independent prognostic value. Furthermore, as suggested in a previous study,[355] the absence of $p21^{cip1/WAF1/sdi1}$ induction following chemotherapy in cells that have high p53 expression may be responsible for the resistance of tumour cells to treatment with various drugs or radiotherapy. Consequently, the identification and knowledge of all

agents that are able to induce $p21^{cip1/WAF1/sdi1}$ through alternative pathways might be relevant to the chemotherapeutic treatment of cancer.

5.3.3 bcl-2 immunoexpression

Information on bcl-2 protein expression in nephroblastomas is very sparse. The present study noted heterogeneity in bcl-2 expression but staining was seen predominantly in blastemal and epithelial cells similar to the findings of other studies.[395,530] In a study of favourable histology nephroblastomas, bcl-2 staining was found to be characteristic of blastemal elements but of no prognostic significance.[530]

The present study found that bcl-2 immunoexpression was significantly associated with histological risk group but not with histological classification, clinicopathological stage or outcome. High risk histology tumours show some degree of resistance to chemotherapy, the exact mechanism of which is still not fully understood. The finding of more frequent high bcl-2 expression in high risk tumours suggests that bcl-2 may play a key role in the development of chemoresistance in these tumours. Furthermore, an interesting and statistically significant finding in the present study was the greater likelihood of bcl-2 immunoexpression in tumours treated with preoperative chemotherapy. The role of bcl-2 in conferring chemoresistance has been investigated in several tumours such as oesophageal squamous cell carcinomas, acute myeloid leukaemias and neuroblastomas.[531-534] It is conjectural and debatable as to the bcl-2 status of the nephroblastomas in our study before chemotherapy was instituted. It is felt that the pre-chemotherapy bcl-2 status of the tumours is more than likely the same as that seen after treatment,[396] although our study contradicts this finding. The high bcl-2 levels in pretreated tumours seen in the present study may be due to the chemoresistance of the bcl-2 positive clones and the ablation of the bcl-2 negative clones.

The general consensus from the literature indicates that elevated bcl-2 levels endow tumour cells with increased survival characteristics, leading to tumours being refractory to chemotherapy. The bcl-2 positive nephroblastomas are therefore more chemoresistant. Bcl-2 positive cells in primary and metastatic prostate carcinomas were found to be refractory to hormone treatment.[535] These authors suggested that

bcl-2 enabled prostate carcinoma cells to survive in an androgen-deprived environment by inhibiting apoptosis. Furthermore, in bladder carcinomas survival analysis showed a trend between lower survival probability and expression of bcl-2 protein, however, a multivariate analysis showed that bcl-2 expression had no independent prognostic value.[536] The above postulate regarding the effect of elevated bcl-2 levels needs to be validated further by prospective studies. The potential application in the future management of nephroblastomas could mean that bcl-2 positive tumours warrant separation from bcl-2 negative tumours and should perhaps be given more and/or different chemotherapeutic agents and should be more carefully followed up.

Two studies failed to demonstrate any prognostic significance for bcl-2 immunoexpression.[396,409] However, Ghanem *et al.*[410] found increased bcl-2 expression in the blastemal component of tumours with increasing clinicopathological stages. In contrast, a decrease in bax expression was observed in the blastemal component of tumours with increasing clinicopathological stage. They concluded that blastemal bcl-2 expression and bcl-2/bax ratio were indicative of clinical progression.

In contradiction to the above findings there have been studies, which found that bcl-2 expression was associated with favourable outcome. In a study of non-small cell lung carcinomas, a higher 5-year survival rate was found in bcl-2 positive tumours compared to negative tumours.[401] In ovarian carcinomas strong bcl-2 expression correlated with low grade tumours. Furthermore, patients with p53 and bcl-2 positive tumours had a significantly better prognosis than patients with p53 and bcl-2 negative tumours.[537] Differentiated thyroid carcinomas expressed bcl-2 but anaplastic thyroid carcinomas were bcl-2 negative.[538,539] Bcl-2 staining in breast carcinoma was significantly associated with small tumour size, and low tumour grade and it has been suggested that bcl-2 immunoexpression might be a marker of prognostic value.[408,540]

The present study found that low levels of p53 immunoexpression are associated with low levels of bcl-2 expression. Since p53 immunoexpression has been shown to correlate with *p53* mutations, it can be inferred that tumours with low p53

immunoexpression have wt p53 protein. Wild-type p53 is known to repress bcl-2 transcription.[288-290] Hence the association of low p53 immunoexpression with low bcl-2 expression suggests that bcl-2 repression by p53 is an important feature in these tumours. This will favour the initiation of apoptosis.

An interesting finding was that immunohistochemical localisation of bcl-2 within the developing kidney corresponds closely to that observed for the transcription factors Pax8,[399], and WT1 [43] suggesting that either one, or both, of these factors may play a role in the observed upregulation of bcl-2 expression. At this stage it is unclear how these factors interact with bcl-2 in the pathogenesis of nephroblastoma. Further studies comparing the expression of these factors in relation to bcl-2 expression may expose the role of bcl-2 in the development of nephroblastoma.

It is feasible that bcl-2 may participate in nephroblastoma tumourigenesis since bcl-2 plays an important role in normal renal morphogenesis and nephroblastoma is said to parody renal development. Further molecular investigation of this aspect may unlock the role of bcl-2 in the genesis of nephroblastomas. However, the findings of bcl-2 immunoexpression emanating from this study have several interesting possibilities that could have relevance to management protocols of nephroblastomas.

5.3.4 pRb immunoexpression

The majority of nephroblastomas showed high pRb immunoexpression. Low expression of pRb was seen in 34 preoperatively treated cases. Unlike p53 immunoexpression which has a strong association with the underlying status of the gene, pRb immunoexpression is not a good indicator of the status of the gene. Complete loss of pRb may be due to deletions whereas a decrease in pRb may be due to point mutations.[365] However, it must be noted that the relation between *Rb* mutations and pRb immunoexpression is not clear.[365] Inactivation of the *Rb* gene has usually been associated with loss of the gene product.[541] However, tumours have been described, such as bladder carcinoma,[542] small cell lung carcinoma,[543,544] and prostate carcinoma[545] where *Rb* inactivation due to point or other small mutations is associated with stable protein products of altered molecular weight. These mutant proteins are thought to be without function, as demonstrated

by their inability to be phosphorylated,[543,544] or bind SV40 large tumour antigen,[543,544] and in general the inability of such proteins to localize in the nucleus.

A significant proportion of tumours that were treated with preoperative chemotherapy showed low pRb expression compared to the group not treated with preoperative chemotherapy. The exact mechanism for this decrease in expression is not clear but may be mediated by chemotherapy induced DNA alterations. The exception to the above finding was in diffuse anaplastic tumours which all showed high expression of pRb, despite all of them being treated with preoperative chemotherapy. There was indeed a significant association between pRb expression and histological classification. The normal expression of pRb in anaplastic histology tumours is not surprising since the poor outcome of this variant is due to its chemoresistance rather than proliferative ability.[161,413]

The significance of pRb immunoexpression in prognosis is unclear because of conflicting results. In some neoplasms pRb expression has been related to prognosis,[366,367] while other studies could not confirm this.[368,369] In nephroblastomas the role of pRb expression requires further investigation.

5.3.5 Cyclin A immunoexpression

The majority of nephroblastomas expressed cyclin A and this was seen predominantly in blastemal cells. Although in most cases with blastemal expression the distribution was uniform, in a few cases the expression was limited to the periphery of blastemal islands, close to the transition to stroma. It is not clear why this distribution pattern occurred in some cases but it may be due to the microenvironment and potential blastemal-stromal interaction.

