

**MOLECULAR ANALYSIS OF THE HOMEBOX GENE
BARX2, A CANDIDATE TUMOUR-SUPPRESSOR GENE IN
OVARIAN CANCER.**

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Declaration

In accordance with the regulations of the University, I declare that this thesis has been composed by myself entirely, and that the work presented is my own, except where I have indicated the contribution of others.

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Abstract

Ovarian cancer is the most common cause of death from gynecological malignancies in women in the UK and China. This is related to its late presentation that results in an extremely poor prognosis. Previous studies have demonstrated that disruption of multiple genetic loci is associated with carcinogenesis. Loss of heterozygosity (LOH) on a chromosome can suggest the presence of a tumour suppressor gene (TSG) at that locus. LOH at chromosome 11q between D11S1894 and D11S1309 occurs frequently in ovarian and colorectal cancer and is associated with progression and poor prognosis in ovarian cancer. Therefore, there may be important TSGs involved in the progression of ovarian cancer on this region. The Barx2 gene, a homeobox transcription factor, is located within this minimal region and expressed at the site of epithelio-mesenchymal in developing mouse. It has also been identified as a regulator of cell-adhesion molecules (CAMs). Therefore, Barx2 is worthy of consideration as a candidate tumour suppressor gene.

Previous LOH studies have demonstrated that LOH at chromosome 11q between D11S1894 and D11S1309 occurs frequently in ovarian and colorectal cancer (using same polymorphic markers and got same results in ovarian and colorectal cancer in our lab). Because there was shortage of ovarian tumour/blood pairs samples in that time, in order to further refine the TSG region at chromosome 11q between D11S1894 and D11S1390 (4.5Mb) and also confirm location of Barx2, 14 polymorphic loci were investigated using microsatellite analysis in colorectal cancer tumour/blood pairs. Of 39 colorectal cancer specimens, 28(71.18%) demonstrated LOH at one or more marker, 10 (25.6%) demonstrated LOH at all marker and 18 (46.2%) had partial LOH. Three distinct LOH regions were detected, between GATA72A01 and D11S4131 (less 200Kb), between D11S912 and D11S4150 (less 200Kb), and between D11S1320 and D11S4085 (2Mb), suggesting possible sites for TSG. There was no correlation with clinicopathological features. This LOH data not only confirmed the previous studies but also narrowed further this minimum region from 4.5Mb to less 200Kb and 2 Mb. This

study has therefore more precisely defined the regions of chromosome 11q likely to contain TSG.

The Barx2 gene is located within this minimal region. In this study, we present evidence from *in vitro* expression studies that, following transfection, human Barx2 inhibits matrigel invasion in the ovarian cancer cell lines OAW42 and OVCAR3. OAW42, a Barx2 non-expressing cell line, following transfection, was also inhibited for migration in response to a collagen IV signal and adhesion to collagen IV. In two sequential cell line series derived from patients with ovarian cancer pre- and post-platinum chemotherapy, the Barx2 gene was downregulated following treatment. Transfection of the Barx2 gene into a platinum resistant cell line, PEO1 cDDP that is derived from PEO1 by *in-vitro* cisplatin exposure and has downregulated expression of Barx2, showed that Barx2 reversed cisplatin resistance. We also show that Barx2 expression levels are lower in clear cell/endometrioid ovarian adenocarcinoma histological variants that are less likely to respond to cisplatin. But we were unable to detect evidence of somatic methylation or inactivating mutations in the coding region of Barx2 gene in ovarian cancer. Our data suggested that the Barx2 gene is a candidate suppressor of tumour progression, and a modulator of cisplatin resistance in ovarian cancer. Furthermore, this represents the first report of Barx2 gene expression associated with modulation of platinum sensitivity

LIST OF ABBREVIATIONS

5-MT	5-methyl transferase
ALF	Automated laser fluorescence
AMV	avian myeloblastosis virus
Antp	Antennapedia
APC	adenomatosis polyposis coli
APLP2	Amyloid precursor-like protein 2
ATF2	cAMP responsive element binding protein 2
ATM	Ataxia-telangiectasia (A-T) mutated
ATX	autoxin
BAD	BCL2-antagonist of cell death
BAX	BCL2-associated X protein
Barx	BarH-like homeobox
BBR	Barx basic region
bFGF	basic fibroblast growth factor
bp	base pairs
Brn	brain POU domain, class 4, transcription factor
BSA	Bovine serum albumin
BSO	Buthionine sulfoximine
CA-125	ovarian cancer antigen CA-125
CAMs	celladhesion molecules
CC	clear cell carcinoma
CC3	cytochrome c 3
CDH6	cadherin-6
cDDP	cisplatin
cDNA	Complementary deoxyribonucleic acid
Cdx1	caudal type homeo box transcription factor 1
Cdx2	caudal type homeo box transcription factor 2
C/EBP	CCAAT Enhancer binding protein

CGH	comparative genomic hybridisation
CHK1	checkpoint 1 gene
Ci	Curies
CLP	calmodulin-like protein
CNAs	copy number abnormalities
GOG	Gynecologic Oncology Group
CRE	cyclic AMP response element
CREB	cAMP responsive element binding protein
CsA	cyclosporin A
CSF-1	macrophage-colony-stimulating factor
dCTP	deoxycytidine triphosphate
DHEA	dehydroepiandrosterone
DHPLC	Denaturing high pressure liquid chromatography
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dUTP	Deoxy-uridine triphosphate
ECM	extra-cellular matrix
EGFR	epidermal growth factor receptor
EGF	epidermal growth factor
EM	endometrioid carcinoma
ER	estrogen receptor
FAP	familial adenomatous polyposis
FIGO	International Federation of Obstetric and Gynecology
g	Grams
GBX	gastrulation brain homeobox
GSK3	glycogen synthase kinase 3
GST	glutathione-S-transferases
GSH	glutathione
H19	Histone 19 gene
HAD	heteroduplex analysis
HBS	homeodomain binding sites sufficient

HD	homeodomain
HGF	hetatocyte growth factor
hm B	hygromycin B
HNPCC	hereditary non-polyposis colorectal cancer
Hox	Homeobox
LEF	lymphoid enhancer factor
ICRF	Imperial Cancer Research Fund
IGF	insulin-like growth factor
IGSF	Immunoglobulin gene superfamily
IL-6	interleukin-6
Kb	kilobases
KCNJ1	potassium inwardly-rectifying channel subfamily J 1
KIP	DNA-PKcs interacting proteins
LOH	loss of heterozygosity
LMP	low malignant potential
LRP	lung cancer multidrug resistance protein
MCS	multiple cloning site
MEN1	Multiple endocrine neoplasia gene 1
MDCK	madin derby canine kidney
MDR1	multidrug resistance 1 gene
MHCs	microcell hybrid clones
min	minutes
ml	millilitres
MMPs	matrix metalloproteases
MMT	mix mesodermal tumour
MRC	Medical Research Council
mRAN	messenger RNA
MTR1	MLSN1 - and TRP -related gene 1
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide
Myb	avian myeloblastosis viral oncogene homolog

Ng-CAM	neuron-glia CAM
N-CAM	neural CAM
NKX3.1	NK homeobox (Drosophila), family 3, A
NPAT	nuclear protein, ataxia-telangiectasia locus
OC	oral contraceptive
Oct	POU domain, class 2, transcription factor
OD	Optical density
OSE	ovarian surface epithelial
PAs	plasminogen activators
PAX	Paired box
PBS	Phosphate buffered saline
PBX	pre-B-cell leukemia transcription factor
PCR	Polymerase chain reaction
PFGE	Pulsed-Field Gel Electrophoresis
Pgp	P-glycoprotein
PGR	progesterone receptor gene
PI3	phosphatidy inositol 3 kinase
PIG8	p53 induced gene 8
Pit-1	POU domain, class 1, transcription factor 1
PPP2R1B	protein phosphatase 2 (formerly 2A),regulatory subunit A (PR 65), beta isoform
PR	progesterone receptor
pRB	retinoblastoma protein
RRE	ras/raf responsive transcriptional element
RAN	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse-transcription PCR
SCLC	small-cell lung cancer
sec	seconds
SER	serous carcinoma
SPN	suppressin (nuclear deformed epidermal

	autoregulatory factor-1(DEAF-1)-related)
SRO	Shortest region of overlap
SSA	aristapedia
SSCP	Single stranded conformational polymorphism
TCCs	transitional cell carcinoma
TCF	T-cell factor
TdT	Terminal deoxynucleotide transferase
TGF- α	transforming growth factor alpha
TGF- β	t ransforming growth factor beta
TIMPs	tissue inhibitors of metalloproteases
tPA	tissue-type plasminogen
TSG	tumour suppressor gene
TSG101	tumour susceptibility gene 101
TSSC	Tumour-suppressing subchromosomal transferable fragment cDNA
TUNEL	Terminal deoxynucleotide transferase mediated deoxy- Uridine triphosphate Nick-End labelling
uPA	urokinase plasminogen
UV	Ultra violet
VGEF	vascular endothelial growth factor
WT	Wilms' tumour
YAC	yeast artificial chromosome

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1. INTRODUCTION

1.1 Epithelial Ovarian Cancer :Causation and Distribution

Ovarian cancer is the most lethal of gynaecological malignancies, and the seventh most common cancer in women worldwide, after breast, cervix, colon/rectum, stomach, corpus uteri, and lung cancers. Each year, an estimated 5,000 women in the UK are diagnosed with ovarian cancer. During any given year, approximately 4,200 cases in the UK die from this disease. In the UK, it is the fifth commonest cause of cancer death in women (Chang J, et al. 1994.). Approximately 1 woman in 70 will develop ovarian cancer, and 1 women in 100 will die from it. In China, the mortality rate of ovarian cancer was 0.67/100,000 per year during 1990's. Compared with 1970's, it has increased nearly five folds and is the sixth commonest cause of cancer death in Chinese women (Lee ND, et al.1997). This high mortality rate is due to presentation at an advanced stage of the disease as symptoms of the disease are insidious in onset and non-specific in nature. Over 75% of women with the ovarian cancer have tumour spread beyond the pelvis at the time of diagnosis and this is the reason why the overall five-year survival rate is very poor alive; only about 25% to 30%.

The incidence rate of ovarian cancer is age-related, it is uncommon in women younger than the age of 40 years, after which incidence rates increase sharply until the eighth decade of life, then decrease slightly. The age-specific incidence rates increase from 2 to 3 cases per 100,000 in the third decade to 59 cases per 100,000 in the eighth decade (Adami HO, et al.1994). There is a difference in incidence rates of ovarian cancer in different geographical areas. There is a particularly high incidence in Scandinavia, followed by Northern Europe and North America. Despite being industrialised, Japan has a particularly low incidence of the ovarian cancer; however, the incidence increases sharply in Japanese migrants to America and in their daughters, suggesting profound environmental components to aetiology (Herrinton LJ et al.1994). However, there has been little change in the incidence rate of ovarian cancer during the last three decades. In high-risk countries such as the United States, there is only a slight increase especially in the older age groups. A trend of increasing

incidence has been reported in previously low-risk countries (Daly M and Oubram G. 1998). There is also a difference in incidence rates of ovarian cancer in different ethnic groups. In USA, it is significantly higher among white and Hawaiian women, intermediate among African-American, Hispanic, and Asian-American women. Presumably, this reflects differences in distribution of risk factors, From 1986-1990, the incidence rate among whites was 50% higher than among African-American, observed in both premenopausal and postmenopausal women (Ries LAG, et al.1997). Both white and African-Americans women have a similar age pattern of incidence, and the known risk factors appear to operate similarly in both groups.

Although analytical epidemiological studies have examined a number of potential risk factors for ovarian cancer, the causative factors that lead to ovarian cancer are still unclear. According to many epidemiological investigations, the aetiology of ovarian cancer mainly includes the following:

- 1) The continuous uninterrupted cell division and regeneration of ovarian epithelium with each ovulation provides opportunity for mutation and malignant transformation. Empirical evidence exists to show the explanation is likely to be correct.

Multiparity, lactation, and the oral contraceptive (OC) use all decrease a woman's risk for ovarian cancer. In data combined from 12 United States case-control studies of epithelial ovarian cancer, the estimated risk of developing ovarian cancer before age 65 for the total population is 0.8%. Among women with no family history of ovarian cancer, the lifetime risk varies from 0.6% among those with three or more term pregnancies and 4 or more years of OC use, to 3.4% among nulliparas with no OC use (Hartge P, et al. 1994). In a large prospective study, parity was the only reproductive factor that had a substantial independent association with ovarian cancer; parous women had a 45 % decrease in ovarian cancer risk relative to nulliparous women, regardless of age at first birth; each birth was associated with a 16% decrease in risk (Hankinson SE, et al. 1995). Other investigation also confirms this trend. Adami et al noted that each additional birth decreases a woman's ovarian cancer risk by between 14% and 22% (Adami HO, et al. 1994). But the other case-

control studies showed those incomplete pregnancies, due either to a spontaneous or induced abortion, either decreases risk slightly or are not associated with ovarian cancer risk in either nulliparous or parous women (Chen MT, et al. 1996). Increased ovarian cancer risk among nulliparas could reflect an association between ovarian cancer and infertility. The pooled analysis of studies of ovarian cancer has shown an increase in risk among women who had used fertility drugs with an odds ratio of 4.0 among women with low malignant potential tumours and 2.8 among women with invasive ovarian cancer, with the highest odds ratio (27.0) for nulliparas with infertility drug use (Harris R, et al. 1992). In another study, infertile women who had taken clomiphene for 12 or more ovulatory cycles were at greater risk of ovarian cancer than infertile women who had taken it for less than 1 year or not at all (Rossing MA, et al. 1994). There is evidence that women with a history of lactation had a lower risk of ovarian cancer than who had not breast-fed. Lactation is known to suppress ovulation in most women. Whittemore et al reported that ovarian cancer risk decreases almost 1% for each month of lactation. The protective effect is strongest in the months immediately after delivery, when ovulation is most likely to be suppressed (Whittemore AS, et al. 1988). Another epidemiological study also reported that pregnancies with lactation appear to be slightly more protective than pregnancies without; The average months of lactation per pregnancy was strongly inversely associated with ovarian cancer risk (Risch HA, et al. 1994).

2) Pituitary gonadotropin stimulation can lead to malignant transformation;

The use of OCs appears to decrease a woman's risk of ovarian cancer by 30% to 60%. In the Oxford Family Planning Association cohort study, the relative risk of ovarian cancer for OC users was 0.4; in comparison with never users (Vessey MP, et al. 1995). The results from analysis of the cancer and steroid hormone study suggested that 5 years of OC use by nulliparous women can reduce their ovarian cancer risk to that of parous women, and that 10 years of OC use by women with a positive family history can reduce their risk to a level below that of women with a negative family history (Gross TP, et al. 1994). With regard the mechanism by which OCs reduce risk of ovarian cancer, the data suggested that OCs suppress

gonadotrophins and in turn, the ovary produces less androgens, estrogens, and progesterone (Daly M and Orams G. 1998). Furthermore, the high gonadotrophic milieu of the post-menopausal state may confer additional risk (Cramer DW, et al. 1983). But recent findings do not support the hypothesis that gonadotrophin stimulation is an aetiological factor in ovarian cancer. A case-control study of serum gonadotrophins and steroid hormones has shown that women with low serum gonadotrophin levels are at increased risk of ovarian cancer, as are women with high androgen levels. Specifically, higher androstenedione and dehydroepiandrosterone (DHEA) levels are associated with increased risk. The risk of ovarian cancer increases with increasing levels of these hormones (Helzlsouer KJ, et al. 1995). Some researchers think that it may be also important to consider that the levels of steroids in follicular fluid and the ovarian epithelial microenvironment may be more important in ovarian cancer pathogenesis than are serum hormone levels (Daly M and Orams G. 1998).

Although the relationship between exogenous estrogens and development of human ovarian cancer is still unclear, many studies have suggested that ovarian cancer is hormone sensitive and that oestrogens, progesterone and their specific intracellular receptor expression are associated with histopathological subtypes and prognosis of ovarian cancer. The actions of these hormones are mediated by specific intracellular receptors that function as hormone-inducible nuclear transcription factors with context-specific, often conflicting effects on proliferation and differentiation of target tissues (O'Malley BW, et al. 1990). Investigations of the relationship between the oestrogens, progesterone and their specific intracellular receptor expression and human ovarian cancer have shown; (a), Oestradiol induced PR expression has been demonstrated in ovarian cancer cell lines, which express ER (Langdon SP, et al. 1994). (b); there is difference in PR expression in different histopathological subtypes of epithelial ovarian cancer. Endometrioid ovarian carcinoma contains relatively more PR than other histopathological subtypes (Slotman BJ, et al. 1988), and PR positivity is associated with well differentiated ovarian cancer in premenopausal women (Munstedt K, et al, 2000) (c); Ovarian cancer has been reported to respond to

antioestrogens in about 10%-20% of cases and to progestins with an average of 36%, within a range between 0% to 60%(Slotman BJ, et al, 1988). (d); Tumour PR content has prognostic significance in ovarian cancer (Hempling RE, et al. 1990), But the evidence for association of tumour ER content with prognosis is conflicting (Eissa S, et al. 1995; Sevelde P, et al. 1990).

3) The ovary is exposed to carcinogens that can travel to the ovary via the vagina and fallopian tubes.

Case-control and record linkage studies have shown decreased risks of ovarian cancer after tubal ligation and/or hysterectomy (Kreiger N, et al. 1997). A case-control study of ovarian cancer in India, that has a high rate of childbearing and early age at first pregnancy and where tubal ligation is widely practiced, showed that the only significant factor to influence ovarian cancer risk was tubal ligation, with an odds ratio of 0.25(Nandakumar A, et al. 1995). Another case-control study showed a significant odds ratio of 0.5 for ovarian cancer after hysterectomy (Risch HA, et al.1994). Therefore the hypothesis is consistent with the idea that surgery prevents exogenous agents from entering the peritoneal cavity and reduces the risk of ovarian cancer.

The use of cosmetic talc in dusting the perineum, in feminine hygiene sprays or on sanitary napkins, condoms or diaphragms has been suggested as a possible risk factor for ovarian cancer. In a case-control study, a significant odds ratio of 1.5 was reported for use of talc, with higher risks in those women who applied it directly to the body, on a daily basis, or for more than 10 years (Harlow BL, et al. 1992). Investigations of women undergoing laparoscopy have suggested that the majority of women have retrograde menstruation (Olive DL and Schwarz LB. 1993). Some data also indicated that talc may be transported by retrograde menstruation through the fallopian tubes to the ovary (Wehner AP. 1994). In view of the fact, talc is contaminated with significant amounts of asbestos; several workers investigated and reported an increased risk of ovarian cancer in women asbestos workers (Heller DS, et al. 1996). Furthermore, asbestos fibre can be found in the ovaries of majority of women with household asbestos exposure, and that fiber burdens were higher in those with exposure histories (Heeler DS, et al. 1996).

4) Hereditary factors:

Case-control studies have demonstrated the importance of a family history of ovarian cancer and a family history of other cancers on the risk for ovarian cancer. The risk of ovarian cancer and other cancer was assessed in first-degree relatives of patient with ovarian cancer from an analysis of 391 pedigree. This analysis showed that overall there was a significant increase in the risk of ovarian cancer (4.5-fold). The risks were 14.2-fold, 5.2-fold and 3.7-fold for relatives of patients diagnosed before 45, between 45 and 55 and after the age of 55, respectively (Houlston RS, et al. 1993). A meta-analysis of family history and risk of ovarian cancer has shown that family histories of ovarian, uterine, breast, and pancreatic cancer were significantly associated with increased risk of ovarian cancer. The relative risk of ovarian cancer was 4.31 for women with a first-degree relative with ovarian cancer, 2.12 for women with an affected second-degree relative, and 1.48 for women with an affected third-degree relative (Kerber RA and Slattery ML. 1995).

5) Other factor:

Galactose metabolism has been proposed as a risk factor for ovarian cancer based on data that galactose is toxic to oocytes, but the result of epidemiological investigation are still conflicting (Cramer DW, et al. 1994;Risch HA, et al. 1994; Herrinton LJ, et al. 1996).

Initial evidence that consumption of animal fat is associated with an increased risk of ovarian cancer (Cramer DW, et al. 1984) was followed with a large case-control study into diet and ovarian cancer risk. This study suggested every 10g of ingested saturated fat per day increased ovarian cancer risk by 20%, whereas the same ingested weight of vegetable fibre reduced ovarian cancer risk by 37%(Risch HA, et al, 1994).

In a record-linkage study of women employed in hairdressing, a small increased risk for ovarian cancer incidence was reported. The increase was significant with a standardised incidence ratio of 1.88(Boffetta P, et al. 1994). This finding may be of further interest because hair dyes contain known mutagens.

1.2 Epithelial Ovarian Cancer: Biology and Pathophysiology

1.2.1 Development of ovarian surface epithelium in embryology

In the embryo, the ovarian surface epithelium originates in the coelomic epithelium that overlies the gonadal ridge. Therefore, it is of mesodermal origin, and developmentally closely related to the underlying stromal fibroblasts. Coelomic epithelial cells penetrate into the fetal ovary and contribute to the development of granulosa cells. Furthermore, the gonadal ridge lies near the regions where invaginations of the coelomic epithelium give rise to the mesonephros and the ovarian rete, and to the Mullerian ducts which are the primordial cells for the epithelia of the Fallopian tubes, uterus, and endocervix. Thus, the embryonic coelomic epithelium in the urogenital region is competent to develop along many different pathways. Common epithelial tumours of the ovary arise from the ovarian surface epithelium, and exhibit a range of histological structures which have appearances similar to other Mullerian derived structures; i.e. serous differentiation (similar to fallopian tube epithelium), endometrioid differentiation (uterine body endometrium), and mucinous differentiation (endocervical epithelium). Ovarian malignant tumour, usually arise from the ovarian surface epithelium. There are anatomical reasons why the ovarian surface epithelium may have an increased tendency to neoplasia. Firstly, the ovarian surface epithelium, in contrast to the peritoneal mesothelium, is in close proximity to the paracrine influence of adjacent ovarian tissue and receptors for many of the hormones and growth factors produced by the ovary in the course of ovum maturation and release are present in surface epithelial cells but not in adjacent peritoneal mesothelium. Surface epithelial cells are often found entrapped in the ovarian cortex and this potentially places the cells under increased mitotic pressure (Hamilton TC. 1992; Salazar H, et al. 1996). Additionally, it may be significant that the surface epithelium in contrast to other adult coelomic epithelial derivatives shows delayed differentiation as manifest by its ability after transformation to differentiate along several pathways. This retention of pluripotentiality may be accompanied by greater proliferative capability with reduced induction of apoptotic death pathways

and therefore perhaps enhanced susceptibility to neoplastic transformation (Auersperg N, et al.1998).

1.2.2 pathology

1.2.2.1 Benign tumours

Benign tumours are almost always serous or mucinous, and generally arise in women between the age of 20 and 60 years (Russel P and farnsworth A. 1997) The tumours are frequently large, sometimes in 15-30 cm dimension. Benign serous tumours are more commonly bilateral, but benign mucinous tumour are almost always unilateral. Benign epithelial tumours are typically cystic, hence the term “cystadenoma” e.g., serous or mucinous cystadenoma. The essential cytological features of them are a single layer of columnar cells, which line the cysts, lack of cellular atypia, a normal nucleocytoplasmic ratio, few mitoses, and no evidence of either microinvasion or invasion into the underlying stroma. Papillae, if present, consist of a fibrovascular core covered by a single layer of mature, tall, columnar epithelium identical to that of the cyst lining. A prominent fibrous component can be present, giving a grossly solid or papillary appearance to areas. In serous tumours, the fluid within the cyst is watery. The serous epithelium is well differentiated, and forms a regular single layer of cuboidal cells with centrally placed nuclei lying on loose stroma. Whereas in mucinous tumours, the cysts are multilocular containing a gelatinous mucinous secretion. The epithelium consists of tall columnar epithelial cells with basal nucle.

1.2.2.2 Borderline tumours (tumours of low malignant potential, LMP)

Tumours of LMP have an excellent prognosis, despite peculiar histologic features suggestive of cancer (Bamhill DK, et al. 1995). The histologic features used to diagnose borderline tumours include; (1), epithelial papillae (especially in serous tumours), with detachment of atypical cell clusters as single or small groups of cells, (2), cellular stratification (especially in mucinous tumours), (3), increased mitotic activity, and (4), nuclear atypia (increased nuclear/cytoplasmic ratio, hyperchromatism, and prominent nucleoli).

1.2.2.3 *Malignant tumours (invasive carcinoma)*

Malignant ovarian tumours often present as solid masses, usually with areas of necrosis and haemorrhage. By the time a carcinoma is found, it has often already spread beyond the ovary and seeded the peritoneum.

The histological hallmarks of malignant tissue are the same as for borderline tumours, with cellular atypia, a high nucleocytoplasmic ratio, and frequent mitoses. There is, however, destructive invasion of the underlying stroma by the malignant adenocarcinoma cells. As part of histopathological diagnosis, an attempt at estimating the degree of differentiation of ovarian adenocarcinomas should be made (Baak JP, et al, 1987). Grade 1 tumours have fine, well-developed papillae nearly throughout with little cellular atypia and relatively few mitoses. Grade 2 tumours disclose areas where sheets of tissue may form greater cellular atypia and mitoses. Grade 3 tumours exhibit large sheets of undifferentiated cells. The mitotic rate and number of atypical mitoses increase progressively in the various grades. Tumours can also entirely lose their histogenic features leaving an undifferentiated adenocarcinoma.

High correlation has been observed between the stage and grade of tumour. Nearly 90% of tumours that extends to the pelvic peritoneum, omentum, or beyond (Stages II B to IV) are grades 2 and 3. In contrast, 72% of tumours confined to the ovaries or the surfaces of the reproductive organ (Stages I to IIA) are grade 1. Both stage and grade were identified as an independent prognostic variable in the multivariate model (Bichel P, et al. 1989; Shimizu Y, et al. 1998).

Serous adenocarcinoma accounts for just under half of all epithelial ovarian cancers and usually display obvious invasion. The tumour may appear as large sheets of cells growing autonomously without stromal support or as broad-to-fine clusters of cells related to papillae that irregularly dissect through the stroma.

Mucinous adenocarcinoma accounts for only 5-10 % of all epithelial ovarian cancers. The tumours present as sheets of cells, often in a cribriform pattern, or as tumour cells dissecting into the stroma. As they become less well differentiated, the cells

often lose their intracytoplasmic mucinous component, and therefore may be difficult to distinguish from serous and endometrioid carcinoma.

Endometrioid carcinomas account for a relatively small proportion (15 %). They resemble adenocarcinomas of the endometrium, and are usually less cystic than serous and mucinous adenocarcinomas. In a quarter of case endometrioid adenocarcinomas may co-exist with endometrial carcinoma, and have identical histology. They can also co-exist with, or arise on, a previous site of endometriosis (Heaps JM, et al. 1990).

Clear cell carcinomas are uncommon, just about 6% of epithelial ovarian cancers. It is characterised by both clear and “hobnail” cells. The clear appearance of the cytoplasm is due to the dissolution of glycogen as the tissue specimen is prepared for microscopic examination. The hobnailed cells are bulbous nuclei that protrude into the lumen well beyond the apparent cytoplasmic limits of the cell. The clear-cells are usually found in sheets of cells that have the appearance of a solid growth. The hobnailed cells and sometimes flat cells are encountered more commonly in a pattern of growth characterized by tubules and cysts.

Undifferentiated epithelial ovarian cancers account for about 3 % of the total, and tend to behave in aggressive fashion (Canzonier V, et al. 1995).

1.3 Epithelial Ovarian Cancer: Clinical Management and Prevention

1.3.1 *Screening for ovarian cancer*

Available potential screening techniques have included pelvic examination, ultrasound examination, CA 125 and other tumour markers, and combined modality approaches. Successful screening should result in a decrease in site-specific morbidity and mortality of the screened population from a disease.

The detection of an asymptomatic pelvic mass on routine physical examination may identify an ovarian cancer before abdominal dissemination, but there are no data on the frequency with which ovarian cancer is detected in asymptomatic women on the basis of an annual pelvic examination.

Ultrasound examination has been used widely for screening for ovarian cancer. However, transabdominal ultrasonography is not sufficiently specific to be useful as a routine screening procedure. A prospective study showed that the odds that abnormal transabdominal ultrasonography could identify the presence of primary ovarian cancer were only one in 67 (Campbell S, et al. 1994). Transvaginal ultrasonography has been proposed as a more specific alternative to abdominal sonography as a screening test because of increased resolution capable of detecting minimal morphologic changes in the ovary (DePriest PD, et al. 1993). On other hand, coupling colour flow imaging with transvaginal ultrasonography may further improve the accuracy of sonography and reduce the unacceptably high rate of false-positive results (Karlan BY, et al. 1994).