There was a strong association between cyclin A expression and preoperative chemotherapy status. High cyclin A immunoreactivity was more frequent in tumours that were not treated with preoperative chemotherapy. In contrast, there was an almost equal distribution of high and low cyclin A staining in the group that received preoperative chemotherapy. A suggestion arising from these findings is that preoperative chemotherapy reduces cyclin A expression. This downregulation of cyclin A may be partly responsible for the decrease in proliferation rate in pretreated

tumours. Cyclin A appears just before DNA synthesis and gradually increases until it peaks in prophase and declines in metaphase when it is degraded.[546] Therefore cells expressing high levels of cyclin A are in late S, G2 or early M phase. It is possible that chemotherapy prevents cells from progressing to the M phase of the cell cycle.

Although overall cyclin A expression was more frequent in the group not treated with preoperative chemotherapy, analysis of the preoperatively treated group revealed interesting results. In this group cyclin A expression was strongly associated with histological classification and risk group. High cyclin A expression was more frequent in the tumours with high risk histology, namely, diffuse anaplastic and blastemal types. The findings in the present study suggest that preoperative chemotherapy might lead to downregulation of cyclin A in intermediate risk tumours but not the high risk tumours and indirectly contribute to the poor outcome associated with these histological types. Further support for the role of cyclin A in high risk tumours is provided by an *in vitro* study which demonstrated that cyclin A may suppress apoptosis mediated by p53 due to DNA damage.[547] Based on the latter it has been suggested that cyclin A should be considered as a potential factor in resistance to chemotherapy or irradiation.[548]

The majority of the cases that showed high p53 expression also showed high cyclin A expression. If the high p53 immunoexpression was reflective of an underlying *p53* mutation, then wild-type p53 promotion of apoptosis and inhibition of cell cycle progression will be impaired. Since wild type p53 blocks progression into the S-phase, an absence of wild-type p53 will promote progression into the S-phase. In this situation the cell will progress through the cell cycle, hence the high cyclin A expression. It must be noted that mechanisms other than an absence or loss of p53 function may also be important for uncontrolled cell division.[252,346,549]

Although a significant result was obtained when cyclin A expression was compared with p21 expression, the results are unexpected and inexplicable. Since p21 is an inhibitor of the cell cycle mainly at the G1 phase, it would be reasonable to expect high p21 to be associated with low cyclin A levels. This study found that the majority of cases with high p21 also demonstrated high cyclin A levels.

Although an association between high pRb and high cyclin A expression was found, a major limiting factor in explaining this relationship is that the primary anti-pRb antibody used in this study does not distinguish between the phosphorylated and unphosphorylated forms of pRb. pRb exists as an unphosphorylated form during G1 phase, whereas in late G1 through to the end of the M phase, it is extensively phosphorylated. During anaphase it undergoes rapid dephosphorylation. The unphosphorylated form of pRb is the functionally active, growth inhibitory form.[376] Since the phosphorylation status of pRb is unknown, it is only possible to speculate about the relationship between these two proteins. pRb phosphorylation is stimulated by cyclin A kinases[219] and is mainly in the phosphorylated form when cyclin A is expressed. Cyclin A, therefore, promotes conversion of pRb from an active (unphosphorylated) to an inactive (phosphorylated) form. Based on the findings of this study, one may assume that in the group with high cyclin A and high pRb, pRb is present in its phosphorylated form.

The majority of tumours that expressed high cyclin A also showed high p-glycoprotein expression and the majority with low cyclin A also showed low p-glycoprotein expression. P-glycoprotein functions as an energy dependent drug efflux pump, removing drugs from the cell. Cells that express p-glycoprotein display resistance to multiple drugs and continue to proliferate. The findings in this study show that the majority of cells with high p-glycoprotein (chemoresistant cells) have a high proliferation rate as indicated by the high cyclin A expression. In this way cyclin A identifies a cohort of tumours with potential chemoresistant characteristics.

The three markers that showed an association with survival in univariate analyses were p53, pRb and cyclin A. Higher expression of these proteins was inversely associated with survival. The only marker that retained this strong association with survival in a joint multivariate analysis was cyclin A.

Overall it seems that cyclin A immunoexpression correlates with poor prognosis. This may be due to its positive regulation of the cell cycle by its role in initiating DNA synthesis, S phase progression and M phase.[550] A similar conclusion was proposed for cyclin A staining in endometrial carcinoma.[548] It was found that cyclin A staining was a useful poor prognostic marker for endometrial carcinoma.

5.3.6 p-glycoprotein immunoexpression

Previous p-glycoprotein immunohistochemical studies using paraffin sections after formalin fixation provided contradictory results.[429,433-436] In one comprehensive study using three monoclonal antibodies (C219, HYB241 and HYB612), p-glycoprotein could not be detected by indirect immunoperoxidase and avidin-biotin complex methods.[429] Other studies have described p-glycoprotein expression in paraffin sections using a range of monoclonal antibodies, C219, C494, JSB-1;[437] C219;[438-440] and JSB-1 and C219.[441] In the present study, p-glycoprotein expression was demonstrated using the C219 monoclonal antibody. The contradictory staining results may be explained by differences in the methodology.

The present study did not find a significant difference in p-glycoprotein expression between the two treatment groups but observed an association with histological classification and risk group within the preoperatively treated group. Although not statistically significant it is noteworthy that the high risk tumours in the preoperatively treated cases, namely, diffuse anaplastic and blastemal types were more likely to express high levels of p-glycoprotein. This is consistent with the suggestion that high risk tumours especially diffuse anaplastic tumours are chemoresistant.[161,412,413] As stated previously, the elucidation of reasons for therapy failure in nephroblastomas is needed before cure becomes a reality for all children with this cancer. The molecular mechanisms of drug resistance in nephroblastomas are still poorly understood. Vincristine, actinomycin D, and doxorubicin are involved in the multidrug resistance (MDR) phenotype.[551] Treatment of tumour cells with drugs of the MDR-type induces the expression of MDR and MRP genes.[552]

Many studies failed to demonstrate significant p-glycoprotein expression in nephroblastomas.[413,418,430] One study found no overexpression of p-glycoprotein in untreated nephroblastomas but approximately half the cases treated with actinomycin D and vincristine showed p-glycoprotein overexpression.[442] In another study, patients not receiving preoperative chemotherapy and patients with classic triphasic nephroblastoma showed no p-glycoprotein overexpression, except in areas of tubular differentiation, as described in the normal kidney.[413] Sola and

associates[553] detected p-glycoprotein in nephroblastomas treated with preoperative chemotherapy. Patients with nephroblastoma treated with actinomycin D and vincristine showed increased p-glycoprotein expression. In the present study, high p-glycoprotein expression was more frequent in high risk preoperatively treated tumours rather than all preoperatively treated tumours. This suggests that histological type is an additional important determinant of p-glycoprotein expression. Furthermore, the variation in the chemotherapy regimen over the study period may account for the inconsistent results in the preoperatively treated tumours.

Since wild-type p53 down-regulates the expression of drug resistance genes, e.g. *MDR1*, *MRP1* and *DNA topoisomerase IIα*,[416-418] p-glycoprotein expression is expected to be elevated in the presence of *p53* mutations. Therefore high p53 immunoexpression was expected to be associated with high p-glycoprotein expression. Although the majority of cases with high p53 staining did show high p-glycoprotein staining this result did not reach statistical significance. The failure to demonstrate p-glycoprotein in a nephroblastoma with a *p53* mutation led to the suggestion that *p53* inactivation may not be sufficient to account for all *MDR1* upregulation.[336,413] In this case the authors speculated that this specific *p53* mutation did not prevent the long-lived mutant p53 from still acting as a transcriptional repressor over the *MDR1* promoter.[413] They concluded that the multidrug resistant characteristics of anaplastic histology nephroblastomas might result from mutation of several genes.

5.4 MICROSATELLITE STUDY

Until recently, molecular biological techniques have been regarded by most to belong to the realm of basic science but this technology is now available to the diagnostic histopathologist. Polymerase chain reaction (PCR) can be used to rapidly amplify any known DNA or RNA sequence. As a result, it is now possible to carry out genetic analysis of almost any tissue sample, including archival formalin fixed, paraffin embedded tissue blocks and stained sections, smears or microdissected cells. The histopathologist is in a unique position to employ these methodologies in diagnosis and research. PCR has allowed histopathologists to better correlate histopathological changes in tissues with infectious agents such as viruses.[445]

In order to achieve the best results using paraffin embedded tissue blocks many factors need to be considered. During the proteinase K digestion phase tissues should be fully digested with little or no remaining precipitate, to efficiently liberate DNA. The amount of prepared sample used can also affect the efficiency of amplification. In this study DNA concentrations were determined to ensure adequate template DNA was available. Furthermore, the purity of the extracted DNA was confirmed by calculation of the OD_{260}/OD_{280} ratio.[504] The age of the paraffin wax embedded tissue blocks has been considered as another potential factor that may affect the extraction of good quality DNA. Despite this, successful amplification from 40-year-old tissue blocks has been reported.[554] In the present study, all tissue blocks were under 20 years old. DNA of adequate purity and quantity was not obtainable in a few cases despite repeated extractions and purification. In some cases, DNA amplification was not successful. If two repeated attempts at DNA extraction and purification did not provide optimal DNA, amplifiable in a PCR using primers for the *insulin* gene, the cases were excluded from the study. Cases were also excluded from the microsatellite component of this study because of insufficient archival blocks, insufficient viable tumour tissue, inability to extract good quality amplifiable DNA or insufficient DNA for completion of analysis of the 17 microsatellite markers. Other factors that may affect the PCR are differences in the quality or age of the chemicals used. This was not a problem in the present study. All chemicals were newly purchased and all reagents freshly prepared.

Another way to overcome the limitations of DNA extracted from fixed and paraffin-embedded tissue is to avoid attempting to amplify DNA fragments greater than around 300-400 bp in PCR.[446,447] Therefore, primer pairs were carefully selected to ensure that none of the amplified products had an expected size of greater than 250 bp.

Seventy cases were finally selected for the microsatellite analysis. The mean and median ages for this patient cohort were 38.2 and 27.5 months respectively, slightly lower than the overall mean and median ages of all patients in this study. The slight female preponderance was retained in this component of the study (1.19:1.0) as was the percentage of low and high disease stages. There were study cohort

included 10 anaplastic tumours, all of which fell into the preoperatively treated group. Although the percentage of unfavourable tumours is slightly higher than most series, this is explained by the selection bias due to exclusion of cases for reasons previously discussed. Forty-eight patients (68.6%) received preoperative chemotherapy. The two treatment groups were analysed separately because of the possible influence of preoperative chemotherapy on the genetic material. The group that did not receive preoperative chemotherapy did not have sufficient cases for valid statistical analysis.

This study was undertaken to determine a baseline of LOH and MSI involving 11p, 16q and MMR loci and to determine if there was any significant association with clinical and pathological features.

5.4.1 Loss of heterozygosity of 11p

The results for the 11p chromosomal region were analysed according to individual polymorphic markers and grouped according to specific chromosomal regions. This was done to facilitate comparison with other studies since different studies have used different polymorphic markers for the 11p region. The number of markers used in different studies also varied. The present study used six markers which is a fair average of the numbers used in most studies.

With regard to 11p, it is also important to note that in the present study tumour specific LOH was investigated but not LOH of the *WT1* gene specifically. The rate of *WT1* LOH is much lower[5,60,88,555] than the frequency of 11p LOH. Previous studies have also reported higher frequencies of LOH for 11p markers between 30-50%.[6-8,35,132,138] It is worth noting that LOH for 11p13 does not necessarily imply a mutation of *WT1*.[73] Some have suggested that there may be other Wilms tumour loci on 11p other than *WT1* and those located at 11p15.[73,138]

Grundy *et al.*[138] analysed 286 nephroblastomas and reported the following frequency of LOH: 11p13 only 3.4%, 11p15 only 7.4% and both 25.7%. All patients in their study were registered in the third or fourth NWTS. These rates are much lower than the findings in the present study, irrespective of treatment group. In the present study the corresponding rates for the preoperatively treated group were;

14.6%, 10.4% and 41.6% respectively and for the group not treated preoperatively these were 22.7%, 0% and 31.8%. The selection of cases in the present study may have influenced this result since many cases were excluded for the various reasons discussed earlier.

High frequencies of LOH for the 11p15 locus have been reported in nephroblastomas. Maw *et al.*[67] recorded a frequency of 44% for LOH at 11p15.5. This study was conducted on a population from New Zealand and Australia. It is not clear whether race or ethnic group might have influenced this result. Karnik *et al.*[556] reported a frequency of 43%, Reeve *et al.* 33.3%[33], Grundy *et al.* 33.1%[138] and Satoh *et al.* 29%[506] for 11p15 LOH. The frequency of 11p15 LOH (± LOH 11p13) in the present study was 52% and 31.8% for preoperatively treated and not treated groups, respectively. The overall frequency inclusive of both treatment groups was 45.7% which is similar to the findings of Maw *et al.* and Karnik *et al.*[67,556] The present study has confirmed that the frequency of 11p15 LOH in this South African population is similar to those reported in other population groups and suggests that this locus has a similar role in African populations and Western populations.

The frequency of 11p13 LOH in the present study was 56.2% and 54.5% in the preoperatively treated and not treated groups respectively. In this study LOH for 11p13 was slightly more frequent than LOH for 11p15. Although the frequency of 11p13 was higher than most studies, Kaneko and co-workers[136] reported a higher frequency of 11p13 alterations (72.7%). Grundy *et al.*[138] found 11p13 LOH in 29.1% of nephroblastomas, Satoh *et al.*[506] reported a frequency of 25% and Skotnicka-Klonowicz et al. 19.6%.[555] It is also possible that this finding is unique for this particular study population consisting predominantly of African patients and is different from most of the other studies performed in Western countries, especially the United States. It is possible that 11p13 LOH is involved in the development of a larger number of nephroblastomas in this population compared to Western populations.

Although LOH for 11p was found to be a frequent occurrence in nephroblastomas in this study population, it did not have any direct prognostic significance. Since *WT1*

maps to 11p13, the high frequency of 11p13 LOH in this study suggests that *WT1* gene alterations are frequent in this population. Schumacher and colleagues[88] in the course of two studies found that the *WT1* mutation frequency was significantly different among the various histological subtypes. Mutations were seen most frequently in stroma-predominant tumours. They concluded that the two-hit inactivation of *WT1* is operative in stromal-predominant nephroblastomas.[89] They also found that 6 of 10 stromal tumours with *WT1* mutations showed LOH of 11p13 markers. Further studies of the *WT1* gene in this cohort of patients will provide more accurate information of *WT1* gene alterations.