Prospective studies have shown that serum CA-125 levels are sufficiently sensitive to identify patients with early-stage disease. However, as previously noted, false-positive test results have been reported in a number of non-malignant gynaecologic condition, such as peritonitis, pancreatitis, and so on (Olt GJ, et al. 1990). Due to the high false-positive rate relative to the low incidence of epithelial ovarian cancer, a single CA-125 assay is not useful in screening for ovarian cancer.

Multimodal screening has been proposed as a useful tool to screening ovarian cancer because any technique alone could not be sufficiently sensitive to identify early-stage disease. A scheme of multimodal screening has been used to screen ovarian cancer and resulted in 99.9% specificity, with sensitivity of 78.6% at 1 year and 57.9% at 2 year. Serum CA-125 was first screened, if it was greater than or equal to 30U/ml, women underwent abdominal ultrasonography, followed by a laparotomy for an ovarian abnormality (Jacobs I, et al. 1993).

1.3.2 Prognostic factors in ovarian cancer

Prognostic factors in ovarian cancer include many features, surgical, pathological, biological, etc. Multivariate statistical methods have been used to analyse prognostic factors, which are truly independent in themselves rather than simply reflecting

association with other prognostic factors. Such analyses suggest that there are several general independent prognostic factors and described below.

1) Surgical prognostic factors;

The 5-year survival of patients with epithelial ovarian cancer is directly related to the International Federation of Obstetric and Gynecology (FIGO) stage (see Table 1-2). Patients with stage I and stage II ovarian cancer have a significantly better outcome than those with stage III or stage IV tumours. Within each stage, however, there are often marked differences in patient survival and subcategorization of stage provides useful prognostic information; capsular invasion, surface excrescence, tumour rupture, ascites, positive peritoneal cytology and degree of peritoneal cytology (Makar AP, et al. 1995; Ahmed FY, et al. 1996). The volume of residual disease following cytoreductive surgery is also directly correlated with survival. Patients who have been optimally cytoreduced have a better prognosis than those undergoing less than optimum resection. In retrospective analyses of the importance of residual volume on survival, the size of the largest residual mass (< 2cm, or >2cm in size), and not the total number of lesions, has emerged as the primary factor correlating with prognosis (Hunter RW, et al. 1992). But one study has been demonstrated that the number of residual masses may be an important prognostic factor as well (Heintz AP, et al. 1988).

1) pathological prognostic factors;

In general, patients with mucinous adenocarcinomas have an overall better survival in comparison to endometrioid or serous adenocarcinomas in same stage and histologic grade (Ansell SM, et al. 1993). Clear cell carcinomas are commonly found to be stage I at diagnosis. There is some controversy regarding their prognostic significance, but there are data to suggest that they have a more aggressive natural history (O'Brien ME, et al. 1993). There are also reports that suggest clear cell tumours are more likely to be resistant to platinum-based chemotherapy (Goff BA, et al. 1996).

The histologic grade of the tumour is a particularly important prognostic factor in patients with early-stage disease. Stage I patients with well or moderately well differentiated tumours have a greater than 70 %-80 % 5-year survival when treated with surgery alone (Young RC, et al. 1990). In advanced-stage patients treated with cisplatin-based chemotherapy, most studies have failed to demonstrate a significant correlation between histologic grade and survival (Friedlander ML. 1998).

On the other hand, flow cytometric analysis of tumoral DNA content can indicate the presence of an euploid tumour populations and is a significant adverse independent prognostic factor (Friedlander ML, et al. 1988).

Although CA125 levels are increased in 75% to 90% of patients with ovarian cancer and have a well established role in following the response to treatment and detecting relapse (Nagele F, et al. 1995), the relationship between CA125 and survival is still ambiguous. The preoperative CA125 level of patients with advanced ovarian cancer does not correlate well with survival, but the decrease of CA125 during the first three cycles of treatment is an important predictor of outcome (Yedema CA, et al.1993).

1) biological prognostic factors;

There is growing understanding of the molecular events associated with the development and progression of ovarian cancer and they have led to numerous reports on the prognostic impact of molecular markers. These molecular markers frequently can be categorized as abnormalities in oncogene products (such as HER-2/neu, p20), tumour suppressor genes (p53, p16, pRB), and measures of drug sensitivity (Pgp, LRP, MPR, GST, BAX) (Bookman MA, et al.1996; Eisenhauer EA, et al. 1999) In addition, there have been a series of reports about markers of proliferation (DNA index, KI-67 index, proliferating cell nuclear antigen), DNA repair (leukocyte platinum, DNA excision repair, helicasep complexes), serum cytokine levels (CSF-1, IL-6), and factors associated with tumour invasion and metastases (NM23) (Friedlander ML. 1998). Despite the large number of reports describing putative prognostic factors, they are not currently used in the routine selection of treatment for patients with either early-stage or advanced-stage disease.

Recently, some studies have demonstrated that p53, HER-2/neu and EGFR are independent prognostic factors (Eisenhauer EA, et al. 1999).

1.3.3 *Clinical staging and primary cytoreductive surgery*

Adequate and complete surgical intervention is mandatory primary therapy for ovarian carcinoma, permitting precise staging, accurate diagnosis, and optimal debulking of the tumour (taking out as much of the cancer as possible).

Many clinical studies underscore the importance of a comprehensive surgical staging procedure (Table 1-1) (Boente MP, et al. 1998). Accurate staging is crucial as it determines subsequent therapy, which will contribute to outcome. The widely used FIGO staging system is presented in Table 1-2.

Table 1-1 Comprehensive Staging Laparotomy for Suspected Early Ovarian Carcinoma

Vertical incision that allows adequate visualization and palpation of structures in the upper abdomen and retroperitoneum
Peritoneal washings (pelvis, parabolic gutters, hemidiaphragms)
Inspection/palpation of all peritoneal and mesenteric surfaces
Biopsy of any lesions or adhesions
Total abdominal hysterectomy and bilateral salpingo-oophorectomy
Infracolic omentectomy
Random peritoneal biopsies (bladder, pouch of Douglas, bilateral pelvic peritoneum, paracolic gutters hemidiaphragms)
Pelvic and para-aortic lymphadenectomy (inspection and palpation only are inadequate)
Appendectomy (optimal)

Table 1-2 Carcinoma of the Ovary: FIGO Nomenclature (1998)

Stage I	Growth limited to the ovaries.
IA	Growth limited to one ovary; no ascites present containing malignant cells. No tumour on the external surfaces; capsule.
IB	Growth limited to both ovaries; no ascites present containing malignant cells. No tumour on the external surfaces; capsule.
IC	Tumour either Stage IA or IB, but with tumour on surface of one or both ovaries, or with capsule rupture, or with ascites present containing malignant cells, or with positive peritoneal washings
Stage II	Growth involving one or both ovaries with pelvic extension
IIA	Extension and/or metastases to the uterus and/or tubes
IIB	Extension to other pelvic tissues
IIC	Tumour either stage IIA or IIB, but with tumour on surface of one or both ovaries; or with capsule rupture, or with ascites present containing malignant cells, or with positive peritoneal washings
Stage III	Tumour involving one or both ovaries with histologically confirmed peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes. Superficial liver metastasis equals Stage III. Tumour is limited to the true pelvis, but with histologically proven malignant extension to small bowel or omentum.
IIIA	Tumour grossly limited to the true pelvis, with negative nodes, but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces, or histologically proven extension to small bowel or mesentery.
IIIB	Tumour of one or both ovaries with histologically confirmed implants, peritoneal metastasis of abdominal peritoneal surfaces, not exceeding 2 cm in diameter; nodes are negative.
IIIC	Peritoneal metastasis beyond the pelvis >2 cm in diameter and/or positive retroperitoneal or inguinal nodes.
Stage IV	Growth involving one or both ovaries with distant metastases. if pleural effusion is present, there must be positive cytology to allot a case to Stage IV. Parenchymal liver metastasis equals Stage IV.

If there is obvious evidence of tumour spread, debulking of residual tumour masses to under 2 cm maximum diameter should be undertaken as there is evidence that this results in better response and survival with subsequent chemo- or other therapy. This is thought to be due to the following: a), the resection of large bulky masses removes the portions of tumour and it will be of benefit for increasing the chemosensitivity of residual tumour nodules (Hoskins WJ. 1991). b), the Gompertzian model suggests that cytoreduction causes a high percentage of resting tumour cells to migrate into the pool of actively dividing cells with a consequent increase in chemotherapy sensitivity (DeVita VT, et al. 1993). c), after removal of large tumour masses, leaving fewer cancer cells would be more easily eradicated by postoperative chemotherapy (Hoskins WJ.1993).

1.3.4 *Chemotherapy*

Ovarian cancer is a fairly chemosensitive tumour, and chemotherapeutic agents from a wide variety of different classes have shown activity (McGuire WP, et al.1998). The best effect of chemotherapy are likely to be achieved after optimal tumour cytoreduction, using combination regimens for chemotherapy. The platinum compounds remain the most active agents in ovarian cancer and are the cornerstone of combination regimens. In the past decade a series of novel compounds were identified to be clinically active in recurrent ovarian cancer. Currently the standard of care for most patients with ovarian cancer consists of combination of paclitaxel and carboplatin (Ozols RF, et al. 1997).

1.3.4.1 *Platinum compounds*

Platinum analogues function as cytotoxic drugs by binding to amino or hydroxyl groups of nucleoside bases and forcing DNA intra-strand and inter-strand crosslinks. This DNA crosslinking results in interference with replication/repair mechanisms. The Gynecologic Oncology Group (GOG) performed the largest comparative trial of a cisplatin-containing regimen versus a non-cisplatin-containing regimen. The results

showed that the complete response rate for the platinum-containing regimen was 51 %, compared to 26 % for non-cisplatin-containing regimens, and the cisplatin-containing regimen was superior with regard to response duration, progression-free interval, and overall survival (Omura G, et al.1986).

Carboplatin is a second-generation platinum compound which was less nephrotoxic, neurotoxic, and emetogenic than was cisplatin (Canetta R, et al.1988). There have been many randomized trials comparing single-agent carboplatin with cisplatin or in combination regimens in patient with ovarian cancer (Go RS and Adjei AA. 1999). Most of these results showed equivalent efficacy and two meta-analyses also showed that there was no significant differences in survival, with 5-year survival rates of 15 % and 19 % for cisplatin and carboplatin, respectively (Aabo K, et al. 1998; Stewart LA, et al. 1992).

1.3.4.2 *Paclitaxel*

Paclitaxel is the prototype drug of a new class of agents. The cytotoxicity of paclitaxel is due to its unique effects on microtubules. Paclitaxel can bind preferentially to the beta subunit of tubulin, leading to stable polymerized microtubules that inhibit the dynamic reorganization of the microtubule network. Paclitaxel arrests cell in G2/M (Dumontet C, et al. 1999).

Paclitaxel was reported to have significant activity in advanced ovarian cancer (Kohn EC, et al. 1994; Eisenhauer EA, et al.1994). A series of clinical trial exploring the activity of paclitaxel as a single agent or in combination with platinum compounds was followed by prospective randomized trials comparing cisplatin plus paclitaxel versus cisplatin plus cyclophosphamide (Rowinsky EK and Donehower RC. 1995; McGuire WP, et al.1996; Eisenhauer EA, et al. 1994). The Gynecologic Oncology Group (GOG) protocol 111 and Ovarian Cancer (OV) protocol 10 showed that complete response rate of cisplatin plus paclitaxel were 51 % and 50 % comparing with 31 % and 36 % of cisplatin plus cyclophosphamide respectively. Overall response rate of cisplatin plus paclitaxel were 73 % and 77 % versus 60 % and 66 % of cisplatin plus cyclophosphamide respectively. The median progression-free

survival (month) of cisplatin plus paclitaxel were 18 month and 16 month comparing with 13 and 12 months for cisplatin plus cyclophosphamide respectively; median overall survival (month) of cisplatin plus paclitaxel were 38 months and 35 months comparing with 24 and 25 months of cisplatin plus cyclophosphamide respectively (McGuire WP, et al.1996; Sturt G, et al. 1998). In addition, three randomised trial of carboplatin plus paclitaxel vs cisplatin plus paclitaxel showed equivalent responding rates and reduced toxicity (Go RS and Adjei AA. 1999). Based on the superior therapeutic index, paclitaxel plus another platinum compound, carboplatin, has been considered the standard of care for treatment of patients with ovarian cancer (Neijt JP, et al. 1997; Ozols RF .1999)

1.3.4.3 *Second-line chemotherapy*

The optimum treatment of patients who have recurrent disease remains a major management problem in ovarian cancer. Although most patients with ovarian cancer can achieve a clinical CR after completion of cytoreductive surgery followed by paclitaxel plus a platinum compound, most of them still recur with disease and fail to respond to initial chemotherapy. A series of new agents have been shown to have activity in the second-line treatment of patients with recurrent ovarian cancer including Topotecan and liposomal doxorubicin. Patients with drug-sensitive tumour have a high re-respond to drugs that have been used in their initial induction regimen. The re-respond for retreatment with a platinum compound, and presumably paclitaxel, is a direct function of the length of the disease-free interval. The exception may be those patients who have had an extremely long disease-free interval, perhaps greater than 1 to 2 years, in whom combination chemotherapy may be the preferred treatment at the time of recurrence. Most patients with recurrent drug-sensitive disease are usually treated with either paclitaxel or platinum compound then crossed over to the other drug as necessary. Retreatment with these two agents in recurrent drug-sensitive tumour has not been shown to be superior to using some of the newer agents to be disease-free as the first chemotherapy option for recurrent disease. It has also been shown that a higher response-rate will be obtained with most second-

line agents in drug-sensitive patients compared with drug-resistant patients. There are summarised by McGuire and Ozols (see Table 1-3) (McGuire WP and Ozols RF. 1998).