Several target genes seem to be activated by WT1 in vivo, including *p21*,[101] *bcl-2*,[102,103] and *CDH1* (E-cadherin).[105] *CDH1* is located at 16q22.1 as well as two related cadherins genes, P-cadherin and cadherin-11. Seven tumours had LOH for both the 11p13 locus and the 16q22.1 locus. These seven cases potentially had LOH for *WT1* and *CDH1*. Of these patients two had stage I disease, both of whom are alive, two had stage II disease and were lost to follow up, one had stage III disease and died of disease, and two with stage four disease died. Four of these tumours had intermediate risk histology and three had high risk histology. This cohort of seven patients did not have a significantly different outcome compared to the rest of the patients.

The present study found a significant association between LOH at 11p12-11p15 and patient age. Grundy *et al.*[138] reported an association between LOH at 11p13 and 11p15 and age at diagnosis but their findings were opposite to those of the present study. Grundy and co-workers[138] found that LOH was associated with younger mean age but the present study found that the mean age of patient with LOH at 11p12-11p15 was higher than those without LOH at this locus.

There was no evidence of a gender predilection for 11p LOH in the present study. Although in another study LOH of *WT1* gene was observed twice as frequently in Wilms tumours in boys (8 cases) than in girls (4 cases), this difference was not significant statistically.[555]

There was no significant difference in the frequency of LOH in the two treatment groups when analysed per marker or per region. In the present study 69.6% of tumours that were treated preoperatively and 54.5% of tumours that were not treated preoperatively harboured LOH at 11p, an overall frequency of 64.7%. Since 11p alterations play a role in the development of nephroblastomas rather than its progression the above finding is not unusual. Also not surprising was the variation in frequency of LOH for the different markers since the markers were covering different loci on 11p.

The cases with LOH for three of more markers had low stage disease and were disease free at last follow-up. Case #102 had favourable histology, stage I disease and was disease free at 74 months of follow up. This case showed loss for all six markers. Loss of large segments of genetic material has been described. Since the 11p polymorphic markers used in this study actually span the short arm of chromosome 11 from 11p12 to 11p15, it is possible that these cases with LOH for three or more markers, especially case #102 have large deletions involving 11p. Loss of large segments of 11p did not correlate with adverse prognosis. Once again, this finding supports the present understanding that 11p LOH plays a role in tumour development but not progression.

The present study found no association between 11p LOH and stage or progression of disease. This is similar to the studies by Skotnicka-Klonowicz *et al.*[555] and Grundy *et al.*[138] The former study found that the frequency of LOH in cases without recurrences and metastases and in cases with recurrences and metastases was similar. Grundy *et al.*[138] also found no association between tumour stage and 11p LOH, similar to the findings of the present study. They suggested that stage is likely to be determined by other factors not associated with the aetiology of the tumour. They also found that 11p LOH was less frequent in anaplastic tumours. The present study found conflicting results with 80% of anaplastic tumours harbouring LOH at 11p. It is interesting that 7/8 anaplastic tumours that showed 11p LOH expressed high levels of p53 protein, which is accepted as an indicator of underlying *p53* mutation. The two cases of anaplastic tumour that did not harbour 11p LOH expressed low levels of p53. In summary, the present study suggests that anaplastic tumours with 11p LOH are associated with high p53 immunoexpression

(*p53* mutation). The statistical significance could not be determined because the number of anaplastic tumours without 11p LOH was too small. Contrary to a previous suggestion and based on the findings in the present study it is possible that some anaplastic tumours do indeed represent progression from non anaplastic tumours.[138] This should be explored further in a larger series of cases. The outcome of the anaplastic tumours was not influenced by the presence of 11p LOH. All patients with anaplasia irrespective of the 11p LOH status had a poor outcome.

The findings of this study confirm the existence of Wilms tumour loci on chromosome 11p. It also, for the first time, reports on the frequency of 11p LOH in nephroblastomas in a unique South African population.

5.4.2 Loss of heterozygosity of 16q

The overall frequency of 16q LOH in the present study was 66.7%. The rates were 65.9% and 68.2% for the groups treated and not treated with preoperative chemotherapy, respectively. This overall frequency of 16q LOH is significantly higher than those previously reported, mainly from Western countries. LOH for polymorphic markers on 16q have been found in 10-25% of Wilms tumours.[6,14,67,131-134,140,557] A 20% frequency of 16q LOH was found in a study of 45 informative cases of nephroblastoma using 9 markers for 16q.[67] One study found LOH 16q in 21.2% of cases, and that the occurrence of LOH 16q was significantly lower in tumours removed in stage I than in stage IIN- or IIN+ and III.[133] This study, however, used just one polymorphic marker, D16S400, limiting the area of analysis to 16q24. A study by Grundy *et al.*[134] reported that 16q LOH tended to occur in higher stage disease. However, another study similar to the present one, did not find any direct association with stage.[132,140]

Another study using 11 markers detected 16q LOH in seven tumours, a frequency of 25%.[131] These researchers found that the common region of LOH was distal to the 16q12-13 marker. A larger follow-up study, as part of NWTS-4, involving 232 patients, found a frequency of LOH at 17%, and used polymorphic markers that mapped to the 16q22-qter region.[132] In the study by Coppes *et al.*,[6] using two polymorphic markers mapping to 16q22.2 and 16q24.3, LOH was identified in 20%

of tumours. A study from the United Kingdom which shared two common markers (D16S413 and D16S520) with the present study found LOH in 15% of tumours.[134] They also reported that the frequency of LOH at D16S520 was twice that for D16S413. The present study showed similar results with 15 cases showing LOH for D16S520 and 7 for D16S413. Mason *et al.*[14] using a minimum of seven 16q microsatellite markers, two of which were common to the present study, found LOH in 18.8% of cases. Wittman *et al.*[557] reported a similar rate of 18.4%. The higher frequency of 16q LOH in the present study may reflect a real increase in this population group. Alternatively, this high rate may be the consequence of the exclusion of completed necrotic low risk tumours from the study. This group of tumours is associated with a good prognosis and is likely not to have exhibited 16q LOH.

In the present study there was no significant association between frequency of 16q LOH and patient age. However, Grundy *et al.*[140] found that LOH was less frequent in younger patients. Although in the group treated with preoperative chemotherapy, 16q LOH was more frequent in females than males, this was only marginally significant. When cases from both treatment groups were combined there was no significant association with gender. No association with gender was reported in another study.[133] There have been reports of genetic changes being induced by chemotherapeutic agents[173], however, in the present study there was no significant difference in the frequency of 16q LOH in preoperatively treated tumours compared to those not treated preoperatively.

The present study found a significant association with histological classification for two different polymorphic markers, D16S496 and D16S520 which map to 16q22.1 and 16q24.2-qter respectively. LOH for these markers did not influence the outcome. A recent study which provides new information and key insights into 16q alterations in nephroblastomas found 16q LOH in 18.9% of nephroblastomas and a significantly greater frequency of 16q LOH in anaplastic tumours compared to favourable histology tumours (32.4% vs 17.4%) but this did not affect the outcome in patients with anaplastic tumours.[140] Wittman *et al.*[557] also found a four-fold increase in the frequency of 16q LOH in anaplastic tumours compared to non-anaplastic tumours (66.7% vs 16.8%). Furthermore the present study found that the

strong association of these two markers was retained when analysed for the broader histological risk groups. This supports the poor prognostic outcome linked to the retention of blastemal histology following preoperative chemotherapy. Although 16q LOH was more frequent in anaplastic histology tumours (80%) compared to non anaplastic histology tumours (66.7%) in the present study, this finding did not reach statistical significance. Similarly high risk histology more frequently demonstrated 16q LOH compared to intermediate risk histology (81.8% vs 69.0%) but again this was not a significant association. These findings support the presence of tumour progression genes at these specific loci, 16q22.1 and 16q24.2.

LOH at 16q21-22 was the most frequent in the present study, differing from another study which found that the telomeric 16q24-qter region was the most common site of allelic loss[134] but consistent with others who described the larger 16q21-qter as the common region for LOH.[4,67] Mason *et al.*[14] analysed 96 nephroblastomas to more precisely define the pattern of 16q LOH and identified the smallest consensus region of deletion as 16q23.2-q24.2. However, Newsham *et al.*[137] suggested that the common region of allelic loss is localised to 16q12.1-16q21. A more proximal locus at 16q11-13 had also been considered.[4] There are several genes present at 16q21-22 which may contribute to tumour progression. The *E2F4* gene, which encodes a transcription factor involved in cell cycle regulation has been considered to function as a potential tumour-suppressor gene, whose loss contributes to the biological aggressiveness of the tumour.[558] One of the markers (D16S496) mapping to this region (16q22.1) showed the most statistically significant correlation with histological classification. Evaluation of the *E2F4* gene in nephroblastomas may help to confirm and further explain its role in nephroblastoma progression. As alluded to earlier, three related cadherin genes, E-cadherin, P-cadherin and cadherin-11, are also situated at 16q22.1. Previous studies have implicated the E-cadherin system in controlling nephroblastoma aggressiveness.[61] The E-cadherin complex participates in cell-cell adhesion and maintenance of normal cellular architecture by interacting with both β- and γ-catenins that bind to actin via α-catenin. The finding of β-catenin mutations in nephroblastomas lends support to the suggestion that E-cadherin plays a role in tumour progression.[61,96] Although one study found a statistically significant reduction in E-cadherin staining in tumours with

LOH for 16q, it failed to demonstrate a significant connection between E-cadherin expression and aggressiveness in nephroblastomas.[130] The same researchers found that the average P-cadherin staining score was higher in the group with 16q LOH compared to the group without 16q LOH, arguing against P-cadherin being a candidate nephroblastoma tumour suppressor gene.

Unfortunately, the present study could not reliably assess the relative risk of relapse since a significant proportion of patients were lost to follow up and this group would certainly have had cases that relapsed. Several studies have found that 16q LOH correlates with risk of relapse. Grundy *et al.* found a significantly increased relative risk of relapse in cases with LOH 1p and 16q.[140] Additional analysis suggested that much of the adverse effect associated with LOH at these loci was confined to the group of patients that had LOH for both loci. The authors considered the involvement of these two regions to be more likely the result of a single chromosomal mechanism. A recent German study also found a significant association between 16q LOH and relapse.[557] Interestingly, the association was more significant when loss of the whole arm was considered.

In the present study 18 of the 48 tumours treated with preoperative chemotherapy and 8 of the 22 tumours that were not treated with preoperative chemotherapy showed LOH for both 16q and 11p loci. Inclusive of both treatment groups twenty six of the 70 (37.1%) cases showed LOH for both 16q and 11p loci. These findings are not dissimilar to those reported in three previous studies which investigated both 11p and 16q LOH in nephroblastomas. The first study found LOH for both chromosomes in three of nine tumours[67], the second in three of six tumours[6] and the third in two of four tumours.[134] The cases with LOH for both 11p and 16q loci were not associated with stage, histological classification or risk group. The presence of a significant number of cases with LOH for one chromosomal site suggests that it is unlikely that alterations on both chromosomes are required for tumour development. A more clear understanding of the interaction between these two chromosomal regions may become apparent when specific genes at these loci are investigated together.

5.4.3 Loss of heterozygosity of MMR markers

This study was the first to investigate nephroblastomas using polymorphic microsatellite markers for the mismatch repair genes. However the small number of informative cases for five of the MMR microsatellite markers limited any significant conclusion being drawn from these findings. For example, although LOH in the 2p16 and 1p13 regions appeared to be frequent occurrences, any valuable conclusion has to be deferred until a larger number of informative cases are analysed. The low informativity for the bat markers were also found in another study performed on a similar race group.[507] Although this suggests that this particular population may show low informativity for these markers, it is not clear whether this is unique for this race group. Further studies may be valuable in clarifying this observation.

In the tumours that were treated preoperatively LOH of MMR markers are relatively less frequent than LOH of 11p and 16q regions (39.6% vs 69.6 and 65.9% respectively).

An interesting observation was that tumours with LOH for both 2p16 markers (bat26 and D2S123) were more likely to show anaplastic histology. Although it is tempting to suggest that 2p16 may harbour gene/s that play a role in the development of anaplasia, not all anaplastic tumours showed the same allelic loss. It is very likely that anaplasia, like nephroblastoma in general, is the result of complex genetic alterations, and not just a single gene effect. The 2p16 locus should be further explored in nephroblastomas.

In nephroblastomas LOH for MMR genes did not appear to be associated with either clinical or pathological features.

5.4.4 Microsatellite instability

A previous study which reported the presence of MSI in a subset of nephroblastomas suggested that defects in the mismatch repair mechanism may play a role in the pathogenesis of nephroblastomas.[14] The present study sought to determine the frequency of MSI in nephroblastomas occurring in a South African

population and whether the presence of MSI was associated with clinicopathological features.

Altogether 17 polymorphic microsatellite markers were employed in this study. Microsatellite analysis of the combined results for the three sets of markers revealed no cases of high frequency MSI (MSI-H), 27 cases with MSI-L and 43 cases that were microsatellite stable. The frequency of MSI, calculated from the data of all 17 markers, for the preoperatively treated and not treated groups was 35.4% and 45.5% respectively. The majority of these cases (n=21) showed MSI for just one of the 17 markers (5.9%). This is a very low frequency of MSI per tumour. The latter is of significance because in the context of colorectal carcinoma some have argued that MSI-L should be included with MSS tumours.

While the clinical significance and implication of MSI-H in colorectal cancer is well established, that for MSI-L is still controversial.[17] Some authors believe MSI-L colorectal carcinomas should be grouped together with MSS tumours, while others feel that MSI-L tumours exist as a distinct entity.[17] Whether this debate applies to tumours that are not part of the HNPCC syndrome also requires evaluation. It seems unlikely that MSI-L nephroblastomas form a distinct entity with different clinical characteristics. The MSI-L cases in this study did not have any consistent clinical or pathological features. On the other hand MSI-H is known to play an important role in morphogenesis of tumours in multiple sites and tissue types.[17] As a result pathologic and histologic features may provide clues, albeit of poor sensitivity and specificity, to the presence of underlying MSI-H. In colorectal cancer, however, MSI-H is associated with specific histopathological features.[559] The present study has demonstrated that MSI is an unlikely event in nephroblastomas. Since MSI-L does not necessarily correlate with mutations of the MMR genes, it is likely that mutations of the MMR genes are not important events in nephroblastoma development.

Only one case (case #2) showed MSI for 4 of the 17 markers (23.5%). This patient was female, 5 years old, had a favourable histology tumour, stage IV disease and died of disease within a month of diagnosis. Of the two cases with MSI for three markers, one (case #1) had stage I disease and was disease free 16 years post

diagnosis. In contrast the other patient died with metastatic disease after 34 months.