Table 1-3 New Active Agents in Recurrent Ovarian Cancer

Agent	Response (%)	Comment
Prior treatment did not include paclitaxel		
Paclitaxel	19 to 40	Multiple studies
Topotecan	14 to 23	Lower response rates in platinum-resistant patients
Vinorelbine	22	Duration of response > 19 weeks
Gemcitabine	29	Activity in platinum-resistant patients
Docetaxel	40	Neutropenia and fluid retention common
Prior treatment with paclitaxel and patinum		
Liposomal doxorubicin	26	Median survival 11 months
Oral etoposide	27 to 35	Higher response rate in platinum-sensitive patients

1.3.4.4 Drug resistance

Acquired drug resistance is the major factor that limits the effectiveness of chemotherapy in patients with advanced ovarian cancer. Patients with recurrent disease after an initial response to chemotherapy rarely, if ever, are cured with chemotherapy. In part, this is related to the broad cross-resistance that develops toward antineoplastic agents from different pharmacologic classes. On the other hand, about 20 % to 25 % of patients have intrinsically drug-resistant tumour at the time of diagnosis and never respond to any chemotherapy regimen.

The biochemical and molecular mechanism associated with drug resistance in ovarian cancer have been extensively studied. But the mechanism of multidrug resistance remains unclear. Many investigations have demonstrated that resistance to alkylating agent and platinum is multi-factorial (Lehnert M. 1996), and includes;

a) Altered drug transport and decreasing drug accumulation in cancer cell. Increased expression of the human MDR1 gene product, P-glycoprotein (P-gp) is one mechanism in multidrug resistance. P-gp is an ATP-dependent drug efflux pump that confers resistance to wide variety of naturally derived drugs such as vinca and taxanes, but not platinum drugs (Germann UA. 1996). Overexpression of MDR1 mRNA or P-gp has been observed in a variety of tumours and has also been found to be an indicator of poor prognosis in patients treated with chemotherapy (Goldstein L. 1996). In ovarian cancer, the role of MDR1 expression is still conflicting (Holzmayer TA, et al.1992; Kavallaris M, et al.1996; Zhu L, et al.1997).

LRP, a vault protein, also is associated with multidrug resistance (Scheffer GL, et al.1995). LRP-positive patients had poorer responses to chemotherapy and shorter overall survival as compared to LRP-negative patients (Izquierdo MA, et al. 1995).

b) Increased inactivation of drugs has been demonstrated by detoxification enzymes such as glutathione-S-transferases (GST), or direct binding to nonprotein thiols such as glutathione (GSH). A wide variety of detoxification pathways exist that enable a cell to inactivate cytotoxic drugs. The mechanisms that have received the most attention in mediating drug resistance in ovarian cancer include increased levels of GSH, GSTs transferases, and metallothioneins. Spontaneous conjugation of GSH with melphalan, chlorambucil, and cisplatin has been shown in vitro (Ishikawa T, et al. 1993) and increased levels of GSH have been found to be associated with resistance to alkylating agent and platinum (Britten RA, et al.1991; Godwin A, et al. 1992; Hosking LK, et al.1990). The GSTs, a multigene family which catalyzes the conjugation of GSH with electrophilic drug, have been implicated in resistance to alkylating agent, anthracyclines, and platinum (Meijer C, et al. 1990; Black SM, et al. 1990). The results from studies of GSTs in predicting patient response to chemotherapy indicate that neither tumour GST activity nor expression level is predictive of response to chemotherapy in ovarian cancer patients (Ghazal-Aswad S, et al.1996; Tanner B, et al. 1997).

c) Altered drug target. Decreased expression or alteration of a gene or its product may enable a cell to escape the cytotoxic effect of certain chemotherapeutic agents. Several reports have described alterations in β -tubulin in paclitaxel, which can prevent paclitaxel binding and enable a cell to circumvent the cytotoxic effects of this drug (Dumonter C, et al. 1996; Ranganathan S, et al. 1996). Other drug targets whose alterations are associated with chemotherapeutic drug resistance are the DNA topoisomerases. Decreased expression or mutation of the topoisomerase I and topoisomerase II genes has been implicated in resistance to these drugs in derived drug resistant models in vitro (Eng WK, et al. 1990; Wang LF, et al. 1997).

d) Increased DNA repair activity. Tumour cells may be able to develop DNA repair mechanisms that are more efficient than normal, counteracting the genotoxic effects of cytotoxic chemotherapy, and hence contributing to resistance to DNA damaging drugs. Increased repair of platinum-DNA adducts has been shown to be associated with cisplatin resistance in human ovarian cancer cell lines (Parker RJ, et al. 1991), and evidence for increased repair of cisplatin interstrand crosslinks in specific genes and nongene regions in cisplatin-resistant cells has also been shown (Johnson SW, et al. 1994).

In order to decrease the multidrug resistance in ovarian cancer, some drugs have been used to reverse drug resistance. A Phase I trial of buthionine sulfoximine (BSO) plus melphalan has been completed. BSO can be safely administered to patients at doses that produce an 80% reduction in GSH levels at circulating lymphocytes in the majority of drug-resistant patients (O'Dwyer PJ, et al. 1996). Clinical trials of aphidicolin, an inhibitor of DNA polymerase alpha, have demonstrated that the drug is essentially nontoxic and that the necessary plasma levels may be achievable, (Auersperg N, et al. 1998). Clinical trials of Cyclosporin A (CsA), a drug capable of reversing multidrug-resistant phenotype, have undergone Phase I and Phase II evaluation in ovarian cancer patient, with the CsA/carboplatin regimen showing

modest activity in platinum-resistant patient (Chambers SK, et al.1996;Manetta A, et al.1998).

1.4 Molecular Genetics of Epithelial Ovarian Cancer

1.4.1 Molecular epidemiology and genetic linkage

The molecular study of genes involved in carcinogenesis has opened a new field of epidemiology. It is possible by analysis of genes thought to be important in the aetiology of a particular tumour type to make inferences about causation; the relative balance of inherited to somatic events and whether the somatic events are due to environmental or spontaneous processes.

Although clearly defined predisposing/hereditary factors account for only about 5% of patients with ovarian cancer, as judged by familial clustering, and the aetiology may have multifactorial components, among the factors associated with ovarian cancer, none alters magnitude of risk more than a family history of the disease. The familial aggregation of ovarian cancer has been shown in numerous case series and case-control studies. These studies have illustrated statistically significant increases not only in ovarian cancer, but also breast, endometrium, and colon cancers among relatives of women with a primary ovarian cancer (Jishi MF, et al.1995; Zweemer RP, et al. 1999) (see above). The observation of familial patterns of ovarian cancer has prompted attempts to identify genes involved, and the genes BRCA1 and BRCA2 gene are considered as the principal involved genes

1.4.1.1 The BRCA1 gene and ovarian cancer

The BRCA1 gene was identified by positional cloning methods and found to extend over 100kb of genomic DNA with 24 exons and encode a protein of 1863 amino (Miki Y, et al. 1994). The BRCA1 protein sequence includes an amino-terminal RING domain, a negatively charged region in its carboxy-terminus, and C-terminal sequences, partially homologous to some of terminal deoxynucleotidyl-transferases, and eukaryotic ligases (Callebaut I, et al.1997). The BRCA1 protein also interacts

with hRad51, a member of a protein family known to mediate DNA strand-exchange functions leading to normal recombination (Scully R, et al. 1997). It is expressed in many tissues during development, including thymus, breast and testis (Miki Y, et al. 1994), and is strongly expressed in the epithelial cell layer of the adult ovary but expression appears to be reduced in malignant cells (Thompson et al. 1995). It is also expressed in rapidly proliferating cell types undergoing differentiation, and its expression is induced in oophorectomised animals after treatment with 17 beta-oestradiol and progesterone (Marquis ST, et al. 1995). Up to now, the normal function of BRCA1 is still unclear but may contribute to homologous recombination and DNA repair, to embryonic, to transcriptional regulation and to ubiquitination (Welsh PL, et al.2000). Transfection of breast and ovarian cancer cell lines with wild-type BRCA1 inhibits growth, although this is not the case for colon or lung cancer cell lines, demonstrating a tissue specific effect and BRCA1 gene locates at chr 17q where LOH is a common event in ovarian cancer (Takahashi H, et al 1995; Cornelis RS, et al. 1995). BRCA1 therefore has most of the characteristics of a tumour suppressor gene.

More than 100 mutations in the BRCA1 gene have been described, many of which result in premature truncation of protein transcription (Daly M and Ostrams GI. 1998). It has been estimated that frequency of BRCA1 mutations in the general population is 0.04 % to 0.20 %. However, the 185delAG and 5382insC mutations are present in approximately 1 % and 0.1 % of the Ashkenazi Jewish population (Ford D, et al. 1995).

It is clear that female carriers of BRCA1 mutations are at significantly increased risk of breast and ovarian cancer (Sutcliffe S et al.2000). Using data from families with evidence of linkage to BRCA1, it has been estimated that the lifetime risk of ovarian cancer is 40% to 66 % (Ford D, et al, 1994; Easton DF, et al. 1995;). In the Washington study among Ashkenazi Jews, where only their mutations were assessed, the risk of ovarian cancer among mutation carriers was 16 %by age 70 (Ford D, et al. 1995). The highest risk for ovarian cancer was observed among carriers of the 5382insC mutation, and the lowest for the 185delAG mutation (Yazici H, et al.

2000). Another study estimated the probabilities that a woman with breast or ovarian cancer harbours a BRCA1 mutation and proposes offering DNA testing to women with at least a 10% to 15% likelihood of carrying this mutation (Weber B, et al. 1996). These results were summarised by Weber et al (see Table 1-4)

Table 1-4 Estimated Probability of BRCA1 Mutation Based on Family History (from Weber et al. 1996)

Family history	Probability of BRCA1 mutation (%)
single affected person	
Breast cancer at <30 years of age	12
Breast cancer at <40 years of age	6
Breast cancer at 40 to 49 years of age	3
Ovarian cancer at <50 years of age	7
Sister pairs	
Both with Breast cancer at <40 years of age	37
Both with Breast cancer at 40 to 49 years of age	20
Breast cancer at <50 years of age, ovarian cancer at <40 years of age	46
Both with ovarian cancer at <40 years of age	61
Families	
Breast cancer only, three or more cases at <50 years of age	40
Two or more breast cancers and one or more ovarian cancers	82
Two or more breast cancers and two or more ovarian cancers	91

Differential occurrence of ovarian cancer by site of mutation has been a subject of considerable interest and controversy. Findings from a British study indicate that mutations closer to the 5' end of the gene carry a higher risk of ovarian cancer. Specifically, they found that mutations in the 3' third of the gene are associated with a lower proportion of ovarian cancer (Gayther SA, et al. 1995; Gayther SA, et al. 1997). Although transfection of a 3'BRCA1 mutant does not inhibit the growth of breast cancer cells, it still does inhibit the growth of ovarian cancer cells (Holt JT, et al, 1996). Furthermore, this 3' mutation appears to map to BRCA1's putative granin

consensus sequence (Jensen RA, et al. 1996), and this suggests a possible mechanism for mutational differences involved in tumours of different tissue origins. However, other studies could not confirm this result that the intragenic position of BRCA1 mutations has a significant influence on the ratio of breast to ovarian cancer (Peelen T, et al. 1997; Serova O, et al. 1996; Berman DB, et al. 1996). Data from the Jerusalem series showed an opposite trend for increased rates of ovarian cancer with mutations at the 3' end (Levy-Lahad E, et al. 1997).

It has been demonstrated that oral contraceptive use may reduce the risk of ovarian cancer in women with mutation of BRCA1. In a case-control retrospective study, the use of oral contraceptives for 6 or more years was associated with a 60% reduction in risk of ovarian cancer in women with mutation in BRCA1 (Narod SA, et al. 1998).

1.4.1.2 The BRCA2 gene and ovarian cancer

A second gene, BRCA2, was mapped to Chr 13q12-q13 (Wooster R, et al. 1994), and consists of an 11-12 kb messenger RNA encoded by a 27-exon gene spanning 70kb of DNA. It encodes a 3418 aa protein of about 350kd. The gene has no obvious strong homologies; the mouse and human genes share only about 55% to 60% homology. However, it contains 8 copies of a highly conserved repeat element dispersed throughout the gene (Wooster R, et al. 1995). Although the most recent findings support the involvement of BRCA2 in the repair of DNA, the exact function is as yet unclear (Lynch HT, et al. 1998).

To date, some 100 distinct mutations have been described in BRCA2 and as is the case for BRCA1 these are scattered throughout the coding sequence and apart from several distinct founder mutations (Levy-Lahad E, et al. 1997; Thorlacius S, et al. 1997) there are no specific hot-spots. The most frequent type of BRCA2 mutation is frameshift, most commonly deletions. A recent study showed that the frequency of 3414delTCAG mutations appears to be high in Turkish breast/ovarian cancer families (Balci A, et al. 1999). It has been demonstrated that BRCA2 confers a high risk of breast cancer and ovarian cancer (Osorio A, et al. 1998). The lifetime risk of ovarian cancer in BRCA2 mutation carriers is approximately 20 %, but the risk is

predominantly for women above age 50 (Ligtenberg MJ, et al. 1999). The expression of ovarian cancer was notably lower in carriers of mutations of BRCA2 compared to carriers of BRCA1 mutation (Levy-lahad E, et al.1997).

1.4.2 *Structural genetic alteration in ovarian cancer*

1.4.2.1 *Cytogenetics*

There have been many cytogenetic studies in ovarian cancer (Gallion H, et al. 1990; Deger RB, et al.1997; Park SH, et al.2000), and it is clear that the majority of tumours are aneuploid with complex karyotypic changes (Berchuck a, et al. 1992). Among those cytogenetic lesions that are frequently reported are those involving chromosome 1, 3, 6, 7, 8, 9, 11, 12, 17, 19 and X (Jenkins RB, et al, 1993; Pejovic T, et al. 1992; Taetle R, et al.1999; Lamerz R. 1999). One study of chromosome abnormalities in 244 cases of ovarian adenocarcinoma reported that a total of 201/224 cases had fully characterized clonal chromosome abnormalities, of which 134 showed clonal chromosome breakpoints, and non-random occurrence of chromosome breakpoints was detected at region 1p1, 1q1, 1p2, 1q22, 1q3, 3p1, 1q4, 6q1. 6p2, 6q2, 7p1, 7q1, 7p2, 11p1, 11q1, 11q2, 12p1, 12q2, 13p1, and 19q1, suggesting that recurring, nonrandom chromosome abnormalities are important in the pathogenesis and/or progression of ovarian cancer (Taetle R, et al.1999). A specific translocation involving chromosomes 6 and 14 was reported in 8 of 14 cases of papillary serous adenocarcinoma of ovary (Wake N, et al. 1980). Additional reports have also suggested a role for aberrations of chromosome 6, mainly involving deletions from 6q (Trent J, et al.1985). Cytogenetic analysis also has demonstrated partial deletions of Chr 11 affecting both long and short arms (Jenkins RB, et al, 1993; Bello JA, et al, 1990; Pejovic T, et al, 1992).