The frequency of MSI for 11p markers in the group not treated with preoperative chemotherapy was twice that of the group treated with preoperative chemotherapy. All seven tumours in the preoperatively treated group showed MSI for just a single 11p marker only. The group not treated with preoperative chemotherapy besides having twice the frequency of 11p MSI, showed MSI for multiple 11p markers in two cases. This did not hold true for the 16q and MMR markers where all tumours showed MSI for a single marker only. When the MSI results for all three sets of markers were combined, once again the group not treated preoperatively had two tumours that showed MSI for markers from all three sets (11p, 16q and MMR) but there were none in preoperatively treated group. There was one case that showed MSI for four markers, two for 3 markers and two for 2 markers in the group that did not receive preoperative chemotherapy, in contrast to only one of the preoperatively treated cases which showed MSI for two markers. This raised the question whether microsatellite unstable clones were more sensitive to chemotherapy and therefore reduced in the pretreated tumours. Despite the higher frequency of MSI in tumours not treated preoperatively, the frequency did not reach the level to be classified as high frequency MSI. An opposing theory has been proposed based on the information that the cytotoxic agents damage virtually all components of the cell and the subsequent biological effects are attributable to their ability to damage DNA. Consequently, any impairment of the DNA repair systems might lead to loss of response to cytotoxic agents. Based on this theory, one study investigated the role of hMSH2 and hMLH1 expression in treatment responses in solid paediatric cancers[560] and found that weak or absent expression of hMLH1 protein did not correlate with the individual treatment responses. They explained this finding by the use of multiple drugs and radiation, the possible contribution of other members of the mismatch repair system (hPSM2 and hMSH3) and translesional DNA synthesis or replicative bypass. Further investigation of the frequency of MSI in tumours treated and not treated preoperatively may yield useful data, however a larger number of cases are required.

A PubMed search did not reveal any literature on nephroblastomas occurring in patients with HNPCC. There are only two studies that have reported on MSI in nephroblastomas.[14,561] One is a case report of a Japanese male patient who had a nephroblastoma in childhood and colon cancer at the age of 26 years.[561] Both neoplasms showed MSI at the same five microsatellite loci (Mfd27, Mfd47, D18S254, TP53, and BAT40). No germline or somatic mutations were detected in *hMSH2*, *hMLH1*, *hMSH6*, *hPMS1*, and *hPMS2* raising the possibility that there may be mutations of other MMR genes. In the second study MSI was found in 3 of 96 (3.1%) nephroblastomas.[14] Two cases were MSI-H with 14 of 16 markers (87.5%) and 8 of 11 markers (72.7%). The last case showed MSI for just one marker (MSI-L) and the authors excluded this case when reporting on the frequency of MSI in their study. These authors suggested that defects in mismatch repair genes may contribute to the pathogenesis of nephroblastomas.

Case #102 which showed LOH for at least one marker from all three sets of markers evidently had a wide loss of genetic material involving different chromosomal loci. It was expected that this tumour would also show some evidence of microsatellite instability in view of the frequent genetic alterations in this tumour. However MSI was not detected with any of the markers used suggesting that another mechanism of genetic alteration was involved. The majority of other cases that showed frequent LOH were microsatellite stable (MSS), very few showed low frequency MSI. This suggests that MSI is rare even in the presence of frequent LOH consistent with the different mechanisms involved in the aetiopathogenesis of these two genetic abnormalities.

MSI-H at 11p, 16q and MMR markers was not seen nephroblastomas in the present study. MSI-L although frequent in nephroblastomas does not appear to correlate with any clinicopathological features and is not an important genetic alteration with clinical and biological significance. Based on the reports on MSI in nephroblastomas together with the results of this study, it is clear that MSI-H is not a common alteration in nephroblastomas and is unlikely to play a role in its initiation or progression. The implications of MSI for clinical outcome in nephroblastoma are still unclear and await further study. It is possible that in childhood tumours there has not been enough time to accumulate genetic mutations within the tissues at risk,

and that somatically acquired mutations in the DNA mismatch repair genes may occur only very rarely in children.

Survival analysis in the molecular study was limited by the small number of cases. The lack of precision in the analysis was evident by the wide confidence intervals.

CHAPTER SIX

CONCLUSION

6.1 Summary of the findings

The present study, the first of its kind in a South African population, reports the clinicopathological features, immunohistochemical expression of cell cycle related proteins and loss of heterozygosity and microsatellite instability in nephroblastomas occurring in South African children.

In general the biology of nephroblastomas in South African children does not appear to be significantly different from Western countries. A summary of significant clinicopathological findings follows:

- Clinicopathological stage was an independent prognostic factor directly associated with mortality.
- Histological classification and histological risk group were significantly associated with clinicopathological stage.
- Histological classification and histological risk group were associated with mortality.

The present study has shown that high p53 expression is a poor prognostic factor since it was significantly associated with anaplastic histology and high histological risk groups. In addition, patients who were disease free had nephroblastomas that expressed low levels of p53 protein. Nephroblastomas expressing high levels of p53 protein should be carefully re-examined for the presence of anaplasia.

p21 immunoexpression was not related to prognosis and did not show any significant correlation with any of the clinicopathological parameters. Furthermore, p21 induction was not a consistent finding in pretreated nephroblastomas.

This study has shown that high bcl-2 immunoexpression in nephroblastomas was related to high histological risk group and more frequent in tumours that were treated with preoperative chemotherapy. These findings suggest that high bcl-2 levels may play an important role in the development of chemoresistance in a subset of nephroblastomas. A potential application based on the findings of this study is that bcl-2 positive nephroblastoma may warrant separation from bcl-2 negative tumours, perhaps be given more and/or different chemotherapeutic agents and should be more carefully followed up.

pRb immunoexpression was associated with histological classification however this significance was not retained when compared with histological risk group. Anaplastic tumours expressed high levels of pRb suggesting that *pRb* mutations are not important events in the development of anaplasia. Tumours treated with preoperative chemotherapy were more likely to show low levels of pRb expression. Further research is required to determine whether the low expression is due to chemotherapy induced DNA alterations.

High cyclin A levels are associated with high risk histology, namely, anaplastic and blastemal histology tumours. This finding together with the finding that cyclin A has the ability to suppress p53 mediated apoptosis suggests that high cyclin A expression should be considered as a mechanism for chemoresistance in these high risk tumours. Cyclin A immunoexpression in nephroblastomas treated with preoperative chemotherapy was significantly lower than in tumours not treated preoperatively. This also supports the previous suggestion because cyclin A positive cells in intermediate tumours are ablated by chemotherapy while similar staining cells in high risk tumours are resistant. Cyclin A was the only marker that retained its strong association with survival in a joint multivariate analysis.

High risk tumours, consistent with their chemoresistant properties, expressed high levels of p-glycoprotein.

The results of the microsatellite study were influenced by the small number of cases and may explain the high frequency of LOH in this study. Furthermore the clinical significance of the findings was affected by the number of cases that were excluded.

The frequency of 11p15 LOH in this study was similar to most reports from Western countries suggesting a similar role for 11p15 alterations in both populations. LOH 11p13 however was more frequent in the South African population raising the possibility that *WT1* mutations may be more frequent in this population. LOH for the *WT1* was not performed in the present study. LOH of the 11p loci, consistent with other studies, were not associated with prognosis.

LOH 16q was significantly higher in the South African study compared to Western countries. Similar to the frequency of 11p13, this may indicate that LOH at 16q loci are more frequent events in this population. Two loci, 16q22.1 and 16q24-qter, were found to be significantly associated with histological classification and risk group raising the possibility of tumour specific genes at these loci. Further evaluation of this chromosomal region and the *E2F4* gene in nephroblastomas may help to confirm and further explain its role in nephroblastoma progression.

LOH of MMR genes occurred in 39.6% of pretreated nephroblastomas but was not associated with prognosis.

Microsatellite instability is not an important event in nephroblastomas as evidenced by the absence of MSI-H at 11p, 16q and MMR loci. Further investigation of the Wilms tumour locus on chromosome 16q should increase our understanding of the genetic complexity of nephroblastomas. The present study has demonstrated that MSI-H is indeed a rare occurrence in nephroblastoma and does not play an important or consistent role in tumourigenesis. On the other hand, LOH is a frequent finding in nephroblastomas and LOH at the 16q region is a poor prognostic indicator.

6.2 The future

It is accepted that the development of nephroblastoma involves several, probably alternative, genetic pathways, but the identity of most of these genes, or the mechanisms by which they are controlled still remains elusive. The contribution of other genes in Wilms tumourigenesis, including *β-catenin*, *IGF2/H19* and other imprinted genes at 11p15 warrant active investigation.

A detailed knowledge of the intricate molecular pathogenesis of nephroblastomas will be the key to the future identification of new biochemical targets for new treatment modalities. Several breakthroughs have been made in the attempt unravel the complex genetic mechanisms involved in Wilms tumourigenesis. The clinical implications of these findings are gradually becoming apparent. In addition to the implications on treatment and management, the identification of the genetic defects involved in Wilms tumour will allow more precise genetic counselling.

Treatment of nephroblastomas has had many modifications and improvement over the last 3 decades and can be regarded as one of the successes in cancer management. Although more than 85% of children with nephroblastomas are being cured with current treatment regimens, several challenges remain. A common aim of several research groups is to develop effective tumour specific therapies that are devoid of the harmful side effects of present chemotherapeutic agents. Toxicities of treatment must be limited and novel therapies must be identified to improve survival for patients with high-risk disease.

One of the aims of the management of cancers is the stratification of patients into good and poor prognosis groups, so that treatment can be targeted more effectively. Despite some success in nephroblastomas further stratification is required to identify those patients who appear to have a good prognosis by conventional criteria, but who relapse and die when treated by what seemed the most appropriate regimen at the time of diagnosis. Morphological studies identified anaplastic nephroblastomas as a variant with poor outcome due to its resistance to standard chemotherapeutic agents. But some favourable histology (non-anaplastic) nephroblastomas also do not respond well to treatment. Research needs to focus on these treatment-resistant tumours. In order to develop methods of identifying such tumours many different markers need to be studied using various techniques.

One step towards achieving the goals set for the management of nephroblastomas is the establishment of tissue and data banks to coordinate molecular genetic research and facilitate information exchange, and to make optimal use of limited biological materials.

The attempt to construct a detailed molecular description of malignant tumours will depend on the investigation of thousands of genes in large numbers of specimens. It is expected that this will lead to the creation of better tumour classification algorithms, and novel tumour markers that will facilitate an improvement in the prediction of clinical behaviour and will discover targets for new therapeutic interventions.

In an endeavour to achieve the above tissue microarrays have allowed the rapid immunohistochemical analysis of a large number of potential prognostic markers on a large set of tumour samples. The introduction of nucleic acid microarrays has also aided in exploring the new world of the genome. The expression of thousands of genes can be investigated and this technique may also play an important role in the discovery of prognostic markers for nephroblastoma. cDNA microarray analysis in nephroblastoma is already yielding important information on the expression of molecules with a role in chemotherapy resistance.

DNA mismatch repair genes have gained much attention with the discovery of their role in the HNPCC syndrome. Defective mismatch repair can lead to the progressive accumulation of mutations and the development and progression of cancer. There are likely to be many more components in the repair process than what is currently known. The interactions among these genes and the consequences of mutations in these genes still need to be determined. The involvement of MMR in drug resistance is of great clinical relevance and an understanding of how this happens is important in order to find agents that can kill MMR-deficient cells more effectively.

Further studies may provide a foundation for improvement in patient stratification and ultimately for the development of more individualised tumour treatments. In the future the use genetic markers in addition to clinical staging and pathological classification will be routine for determining prognosis and treatment stratification. The constantly growing body of evidence on nephroblastomas promises a better future for affected children.

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APPENDIX 1

POLY-L-LYSINE SLIDE PREPARATION (SIGMA DIAGNOSTICS).

Dilute 50ml of poly-L-lysine in 450ml of distilled water.
Submerge a clean rack of new glass slides into this solution for 5 minutes.
Shake slides after removal and place on thick absorbent paper to remove excess solution.
Allow coated slides to air-dry overnight at room temperature prior to packing.

Note:
Employ a 30 second increment for subsequent racks in step 2.

APPENDIX 2

KITS FOR IMMUNOHISTOCHEMICAL STAINS

DAKO LSAB®2 SYSTEM, PEROXIDASE (Code No: 675)

Manufacturer: Dako Corporation, Carpinteria, CA, USA

Contents:

Biotinylated Link (110 ml), (link antibody)

Streptavidin-HRP (110 ml), (label antibody)

This kit was designed for use with primary antibodies from rabbit and mouse.

Used for: p53, bcl-2, p-glycoprotein.

CATALYZED SIGNAL AMPLIFICATION (CSA) KIT (Code K 1500)

Manufacturer: Dako Corporation, Carpinteria, CA, USA.

Contents:

3% hydrogen peroxide (15 ml)

Protein block (15 ml)

Link antibody (15 ml)

Streptavidin-Biotin complex, reagent A (1ml)

Streptavidin-Biotin complex, reagent B (1 ml)

Streptavidin-Biotin complex, diluent (25 ml)

Amplification reagent (15ml)

Streptavidin Peroxidase (15ml)

Substrate Tablets, DAB chromogen (10 tablets)

Substrate, Tris-HCL buffer concentrate (6ml)

Substrate, 0.8% hydrogen peroxide (2 ml)

This kit was designed for use with mouse primary antibodies. The CSA staining system is known to be an extremely sensitive immunohistochemical procedure and has been shown to be 50-fold more sensitive than the standard labelled streptavidin biotin (LSAB) methods (Instruction insert).

Used for: Retinoblastoma and cyclin A

ENVISION™+ KIT (Code K 4001)

Manufacturer: Dako, Copenhagen, Denmark

Contents: Goat antimouse immunoglobulins conjugated to peroxidase labelled-dextran polymer in Tris-HCl buffer containing carrier protein (110ml)

Used for: p21 (WAF1)

DIAMINOBENZIDINE (DAB) DETECTION SYSTEM (Code K3466)

Manufacturer: Dako A/S Copenhagen, Denmark

Contents:

3.3'-diaminobenzindine (DAB) chromogen solution (5 ml)

Buffered Substrate (110ml)

Preparation: 1 drop of DAB chromogen and 1ml of DAB substrate buffer - mixed well.

Used for: This chromogen system was used in LSAB®2 and EnVision™+ kits.