In-situ hybridisation methods provide refinement to the cytogenetic data, Sonoda et al, observed that most carcinoma specimens displayed numerous imbalances. The most common overlapping sites of copy number increases, in order of frequency, were 8q24, 20q13.2-qter, 3q26.3, 1q32, 20p, 9p21-pter, 12p, and 5p14-pter. DNA amplification was identified in nearly 50 % of the tumours examined. The most

frequent sites of amplification were 8q24, 3q26.3, and 20q13.3, while other recurrent sites of amplification included 7q36, 17q25, and 19q13.1-13.2. The most frequent changes could reflect the amplification of target regions containing genes such as *c-myc* at 8q24, *MYBL2* at 20q13.1, *EVII* at 3q26 (Sonoda G, et al. 1997). One study has shown that copy number abnormalities were more frequent in high-grade ovarian cancer and not present in any of the benign tumours of ovary. In only 31 % cases overall was there concordance between LOH for a chromosome arm and physical deletion as judged by comparative genomic hybridisation (CGH). This suggested that flanking regions which do not show LOH must contribute to the discrepancy between published LOH and CGH analyses of the same regions and demonstrates that concordance occurs mainly where contiguous LOH is long enough to approach the limits of resolution defined by CGH. Of the most frequent 18 copy number abnormalities detected, 13 were instances of increased copy number. The 6 most frequent observable CGH reduction of copy number were 17 pter-q21, 16q, 8p21-23, Xp and whole 19 (Iwabuchi H, et al. 1995). In another study, genetic abnormalities were detected by comparative genomic hybridization (CGH) in 12 ovarian clear cell adenocarcinomas. DNA sequence copy number abnormalities (CNAs) occurred in more than 20% of the cancers. Increases in copy numbers of 8q11-q13, 8q21-q22, 8q23 and 8q24-qter occurred more frequently in disease-free patients than in recurrent/non-surviving patients. However, increases in copy numbers of 17q25-qter and 20q13-qter occurred more frequently in recurrent/non-surviving patients than in disease-free patients. This suggested changes in specific chromosome copy number associated with progression of disease (Suehiro Y, et al. 2000).

1.4.2.2 *Loss of heterozygosity (LOH) in ovarian cancer*

Although LOH does not provide direct proof of a tumour suppressor gene, a high frequency of allele loss in a specific region of a chromosome in a tumour type indicates the possible presence of a tumour suppressor gene or genes. Ovarian tumours have been analysed for LOH across the genome and a number of hotspots for allele loss identified on different chromosomes. However, there are many

ambiguities and difficulties in the interpretation of LOH data. First of all, many of the studies have analyzed only small numbers of tumours and these may or may not have included some benign or borderline tumours. Second, because some authors have used microdissected tumour tissue for LOH analysis, if a sample contain a high percentage of contaminating stromal tissue, LOH in the tumour cells may be masked. Third, there is often considerable variation in composition of the tumour with respect to histological subtype, tumour stage and grade, all of which might be expected to influence the outcome of any LOH analysis. Finally, many studies use only one or two polymorphic markers per chromosome arm and there is often great variation between studies in the marker used. Direct comparisons between studies are therefore very difficult and often lead to conflicting and confusing results.

To date, more than 100 LOH studies have looked at epithelial ovarian cancer, and allelic losses for polymorphic DNA markers in epithelial ovarian cancer have been observed on every chromosomal arm (Lynch HT, et al. 1998). Allelotyping studies of ovarian cancers revealed that more than 30% of the tumours studied showed LOH on chromosomes 1p, 3p, 5q, 6,7 9, 8p, 11, 16q, 17, 18q 21q, 22q, and X (Wertheim I, et al.1996; Zborovskaya I, et al. 1999; Huang LW, et al; 2000), with the highest LOH rates on 17p, 17q, 22q, 18q, 8p, 9q, 1p, 9p, 7q, Xp, 6q, 5q, 11q, 13q, 19p, 21q, 3p, 6p, 11p, and 7p, in descending order (Lynch HT, et al. 1998; Zborovskaya I, et al. 1999; Huang LW, et al. 2000). LOH of >60 % has been reported for 11p, 11q, 17p, 17q, 18q, and 22q (Zborovskaya I, et al. 1999).

The relationship between LOH and specific tumour suppressor genes in epithelial ovarian cancer has also been reported. The establishment of linkage to chromosome 17q21 in families with an inherited predisposition to early onset breast and ovarian cancer suggested initially that the high rates of LOH from 17q in sporadic tumours may reflect the inactivation of BRCA1. However, with more detailed linkage analysis in families, extensive deletion mapping in sporadic tumour, and mutation analysis in BRCA1, the results show somatic mutations of BRCA1 are rare in sporadic tumours and they may not play a major role in tumorigenesis in sporadic breast and ovarian cancer (Futreal PA, et al. 1994; Merajver SD, et al, 1995; Sorlie T, et al.1998).

Recent studies showed that aberrant cytosine methylation of the BRCA1 promoter was directly correlated with decreased levels of BRCA1 expression in human breast and ovarian cancer (Baldwin RL, et al. 2000; Bianco T, et al. 2000). BRCA1 methylation was strikingly associated with the medullary and mucinous subtypes of breast cancer, which are overrepresented in BRCA1 families. In ovarian neoplasms, BRCA1 methylation was found only in tumours with LOH. The BRCA1 promoter was unmethylated in other tumour types. These data suggest that epigenetic silencing of the BRCA1 may be one mechanism of transcriptional inactivation of BRCA1 in sporadic breast and ovarian carcinogenesis (Esteller M, et al. 2000)

In other studies, investigators have found that approximately 30 % of the ovarian tumour they examined showed changes affecting p53 genes, (Tsao SW, et al. 1991; Kohler MF, et al. 1993), mutation of p53 on 17q13.1 is one of the commonest genetic alterations described thus far in epithelial ovarian carcinoma (approximately 50 %) (Kupryjanczyk J, et al.1995). LOH from the short arm of chromosome 17 has often been assumed to represent inactivation of the p53 gene at 17p13.1. However, losses at 17p13.3 were demonstrated in Stage I ovarian cancers and borderline tumours. In the latter case, the LOH at 17p13.3 were not accompanied by LOH at p53 (Phillips NJ, et al. 1996). Recently a study showed that absent over-expression of p53 was significantly associated with LOH at 17q in ovarian carcinoma (Caduff RF, et al. 1999).

Mutation and expression of another tumour suppressor gene, p16, have also been studied in ovarian cancer (Rodabaugh KJ, et al. 1995). Despite the identification of 50 % homozygous deletion rate in ovarian cancer cell lines, neither mutation nor abnormal expression of the p16 gene has been found in ovarian tumour tissues (Shigemasa K, et al.1997). Recently, the studies have shown that p16 is frequently methylated in ovarian cancer. Therefore, p16 silencing may be important for the development of ovarian carcinomas (McCluskey LL, et al. 1999; Wong YF, et al. 1999).

1.4.3; Alterations in tumour suppressor genes, oncogenes, and growth factors of ovarian cancer

1.4.3.1 p53 in ovarian cancer

The p53 tumour suppressor gene is the most frequently inactivated gene described thus far in ovarian cancer. It encodes a 53-kd nuclear phosphoprotein that is expressed at low levels in virtually all normal cells, and is composed of 11 exons, the first of which is non-coding and localised 8 to 10 kb upstream of exons 2 through 11 on the short arm of human chromosome 17. There are five conserved domains within the coding regions of the gene, which contain 80 % to 90 % of described functional p53 mutations. The functions of normal or wild-type p53 have been shown to be involved in 1) DNA damage response pathways; 2) cell-cycle arrest at the G1-S checkpoint before DNA replication and at the G2-M checkpoint prior to mitosis (facilitates DNA damage repair and prevents mutation and aneuploidy, respectively), and 3) inducing apoptosis in cases where irreparable DNA damage has occurred (Lee G, et al. 1995; Siddik ZH, et al. 1998; Kupryjanczyk J, et al. 2000).

Functional p53 mutation usually affects trans-activation domains. More than 90 % of the substitution mutations reported so far in malignant tumours are clustered between exons 5 and exons 8 and most are localised in the evolutionarily conserved regions. Most p53 mutants divide into two classes: 1) DNA contact mutants; that are unable to bind specific DNA target sequences because of substitution of crucial DNA residues, including mutational hot spots; 2) structural mutants; that lose specific protein function. Central core mutations are the main mutations affecting trans-activation. It has been suggested that greater than 60 % loss of p53 function is needed to result in a tendency towards neoplastic transformation or growth (Pfeifer GP. 2000; Spandidos DA, et al. 2000; Wallace-Brodeur RR and Lowe SW. 1999).

Mutation of p53 is observed in more than 50% of ovarian cancer, and is observed at multiple structural sites, but there is no single site or codon that is distinctive or unique to ovarian cancer (Kohler MF, et al. 1993). Over-expression of p53 has not been reported in borderline tumours of the ovary and appears to be correlated with an

increasing histologic grade, and it is clear that the frequency of over-expression of mutant p53 is significantly higher in advanced stage III/IV disease relative to stage I cases (Berchuck A, et al. 1994). Therefore, it is possible that loss of p53 may confer an aggressive phenotype associated with more rapid progression. However, the prognostic significance of p53 expression in ovarian cancer remains to be fully determined, although some studies suggest that over-expression of p53 is associated with an unfavourable outcome. In the largest study, investigators examined p53 expression in 284 patients with epithelial ovarian cancer and detected p53 immunoreactivity in 177 (62 %). In a univariate analysis, but not in a multivariate analysis, p53 expression was associated with decreased survival. In a subset of patients with early-stage disease, p53 expression approached statistical significance as an independent prognostic factor (Hartmann LC, et al. 1994). Recently, one study also showed that the p53 mutation frequently occurs in recurrent ovarian cancer and that alteration of p53 status affects salvage chemotherapy and may therefore predict outcome (Irie T, et al. 2000).

It has been suggested that loss of functional p53 might confer a chemoresistant phenotype because p53 plays a role in chemotherapy-induced apoptosis. In this regard, several studies have examined the correlation between chemosensitivity and p53 mutation in ovarian cancers in vitro. Some have suggested a relationship between p53 mutation and loss of chemosensitivity, but in other equally valid studies, such a relationship has not been observed (Brown R, et al.1993; Eliopoulos AG, et al.1995; Righetti SC, et al. 1996; Perego P, et al. 1996). It is likely that the status of the p53 gene is just one of a multitude of factors that determines chemosensitivity.

1.4.3.2 *Alteration of oncogenes in ovarian cancer*

The HER-2/neu tyrosine kinase is a member of a family of related transmembrane receptors, which encode a 185 kd transmembrane receptor that is highly homologous with the EGF receptor (Tzahar E, et al.1998). Over-expression of HER-2/neu has been reported in 26 % of human ovarian cancer and has been shown to be associated with poor prognosis in some studies (Berchuck A, et al.1990; Slamon DJ, et al.1989)

but not all studies (Kacinski BM, et al. 1992; Rubin SC, et al.1993). Amplification of HER-2/neu has been demonstrated to be significantly associated with particular histological cell types and higher histological grade (Seki A, et al. 2000). Some studies also showed that over-expression of HER-2/neu may contribute to drug-resistance (Yu D, et al. 1998) and poor response to chemotherapy (Yu D, et al. 2000; Nijman HW, et al.1999). It has been shown that transfection of HER-2/neu into normal ovarian epithelial cells can induce a malignant phenotype in vitro including the ability of cells to grow in an anchorage-independent fashion and to form tumours in nude mice. Conversely, monoclonal antibodies that interact with HER-2/neu can decrease growth of breast or ovarian cancer cell lines that over-express this receptor (Pietras RJ, et al.1998; Rodriguez GC, et al. 1993). Introduction of the viral E1 gene downregulates HER-2/neu expression and inhibits growth of ovarian cancer cell lines that over-express HER-2/neu, and also increases sensitivity to the cytotoxic effect of paclitaxel (Yu D, et al. 1998).

Ras genes encode 21-kd proteins that localize to the inner aspect of the cell membrane and have intrinsic GTPase activity. It is estimated that point mutations in Ras genes are present in about 1/3 of cancers (Kinzler KW and Vogelstein B. 1997). In human cancer, activating mutations in Ras are observed at codons 12, 13, or 16. The encoded amino acids at these three locations appear to play a critical structural role in the active site of Ras, such that missense mutations at one of these codons destroys the ability of Ras to correct GTP to GDP, which then chronically transmits its growth stimulatory signal. In epithelial ovarian cancer, the most frequently observed ras-mutation is that of K-ras codon 12. The incidence of K-ras mutations at codon 12 is 23 % to 63 % (Cuatrecasas M, et al. 1997; Suzuki M, et al. 2000), and associates with mucinous ovarian cancer (about 50 %) (Suzuki M, et al. 2000), and correlated with the malignant potential of the neoplasms (Varras MN, et al. 1999) K-ras mutations are also common in borderline epithelial ovarian tumours, occurring in 20 % to 50 % cases (Mok SC, et al. 1993). This suggests that K-ras mutational activation is an early event in ovarian tumorigenesis.

The alpha 110Kd subunit of phosphatidy inositol 3 kinase (PI3 kinase) is amplified in at least 80 % of ovarian cancer, and associated with increased kinase activity. Inhibition of kinase activity can slow growth of ovarian cancer cell lines, and consistent with the possibility that signalling through this pathway is important for regulation of cell proliferation and/or apoptosis (Shayesteh L, et al. 1999). Recently, the study also showed that PI3 kinase antagonists could inhibit cell growth and tumorigenicity in human cancer cell lines (Razzini G, et al. 2000). The AKT serine-threonine kinases are activated by the products of PI3 kinase and can inhibit apoptosis by phosphorylating BAD and/or caspase 9, and amplification or over-expression of AKT occurs in 14.4 % of ovarian cancers. Furthermore, a significant correlation was observed between amplification and over-expression of AKT and undifferentiated histology and median survival of patients (Bellacosa A, et al.1995). Amplification of the C-myc oncogene occurs in some epithelial ovarian cancers. C-myc was reported to be amplified in 31 % of epithelial ovarian cancers (Aunoble B, et al. 2000). In a recent study in which 51 epithelial ovarian cancers were analysed, a similar incidence of C-myc over-expression was observed (37 %). In this study, C-myc over-expression was more frequently observed in advanced stage serous cancer (Tashiro H, et al. 1999).

1.4.3.3 *Alteration of growth factors in ovarian cancer*

Growth of ovarian cancer can be stimulated by several peptide and lipid growth factors. Peptide ligands that bind to the epidermal growth factor receptor (EGFR) are produced by ovarian cancers including EGF, transforming growth factor alpha (TGF- α), and amphiregulin (Stromberg K, et al. 1994). Three factors have been identified in ovarian tumours and cancer cell lines. TGF- α is reported to be present in 50 % to 100 %, EGF in 28 % to 71 % and amphiregulin in 18 % of malignant ovarian tumours (Bauknecht T, et al. 1990; Morishige K, et al. 1991; Stromberg K, et al.1994). TGF- α and EGF stimulate the growth of ovarian cancer cell lines in vitro, indicating that these factors are mitogenic for this disease (Morishige K, et al. 1991; Crew AJ, et al. 1992). Antibodies against TGF- α can inhibit the growth of ovarian cancer cell lines

that continue to express EGFR, consistent with autocrine growth stimulation (Stromberg K, et al.1992).

The transforming growth factor- β (TGF- β) is involved in cell growth regulation, tissue remodelling, angiogenesis and immune suppression. Three forms of TGF- β have been identified in human systems, TGF- β 1, 2 and 3. TGF- β can inhibit proliferation of normal ovarian surface epithelial (OSE) cells (Henriksen R, et al. 1995). During malignant transformation, expression of TGF- β is lost in 40 %, interrupting potential autocrine growth inhibition (Hurteau JA, et al. 1999). In different cancer cell lines that have been established in culture, exogenous TGF- β can inhibit, fail to affect or even stimulate growth (Berchuck A, et al. 1992). When ovarian cancer cells are isolated directly from ascites fluid, growth can still be inhibited with TGF- β in more than 90 % of specimens (Havrilesky LJ, et al.1995). In primary ovarian cancer, mRNA for three isoforms, TGF- β 1, 2, and 3 has been detected in 46 %, 66 % and 66 % respectively of malignant tumours, the predominant pattern of expression being either dual or triple co-expression (Bartlett JM, et al. 1997). The TGF- β 2 receptor was present in over 90 % of samples. Patterns of expression were similar between malignant, borderline and benign tumours. TGF- β 3 was associated with advanced stage and reduced survival, suggesting that perhaps the influence of this factor on angiogenesis and other features of tumour progression are more significant than direct inhibitory effects on growth (Bartlett et al. 1997). In support of this, an association between TGF- β expression and features of angiogenesis in ovarian tumours has been identified. (Nakanishi Y, et al. 1997).

1.4.4 Attachment Invasion, metastasis and angiogenesis

Although the mechanisms and regulation of metastasis are still unclear, the complex process of invasion consists of multiple steps. These steps mainly include that during tumour progression, tumour cells detach from the primary tumour and then attach to underlying basement membrane, invade the local stroma, degrading the extracellular matrix components, and move into adjacent blood vessels. Then neoplastic cells circulate in the bloodstream until they become attached to the wall of a blood vessel.

This is followed by secretion of proteases to generate access through the vessel wall, after which cells invade surrounding tissues, where they proliferate and form a new tumour mass. In ovarian cancer, locoregional peritoneal dissemination rather than blood-borne metastasis represents the major problem in tumour progression

1.4.4.1 *Tumour cell detachment and adhesion*

In order to invade, a tumour cell first has to detach from its neighbour and then attach to the underlying basement membrane. During the invasive cascade, both cell-cell and/or cell-stroma interactions play an important role. Connection through cell adhesion molecules, integrin, and cadherins stabilize tissue integrity, whereas loss or alteration of these cell surface proteins has been shown to be associated with increased metastatic potential (Varner JA, et al. 1996). Cell polarity and organization during spreading and migration are regulated by cell interaction with extracellular matrix proteins through the integrin family, and with other cells through the cadherins. Activation of these factors passes signals from the outside into the cell and thus directs cell behaviour.

Cadherins are transmembrane glycoproteins that mediate extracellular calcium-dependent cell interaction and are involved in epithelial cell-cell communication. In one study, a decrease of E-cadherin expression in ovarian surface epithelial cells caused a reduction in cell adhesiveness during progression of the transformation of ovarian surface epithelium to ovarian carcinoma (Hoffman AG, et al. 1993). E-cadherin has also been demonstrated to function as a metastasis suppressor molecule in several cell lines (Semb H, et al. 1998; Vleminckx K, et al. 1991).

Integrins are heterodimeric protein complexes, which link cytoskeletal microfilaments (e.g. Actin, vinculin and talin) to extracellular matrix protein such as laminin, fibronectin, collagen and vitronectin. Integrins mediate their binding through a specific tripeptide motif, and this tripeptide has been shown to inhibit tumour cell invasion in-vitro and in-vivo. A balance of integrin expression is thought to be required for optimal invasiveness of tumour cell. Integrin under-expression does not allow tumour cells to adequately attach to the basement membrane (Aznavoorian S, et al. 1993).

Similarly, integrin over-expression may inhibit the capacity of tumour-cells to dis-attach from the basement membrane and therefore render those cells less invasive (Giancotti FG and Ruoslahti E. 1990). until now, integrins have not only been identified as cell adhesion molecules but also are recognized as signalling molecules for regulation of apoptosis (Meredith JB Jr, et al.1996; Kheradmand F, et al. 1998), gene expression (Lafrenie RM and Yamada KM. 1998), cell proliferation (Porter JC and Hogg N. 1998), invasion and metastasis (Joseph-Silverstein J and Silverstein RL.1998), and angiogenesis (Friedlander M, et al.1995).

Over expression of a specific 67kD laminin receptor (67LR) has shown to be important for cancer cell adhesion to and invasion into the extracellular matrix (Liotta LA, et al. 1984) and also with progression of various cancers (Pellegrini R, et al. 1995). Expression of 67LR has been shown to be increased in patients with progressive ovarian cancer (van den Brule FA.1996).

1.4.4.2 *Basement membrane degradation by tumour cells*

To be successful with the invasion process, tumour cells have to secrete enzymes that degrade the extracellular matrix barriers. Overexpression of one or more of these enzymes has been shown to occur in almost all cells of the tumour-host environment. Degradation of the basement membrane is not dependent solely on the amount of the proteolytic enzymes present, but on the balance of activated proteases and their naturally occurring inhibitors (Liotta LA, et al.1991). A positive correlation with tumor aggressiveness has been shown for a variety of degradative enzymes, including heparanase, and seryl-, thiol-, and metal-dependent enzymes (Price JT, et al. 1997).

Many investigations have shown that matrix metalloproteases (MMPs) play an important role in tumour cell invasion across the basement membrane (Huang LW, et al.2000). MMPs are a family of neutral enzymes secreted as latent proenzymes. There are five subclasses grouped according to substrate specificity: interstitial collagenases, gelatinases, stromelysins, membrane type-MMPs, and elastases. Increased MMP activity has been detected and shown to correlate with invasion and metastatic potential in a wide range of cancer, including ovarian epithelial cancer (Liotta

LA, et al.1991). Fishman et al showed that epithelial ovarian carcinoma cells derived from primary ovarian tumours, metastatic lesions, or ascites overexpressed MMP-2 and MMP-9 (Fishman DA, et al.1997). The results of another study showed that MMP-9 expression in tumour cells was found to be significantly enhanced in serous and mucinous ovarian carcinomas compared with benign and borderline tumours (Huang LW, et al.2000). MMP-7 was also found to be overexpressed frequently in mucinous ovarian cancer (Shigemasa K, et al.2000). MMPs are inhibited by a family of five endogenous inhibitors known as the tissue inhibitors of metalloproteases (TIMPs) (Gomez DE, et al.1997). The balance between the levels of activated MMP and free TIMPs determines the balance between matrix degradation and matrix formation. Altering this equilibrium affects the progression of the invasive phenotype. It has been shown that TIMP-2 can inhibit bFGF-induced stimulation of endothelial cell proliferation independent of its ability to inhibit MMP activity (Murphy AN, et al. 1993). MMPs and their inhibitors have been demonstrated to be present in most epithelial ovarian carcinoma and molar ratios of TIMP-1/MMP-9 and TIMP-2/MMP-2 were higher in the adenoma category (Furuya M, et al.2000). TIMP-1 and TIMP-2 have been shown to inhibit tumour-induced angiogenesis in experimental systems (Gomez DE, al.1997).

Plasminogen activators (PAs) are serine-specific proteases that convert inactive plasminogen to active plasmin, a trypsin-like enzyme that degrades a variety of proteins, including fibrin, fibronectin, type IV collagen, vitronectin, and laminin. Plasminogen activator exists in two forms: tissue-type plasminogen activator (tPA), the primary plasminogen activator in plasma, and urokinase plasminogen activator (uPA) uPA is involved primarily in cell-mediated proteolysis during macrophage invasion, wound healing, invasiveness, and metastasis (Conese M and Blasi F. 1995). Production of uPA in normal ovarian epithelial cells was reported as 17-to 38-fold lower than that found in ovarian carcinoma cells (Moser TL, et al.1994). Pedersen et al demonstrated increased uPA-receptor expression in ovarian tumours associated with short overall survival of patients (Pedersen H, et al. 1994). The interaction of uPA and its receptor plays an important role in direct and indirect extracellular matrix

degradation, thus potentiating invasive events (Miyake H, et al. 1999). The action of uPA can be counteracted by several naturally occurring inhibitors of the uPA/plasmin system, such as plasminogen activator inhibitor-1 (PAI-1) and plasminogen activator inhibitor-2 (PAI-2). Elevated expression of uPA and its inhibitor (PAI-1) in tumour extracts of ovarian cancer has been associated with increased invasion, increased incidence of relapse, and shorter overall survival (Schmitt M. et al. 1995). Furthermore, several studies have shown a correlation in which both uPA and PAI-2 levels were associated with a poor prognosis in advanced ovarian cancer, whereas higher levels of PAI-2 only was associated with better prognosis and less disseminated disease (Chambers SK, et al. 1995).

1.4.4.3 *Tumour cell migration*

The process of tumour cell migration is very complex. Studies has shown that proteolysis alone cannot be responsible for the entire invasion cascade. Therefore after degrading the basement membrane, the tumour cell must actively migrate to the adjacent tissue compartment that it will occupy. Cell motility begins with polarisation and orientation of the cell followed by formation of pseudopodia, a membrane extension, at the cell's leading edge. The pseudopodium then forms attachments with the ECM and produces a contractile force to move the cell forward by a series of attachment and dis-attachment steps including cellular receptors that bind the matrix, and specific signals that give direction and urgency to migration, including extracellular matrix components, growth factors, scatter factors, and tumour-secreted factors. Examples of growth factors that stimulate tumour cell motility include the insulin-like growth factors (IGF-I, -II), hepatocyte growth factor (HGF), and the fibroblast growth factors. IGF-I has been shown to induce a chemotactic response in human ovarian cancer (Kohn EC, et al.1990). HGF is a paracrine motility factor that acts at picomolar concentrations to stimulate motility of epithelial cells (Stoker M, et al.1991). The cell surface-associated ectokinase, autoxin (ATX), a potent motility-stimulating glycoprotein, may stimulate motility directly by binding a cell surface receptor (Clair T, et al.1997). Furthermore, some of

tumour cells have to survive the stage of vascular transport and arrest in capillary beds of distant organs to engage in a second round of invasion and extravasation, whereby neoplastic cells exit from the vessel lumen into the surrounding stromal tissue (Quigley JP, et al. 1998).

1.4.4.4 *Angiogenesis*

Angiogenesis, the formation of new blood vessels from a pre-existing vascular network, has been shown to be essential to tumour survival (Folkman J. 1992). Angiogenesis is a multistep process. It involves endothelial cell vascular sprouting, these sprouts then elongate and invade into local stroma toward the tumour. Furthermore, these neovessels develop lumens and connect with existing vascular networks as functional capillaries. Angiogenesis of tumour has correlated with progression-free survival, overall survival, and the presence of locoregional dissemination in ovarian cancer (Paley PJ, et al. 1997).

Angiogenesis of tumour is regulated tightly by a network of inducing and inhibiting factors under physiologic conditions, whereas in pathologic condition, such regulation is altered or absent. The switch of endothelial cells from quiescence to rapid growth is regulated by angiostimulatory and angiostatic signals (Hanahan. 1997). Numerous angiostimulatory or angiostatic factors have been identified.

Vascular endothelial growth factor (VEGF) is the most notable angiostimulatory factor. VEGF is a growth factor that stimulates vascular permeability (Ferrara N. 1995). The functions of VEGF are mainly to induce capillary tube formation; increase vascular permeability, and stimulate endothelial cell migration (Ferrara N et al. 1992). VEGF expression is upregulated in ovarian cancer (Paley PJ, et al. 1997) it has been shown that VEGF is expressed selectively in tumour cells but not in endothelial cells, indicating that VEGF is a paracrine mediator of angiogenesis. Many studies have also demonstrated that there are significant associations between the VEGF expression and invasion, metastasis and angiogenesis in ovarian epithelial cancer, and its expression reflects tumour progression (Gadducci A, et al.1999; Shen GH, et al.2000).

1.5 The Role of Chromosome 11 in Epithelial Ovarian Cancer

The studies have demonstrated that the disruption of multiple genetic loci is associated with epithelial ovarian cancer, and LOH on a chromosome can suggest the presence of a tumour suppressor gene (TSG) at that locus. LOH in epithelial ovarian cancer has been observed on every chromosomal arm (Lynch HT, et al. 1998). The studies of LOH of chromosome 11 have shown that LOH of chromosome is associated with the progression and poor prognosis of ovarian cancer, therefore, there may be important TSGs involved in the progression of ovarian cancer on chromosome 11 (Launonen V, et al.1998; Gabra H, et al. 1996).

Studies of chromosome 11 microcell-mediated transfer have demonstrated that normal chromosome 11 can inhibit the growth of cancer cell (O'Briant K, et al. 1997; Kugoh H, et al. 2000). O'Briant et al used microcell-mediated transfer human chromosome 11 and 11p into a human lung adenocarcinoma cell line and found that all four chromosome 11-containing hybrid clones showed significantly reduced tumorigenicity in nude mice and growth in liquid culture (O'Briant K, et al. 1997). Kugoh et al also reported that human bladder carcinoma cell line that contained chromosome 11 by using microcell-mediated transfer showed a remarkable change in cell morphology. They flattened and ceased growing, or senesced, prior to population doublings (Kugoh H, et al. 2000). These results support the hypothesis that chromosome 11 contains one or more tumour suppressor genes.