APPENDIX 3

THE PCR CORE KIT

(Roche Diagnostics, Mannheim, Germany)

The kit consisted of:

Taq DNA polymerase (250 units, 5 units/µl)

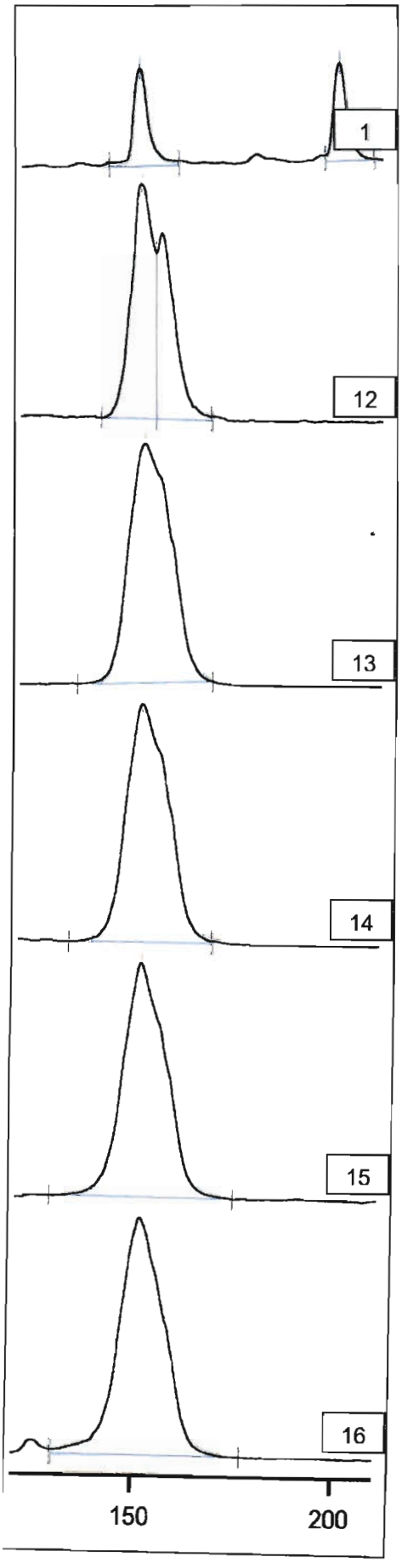
dNTP stock solution (200µl) (containing 10 mM dATP, 10 mM dCTP, 10 mM dGTP and 10mM dTTP)

PCR reaction buffer, 10x concentration (2ml) (containing 1.5 mM MgCl2)

$MgCl_2$ stock solution (1ml 25mM $MgCl_2$)

PCR reaction buffer without $MgCl_2$, 10x concentration (1ml)

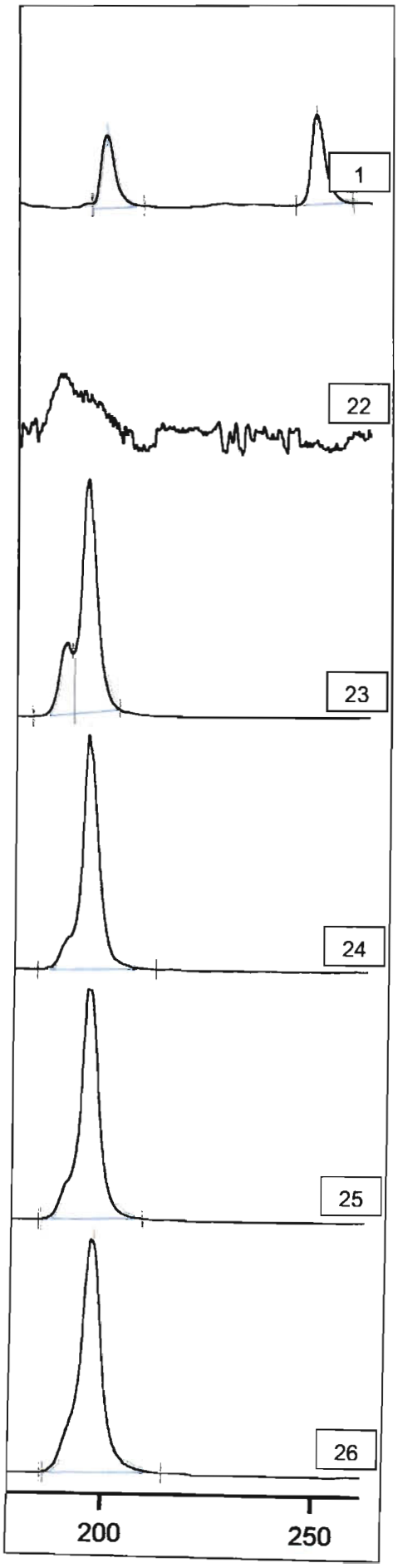
APPENDIX 4



Lane	$MgCl_2$ (µl)	Peak	Peak area	Size (bp)
12	1	1	1820.2	150.3
		2	1200.7	155.5
13	2	1	2279.7	151.3
14	3	1	1232.0	150.9
15	4	1	1099.0	151.0
16	5	1	1236.4	151.4

Appendix 4: Magnesium titration for **D11S902**. 1 µl of $MgCl_2$ was used in the 25 µl PCR. Lane 1: 50bp size marker

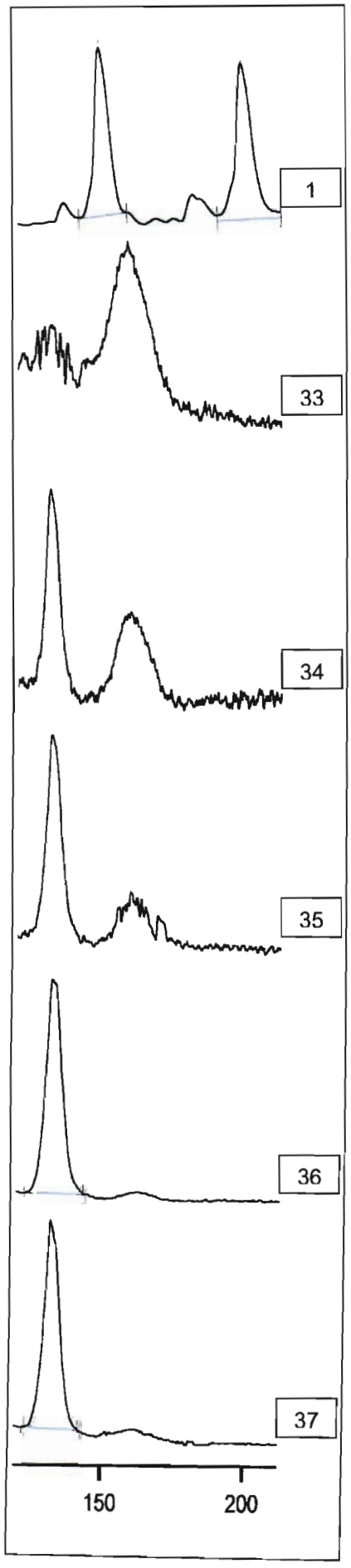
APPENDIX 5



Lane	$MgCl_2$ (µl)	Peak	Peak area	Size (bp)
22	1	-	-	-
23	2	1	95.7	190.4
		2	407.8	195.6
24	3	1	300.2	195.7
25	4	1	344.4	195.8
26	5	1	373.7	196.1

Appendix 5: Magnesium titration for **D11S904**. 2 µl of $MgCl_2$ was used in the 25 µl PCR. Lane 1: 50bp size marker

APPENDIX 6



Lane	$MgCl_2$ (µl)	Peak	Peak area	Size (bp)
33	1	-	-	-
34	2	1	25.8	132.6
35	3	1	30.2	132.3
36	4	1	108.5	132.1
37	5	1	107.0	132.0

Appendix 6: Magnesium titration for **D16S413**. 4 µl of $MgCl_2$ was used in the 25 µl PCR. Lane 1: 50 bp size marker