In ovarian cancer, Rimessi and colleagues transferred chromosomes 3 and 11 into ovarian carcinoma cell line HEY. The result showed that transfer of chromosome 3 induced senescence and growth arrest as well as suppression of tumorigenicity. But the effect of chromosome 11 introduction into HEY cells was markedly different. Cellular morphology and the immortalised phenotype were unaltered. However, the in vitro growth rate and clonogenicity in soft agar were reduced. Tumorigenicity was not consistently controlled by chromosome 11 transfer (Rimessi P, et al.1994). Although this study downgrade the role of chromosome 11 since it does not suppress tumorigenicity in the nude mouse assay, the pattern that Rimessi's group and

O'Briant's group observed in lung cancer cell lines might, in fact, be quite consistent with the action of a late-acting tumour progression suppressor which although it does not determine fundamental neoplasia, has more subtle effects not easily detected using the crude assays available, perhaps reflected here in altered growth rate and cloning efficiency, but nevertheless a powerful determinant of tumour progression.

Recently, YACs, one containing the candidate gene ATM at 11q23.1, and two contiguous YACs overlying most of the 11q25 deleted region, were used to transfect into murine A9 fibrosarcoma cells. Selected transfectant clones were assayed for in vivo tumorigenicity in c-nu/nu mice. All the 11q YAC transfectant clones demonstrated significant tumour suppression compared to the control (Koreth J, et al. 1999) these results define tumour suppressor loci on chromosome 11q by functional complementation.

1.5.1 Loss of heterozygosity of chromosome 11p

Although no specific tumour suppressor genes for sporadic epithelial ovarian cancer have been cloned, LOH studies have implicated a number of chromosomal sites as regions harbouring potential tumour suppressor genes. Loss of normal TSGs may allow for the expression of tumorigenicity or lead to tumour progression.

The frequency of LOH at chromosome 11p in ovarian cancer has been shown to peak at chromosome 11p15 (Lu KH, et al. 1997; Weitzel JN, et al.1994; Kiechle-Schwarz M, et al.1993; Viel A, et al. 1992; Zborovskaya I, et al. 1999; Eccles DM, et al. 1992; Gallion HH, et al. 1992; Zheng JP, et al. 1991; Lee JH, et al. 1990), although not all of these investigations have demonstrated this high level of loss (Zheng JP, et al. 1991; Kiechle-Schwarz M, et al.1993). Detailed mapping of this region identified two separate minimal deleted regions in nonmucinous ovarian carcinomas. An 11-cM region on chromosome 11p15.5-15.3 extending from D11S2070 to D11S988 including the HRAS locus and a 4-cM region on 11p15.1, also defined by the marker D11S1310. The frequency of LOH in these two regions are 43 % and 32 % respectively, with an overall 39 % LOH for both regions (Lu KH, et al. 1997), suggesting two novel candidate TSG loci. The proximal loci at 11p13 exhibits lower

levels of LOH in ovarian cancer (Bruening W, et al. 1993; Vandamme B, et al. 1992; Viel A, et al. 1992). Most of the studies have shown a significant correlation of chr 11p LOH with poorly differentiated (Lu KH, et al. 1997; Kiechle-Schwarz M, et al. 1993; Zheng JP, et al. 1991) and more advanced (Viel A, et al. 1992) ovarian cancer. Lu et al also reported that detection of 11p15.1 is strongly associated with high-grade nonmucinous epithelial ovarian cancer (Lu KH, et al. 1997). Table 1-5 outlines the major studies that have defined regions of LOH of chromosome 11p in ovarian cancer.

Table 1-5 Ovarian Cancer Common LOH Regions on Chromosome 11p

chromosome site	loci	% LOH
11p15	T24-C3	35
11p15	HINS214	29
11p15	HRAS	38
11p15.1	D11S1310	32
11p15.2	PTH	31
11p15.4	CALC	37
11p15.5-15.3	D11S2070- D11S988	43
11p15.5	HBD /INH	30
11p13	S17/FSH/S16/CAT	29

Many candidate genes have been identified within these regions and the relationship between the LOH of chromosome 11p15.5 and 11p15.1 and some candidate tumour suppressor genes have been investigated. For example, the TSG101 gene, a proposed tumour suppressor gene located on Chr 11p15.1, has been investigated in ovarian cancer. Despite 32 % LOH in this region, the study's conclusion was that there was no evidence to suggest that inactivation of this putative tumour suppressor gene played a role in the pathogenesis of ovarian cancers (Carney ME, et al. 1998). The major literature about candidate genes within these regions as well as relationship with ovarian cancer is summarised in Table 1-6

Table 1-6 Candidate Genes within Chr 11p15.5 or 11p15.1 and The Relationship with Ovarian Cancer

Gene	Location at 11p	Major function	Relationship with ovarian cancer
p57	15.5	Blocks the growth of human astrocytomas and induces cell senescence(Tsugu A.,et al.2000)	No study in ovarian cancer
SSSA/RO52	15.5	Putative tumour suppressor gene in lung cancer (Kim YC, et al.2000)	No study in ovarian cancer
H19	15.5	Putative growth suppressor gene regulating IGF-II (Feinberg AP. 1999)	Express and LOH in the majority of serous epithelial tumours. no evidence to show tumour suppressor activity in ovarian cancer (Tanos V, et al.1999; Kim HT, et al. 1998)
TSSC1/TSSC3 TSSC4/TSSC6	15.5	Tumour suppressing subtransferable candidates(Hu RJ, et al. 1997)	No study in ovarian cancer
IGF2	15.5	Autocrine regulator of cell proliferation (Muller S, et al 2000	Loss of IGF-II associating with advanced ovarian cancer (.Chen CL, et al. 2000)
SPN	15.5	Suppressing cell cycle entry, and inhibiting cell proferation(Zang X, et al.1999)	No study in ovarian cancer
MTR1	15.5	Homology to MLN1, which is downregulation in melanoma (Prawitt D,et al.2000)	No study in ovarian cancer
p27/KIP	15.5	Cyclin dependent kinase inhibitor negative regulator of cell proliferation (Desdouets C and Brechot C. 2000)	Frequent downregulation and associated with worse prognosis (Sui L, et al.1999)
TSG101	15.1	Putative tumour suppressor gene (Carney ME, et al. 1998)	No evidence to suggest that loss of this putative tumour suppressor gene play a role in pathogenesis of ovarian cancers (Carney ME, et al.1998).
CC3	15.1	A gene associated with	No study in ovarian

		metastasis suppression. (Baker ME, et al.2000)	cancer
CLP	15.1	Cardiac LIM protein, regulating growth inhibition (Charpentier E, et al. 2000	No study in ovarian cancer
KAI-1	15.2	Putative metastasis suppressor in prostate cancer (Maraj BH, et al. 2000)	Frequent downregulation and lack of mutation in ovarian cancer (Liu FS,et al.2000)
WT1	13	Responsible for tumour suppress in Wilms tumor (OjiY,etal.1999)	No mutation in ovarian cancer(Bruening W, et al.1993)

1.5.2 Loss of heterozygosity of chromosome 11q

Although proximal allele imbalance at 11q13 has been reported for ovarian cancer, the frequency of LOH at 11q13 showed low levels, about 14 % to 25 % (Foulkes WD, et al.1993; Weitzel JN, et al. 1994; Emmert-Buck MR, et al. 1997), suggesting that LOH at this region is less important in ovarian cancer.

Distal allele imbalance at 11q in ovarian cancer was first reported by Foulkes et al at 1993 (Foulkes WD, et al.1993). Since then, other investigators have also reported LOH at distal 11q in ovarian cancer. A notable feature of these studies was the definition of several regions of LOH with the interval 11q22-q25 (Launonen V, et al.1998; Watson RH, et al.1997; Gabra H, et al.1996; Davis M, et al. 1996; Foulkes WD, et al.1993). These results are outlined in Table 1- 7

Table 1-7 Ovarian Cancer Common LOH Regions on Distal Chromosome 11q

chromosome site	loci	% LOH
11q22-23.3	S85/STMT-1/S144/S29 /CD3D /S147	40
11q24.1	S147	67
11q22-23.2	D11S35-D11S933	42
11q23.3-24.3	D11S934-D11S1330	65
11q23.3-qter	D11S925-D11S1336	54
	D11S912- D11S439	49
11q23.3-24.1	D11S1336-D11S1328	34
11q22.3-25	D11S1340-D11S912	61

Furthermore, the relationship between LOH of chromosome 11q22-25 and clinical significance has been investigated. The analysis of LOH from the 11q22-q25 region between D11S35 and D11S968 in 40 ovarian tumours revealed that there were two distinct regions of loss, a large centromeric region between D11S35 and D11S933 (11q22-q23.3) and a telomeric 8.5-Mb region lying between D11S934 and D11S1320 (11q23.3-24.3). Specifically, LOH of the latter region (chr11q23.3-24.3) was significantly associated with poor survival in ovarian cancer patients (Gabra H, et al.1996). This was the first evidence showing this relationship with chromosome 11q22-25 region in ovarian cancer. More recently, another clinical study of LOH of chromosome 11q23-25 in ovarian cancer further confirmed this result. In this investigation, LOH was detected in 61% of the patients. LOH at 11q23.3 seemed to be associated with significantly reduced survival times ($P = 0.005$) and serous tumour histology ($P = 0.036$). LOH of at the more distal 11q24-q25 location correlated with a higher tumour stage ($P = 0.003$), serous tumour histology ($P = 0.015$), and the finding of residual tumour ($P = 0.047$)(Launonen V, et al.1998). These results further indicate that gene associated with prognosis in ovarian cancer would be located at 11q23-25.

Many candidate tumour suppressor genes have been identified within these regions. Some of them have been analysed in ovarian cancer. The major literature about candidate genes within these regions as well as the relationship with ovarian cancer is summarised in Table 1-8.

Table 1-8 Candidate Genes within Chr 11q and The Relationship with Ovarian Cancer

Gene	Location at 11q	Major function	Relationship with ovarian cancer
ATM	22-23	Plays acritical role in maintaining genetic stability` (Khanna KK. 2000)	Not structural alterations in ovarian cancer (Koike M, 1999)
PPP2R1B	23-24	Candidate tumour suppressor gene (Wang SS,et al.1998)	Infrequent mutations of the gene in ovarian cancer(Wu R,et al.1999)
PGR	22-23	Progesterone receptor gene, putative tumour suppressor gene (Lancaster JM.1998)	Inhibitor role on breast and ovarian cancer (Rowe SM, et al. 1995)
IGSF	23.2	Putative tumour suppressor gene immunoglobulin superfamily gene (Gomyo H, et al.1999)	No study in ovarian cancer
NPAT	22-24	Putative tumour suppressor gene (Imai T, et al. 1997)	No study in ovarian cancer
CHK1	23-24	Encoding a protein kinase required for DNA damage checkpoint function (Sanchez Y, et al.1997)	No study in ovarian cancer
PIG8	24	p53-target gene, ply an role in negative cell growth control by functioning as an apoptotic effector of p53 activity (Gu Z, et al. 2000)	No study in ovarian cancer
APLP2	24	Encoding a human sperm membrane protein (Leach R, et al. 1999)	No study in ovarian cancer
KCNJ1	24	Encoding various isoforms of the human poassium channel (Bock JH, et al.1997)	No study in ovarian cancer
SNC19	24-25	Putative tumour suppressor genes (Zhang Y, et al. 1998)	No study in ovarian cancer
MEN1	13	Encoding a putative growth-supptrrсор protein, menin binding JunD, a transcriptional factor belonging to the AP-1 complex. involving in deregulation of the RET-signalling pathways (Calender A.2000)	No study in ovarian cancer

1.6 The Role of Homeobox Genes in Neoplasms

Homeobox genes are a large family of genes that contain a common 183-nucleotide sequence, first identified as a common feature of several classes of genes known to control developmental pathways in *Drosophila* (McGinnis W, et al, 1984). They encode a 60-amino-acid protein domain, The homeodomain, responsible for recognition and binding of sequence-specific DNA motifs (Gehring WJ, et al. 1992), and act as transcriptional modulators. It is the co-ordinate regulation of network of genes, both temporally and spatially, that leads to establishment of normal segmentation and differentiation patterns in the embryo (Hunt P and Krumlauf R.1992).

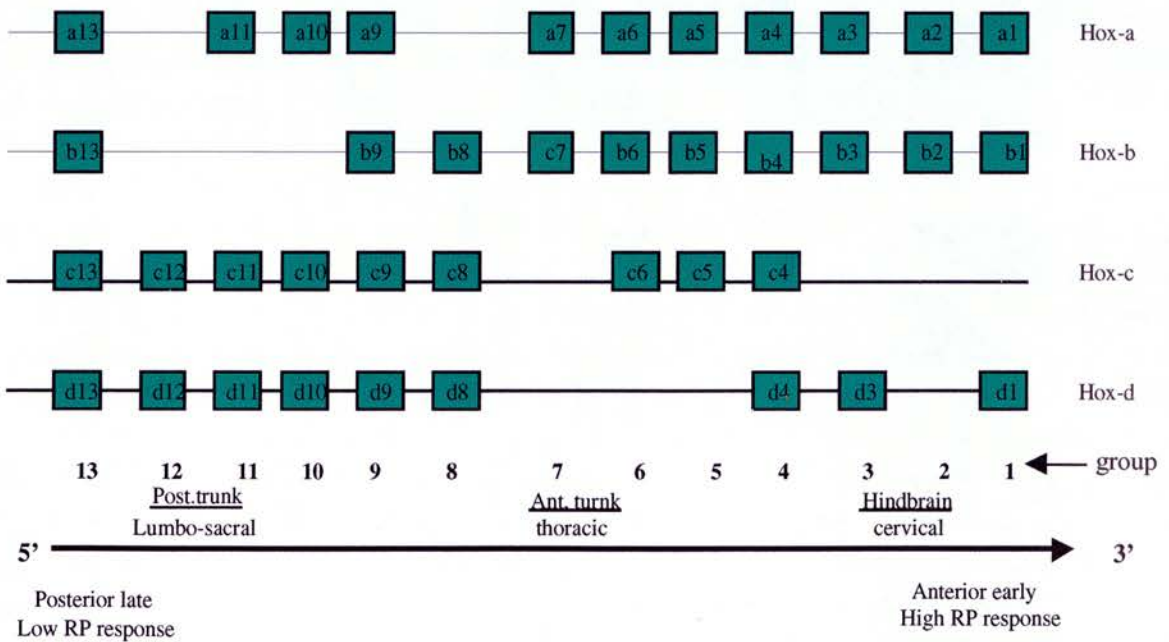
The relationship between homeobox genes and cancer still remains unclear. However, the hypothesis of an association between homeobox genes and the oncogenic process has been strengthened by a number of observations. Many studies have demonstrated that homeobox genes are involved in many cancers such as leukaemia, breast, kidney, lung, colon and so on. First; constitutive expression of some homeobox genes can be oncogenic in mice (Aberdam D, et al.1991). Second; comparing with normal organs, the alteration in expression patterns of several homeobox genes have been reported in variety of malignant lesion (Cillo C, et al. 1999). Third; co-ordinate regulation of homeobox genes has been showed to play an important role in human cancer cell differentiation (Scott MP. 1997). Finally; functional analysis of homeobox genes in cancer cell lines showed that several homeobox genes are upregulated in cancer cell line, but other are downregulated and may function as tumour-suppressor genes (Gao AC, et al. 1998).

1.6.1 Homeobox Genes: Genome Organization and Roles in Development

Different homeobox gene families have evolved that encode the homeodomain (HD) (Duboule D and Morata G. 1994). Among these, the *Drosophila Antennapedia (Antp)* HD defines a consensus sequence referred to as a class I HD and these are also known as the clustered homeobox genes. They are organized in four genomic clusters

of approx 100 kb in length, each of cluster containing several homeobox genes arranged in linear order. To date, 39 of Hox gene shas been defined by virtue of their homology with the genes of this homeotic complex of Drosophila (Deschamps J and Meijink F.1992). The Drosophila clusters are referred to as HoxA, HoxB, HoxC, and HoxD. They are localised to chromosomes 7,17,12 and 2 respectively and comprise 9-11 genes per cluster arranged linearity (Innis JW. 1997) (see Figure 1-1).

Figure 1-1 Schematic Representation of Four Hox Loci



See text for details

In mammalian embryos, clustered homeobox genes start to be expressed at gastrulation. They collectively control the identity of various regions along the body axis from the branchial area through to the tail (Graham A, et al.1989). This action occurs following the rules of temporal and spatial colinearity, with 3' homeobox genes expressed early in development and controlling anterior regions, followed by progressively more 5' genes expressed late in development and controlling more posterior regions (Dekker EJ, et al. 1992). In particular, the 3' homeobox genes of groups 1 to 4 primarily control the development of the embryonic region corresponding to the hindbrain (Lumsden A, et al.1996). Central homeobox genes of group 5 to 8 control the thoracic portion of the body, whereas 5' homeobox genes of 9 to 13 control the lumbosacral region. This spatiotemporal colinearity also holds for *Drosophila* homeotic genes (Duboule D and Morata G. 1994). The physical organization of clustered homeobox genes appears essential for their expression and responsible for major biological function probably yet to be identified. The entire clustered homeobox genes are expressed in the embryonic central nervous system (Giampaolo A, et al. 1989). The many functions of Hox genes in development are outlined in Table 1-9

Table1-9 The Function of the Clustered Homeobox Genes in Normal Development

Gene	Function
Hox A1, B1 and D1	Playing a key roles in anteroposterior axis formation and being detected extensively throughout the embryo in the neural tube adjacent mesenchyme. paraxial mesoderm. somites and gut epithium and bcaudal region of the embryo (Mark M, et al.1993).
HoxA2 and B2	Controlling dorsoventral rat patterns of neruronal development in rostral hindbrin and normal sternum morphology. (Davenne M, et al.1999).
HoxA3 and D3	Neural-crest-derived and somatic – mesoderm-derived tissue (Manley NR, et al. 1997).
Hox A4, B4, C4, and D4	Plays a main role in conferring positional information along the anteroposterior axis to specify the identity of the third and seventh vertebrate.(Graham A, et al. 1988; Horan GS, et al 1994; Kuliev A, et al .1996;)
Hox A5, B5 and C5	A role in the generation of tissues derived from or induced by the embryonal mesoderm (Fuller JF, et al.1999).
Hox A6,B6 and C6	Acting as genetic controller of multiple genes involved in development and cell differentiation (Malicki J,et al.1990).
Hox A7 and B7	A role in the assignment of positional identity along the axis of the embryo(MahonKA, et al.1988), and regulating gene transcription in hematopoietic and lymphoid tissues (Deguchi Y, et al.1991).
HoxB8, C8 and D8	Being necessary for formation of limbs(Izpisua-Belmonte JC, et al. 1990). and playing a key role in regulation of cartilage differentiation (Yueh YG, et al.1998).
HoxA9, B9, C9 and D9	Having both specific and redundant functions in lumbosacral axial skeleton patterning and in limb morphogenesis at the stylopodal level. (Fromental-Ramain C, et al.1996; Chen F, et al.1997), controlling development of the mammary gland in response to pregnancy (Chen F, et al. 1999), and playing a role in T-cell development(Izon DJ, et al. 1998; Lawrence HJ, et al.1997).
Hox A10, C10 and D10	Having a key role in the morphogenesis of the urogenital mesenchyma(Kolon TF, et al.1999),in regulating endometrial development during the menstrual cycle (Osborne J, et al. 1998) and formation of hindlimb (Peterson RL, et al.1993).
HoxA11 and D11	A role in endometrial development, implantation, and maintenance of pregnancy (Morgan, et al.1992) and invloved with spleen development(Davis AP, et al.1995).
HoxC12 and D12	Role for the Hox-D12 gene during endoderm differentiation in teratocarcinoma embryoid bodies (Labosky PA, et al.1993).
HoxA13, B13, C13 and D13	Playing a key role in limb development)(Godwin AR and Capecchi MR.1998)

Other type of homeobox genes are non-clustered or divergent homeobox gene, named after their *Drosophila* homologs: *paired*, *empty spiracles*, *orthodenticle*, and *muscle segment homeobox* genes (Duboule D, 1994). They are scattered throughout the genome and fall into a number of groups on basis of the similarity of sequence. To date, more than 150 non-clustered genes have been cloned and identified (Manak JR and Scott MP. 1994) The main roles of divergent homeobox genes in development are outlined in Table 1-10

Table1-10 The Function of the Divergent Homeobox Genes in Normal Development

Gene	Function
POU family	Multiple function in the differentiation and proliferation of cell during embryogenesis.
Pit-1	As a transactivator of the GH and PRL genes, and as a regulator of the TSH beta gene (Pellegrini- Bouiller J, et al.1997).
Oct-1	Involviend in cell cycle regulation (Verrijzer CD, et al.1990).
Oct-2	Stimulating B-cell-restricted expression of immunoglobulin genes through binding sites in immunoglobulin gene promoters and enhancers (Corcoran LM and Karvelas M. 1994).
Oct-3/4	Hierarchy of regulator genes (Ovitt CFand Scholer HR. 1998).
Oct-6	A major transcription regulator in Schwann cell differentiation (Blanchard AD, et al. 1996)
Brn genes	Play a role in nervous system during embryogenesis (Hagino-Yamagishi K, et al.1998).
PAX family	Involved in the control of development and expressed in the CNS and paraxial mesoderm and its derivatives (Torban E and Goodyer P.1998; Eccles.1998).
LIM family	Play a general role in dorsal-ventral patterning of appendages and the central nervous system (Ahlgren U, et al 1997).
Cdx1	A role in the terminal differentiation of the intestine (Bonner CA, et al. 1995).
Cdx2	Play an important role in the proliferation and differentiation of intestinal epithelial cells (Valcanis H, et al.1997).
APC	A function related to signalling at the adherent junction and possibly with other more complex roles in cells committed to terminal differentiation (Midgley CA, et al.1997). Being a tumour suppresser gene (Polakis P.1999).
PBX genes	Involved as regulator with Hox genes in segmental identity (Vollbrecht E, et al.1991).
GBX genes	Being required for normal development of the mid/hindbrain region, and its function appears to be necessary at the neural plate stage for the correct specification and normal proliferation or survival of anterior hindbrain precursors (Wassarman KM, et al.1997).
Barx2	Involved in coordinated gene expression during embryonic development and as transcriptional regulator of genes encoding several cell adhesion molecules.

1.6.2 The relationship between homeobox genes and cancer

It is now well established that many cancers arise from a disruption of the normal programme of cell growth and differentiation. Therefore, based on the role of homeobox genes as important development regulatory genes, it has been proposed that homeobox genes may be important in oncogenesis (Blatt C. 1990; Castronovo V, et al. 1994).

1.6.2.1 The Hox genes in cancer

1.6.2.1.1 The expression pattern of Hox genes in solid tumours

1.6.2.1.1.1 The expression of Hox genes in renal cell carcinomas

Hox B5 and Hox B9 are expressed in the normal adult kidney; however, the majority and renal cell carcinomas tested do not show expression of these Hox genes (Cillo C, et al. 1992; Barba, et al. 1993). Hox C11 is not expressed in normal kidney; however, Hox C11 transcripts are present in renal cell carcinomas (Cillo C, et al. 1992). Whereas HoxD4 transcripts are normally found in adult kidney, only a subset of these transcripts are frequently found in the renal cell carcinomas (Barba D, et al. 1993). Furthermore, in Wilms' tumor, there are also different expression patterns compared with normal adult kidney. Two abundant HoxC11 transcripts were detected, whereas HoxC11 expression is generally undetected in normal kidney (Cillo C, et al. 1992). Another study showed that the epithelial Wilm's tumour expressed only an aberrant 0.3 kb HOXD10 transcript (Redline RW, et al. 1994).

1.6.2.1.1.2 The expression of Hox genes in colorectal carcinomas

In the normal colon, Hox A1 through 4 are usually expressed; but they are often silent in colorectal carcinomas, and Hox D11 is overexpressed in colorectal carcinomas compared with normal colon (De Vita G, et al. 1993; Barba D, et al. 1993). Hox B7 and Hox D4, both of which are expressed in the normal colon,

demonstrate altered transcript patterns in some colorectal carcinomas (De Vita G, et al.1993). HoxA7 and HoxD11 exhibit altered expression in metastatic lesions compared with primary colorectal and normal intestinal mucosa. Novel transcripts are expressed or different amounts of mRNA is observed in metastatic as oppose to primary colon cancer. In contrast, HoxA13 and HoxB9 display identical expression pattern in normal colon, primary colorectal cancer and corresponding liver metastases when these are derived from a single patient, whereas these genes are silent in the corresponding normal liver (Barba D, et al.1993). So in the same tumour model, Hox genes may exhibit an organ-specific function. Furthermore, human HOXB6, B8, C8 and C9 are overexpressed at various stages of colorectal carcinomas and HOXB8 has been shown to interact with the tumour suppressor genes involved in colorectal cancer such as APC (Vider BZ, et al. 1997).

1.6.2.1.1.3 The expression of Hox genes in lung cancer

Studies have analysed the expression of the whole Hox gene network in human small-cell lung cancer (SCLC) because this tumour type originates from the neural crest. Attention was paid to the relationship between the expression pattern of Hox genes and tumour progression as well as the histological type. The results showed: a) in primary and metastases of classic histology, no major differences in HOX genes expression with regard to both the number of active genes and the transcript classes were identified. Only minor differences at the level of individual genes, mostly concentrated at the 5' end of the HOXC and D loci were identified in SCLC. The over-all number of active HOX genes was remarkably constant. b) In the primary tumour derived from variant histology, virtually the entire HOXB locus was expressed but HOXB4 was silent, two blocks of 3 genes in the HOXC locus (C8, C9 and C10) and in HOXD locus (D9, D10 and D11) were silent in half of the samples. Compared with the primary tumour, metastases showed dramatic changes. Two Hox loci, HOXB and C, were almost completely silent, in which HOXB4 and HOXC5 were barely detectable. c) Comparing classical with variant SCLC in the metastases, 25 out of 38 HOX genes were switched on in the classical type whereas the variant

type displayed only 19 active genes. HOXA1, A6 and A10 were expressed in the classical type but turned off in the variant type. HOXB5 and B6 were silent in the classical type but active in the variant. Conversely, the HOXB7 was active in classical but silent in variant type. HOXD9 and D11 were expressed in the classical but were switched off in the variant (Cillo C.1994). These results indicated that classical and variant histologic type and different tumour progressive stage displayed differential patterns of HOX gene expression and highlighted that HOX genes are switched off during variant SCLC progression. Another report analysed the expression of HOX genes in primary and metastatic human small-cell lung cancer (SCLC) xenografted in nude mice, in order to investigate whether HOX gene expression correlates with the histology and stage of SCLC progression. The results showed that different SCLCs display differential patterns of HOX gene expression. Furthermore, in SCLC, the number of actively expressed HOX genes might be substantially lower in metastatic cancers than in primary tumours. The alteration in HOX gene expression in SCLCs mainly concerns the HOX B and C loci (Tiberio C, et al.1994). This finding suggests that downregulation of HOX genes may play a role in small-cell lung cancer progression, possibly through their implication in tumour suppression.

1.6.2.1.1.4 The expression of Hox genes in breast cancer

The first report regarding the role of Hox genes in breast was a study in normal and neoplastic mouse mammary gland. These results showed that the members from each of the four major Hox gene clusters were expressed in normal mice mammary but in mammary adenocarcinomas and in transplant lines of the benign, precancerous tissues from which the cancers arose, Hoxc-6 was expressed at low levels in the precancerous tissue and was not expressed in cancers. In contrast, Hoxa-1 was expressed only in cancers, not in normal gland or in precancerous mammary tissues (Friedmann Y, et al.1994), suggesting that Hox genes may play a role in a late stage in the stepwise development of mammary malignancies. The study on expression of HOX genes in human breast cancer cell lines revealed that HOXA1, HOXA10, HOXB6 and

HOXC6 are expressed in breast cancer cell lines, and these genes are involved with a role in the development and progression of human breast cancer (Castronovo V, et al. 1994). Furthermore, HOXA1 was over-expressed in a variety of human breast cancer lesions (Chariot A and Castronovo V.1996). So HOXA1 may be required for the establishment of breast cancer cells and alteration of HOXA1 expression could play a role in breast cancer progression.

1.6.2.1.1.5 The expression of Hox genes in gynecologic tumours

mRNA expression of HOXD10 relative to beta-tubulin is significantly lower in endometrial carcinomas than in normal endometrium. Furthermore, the ratio of HOXD10 to beta-tubulin expression varies inversely with the histologic grade of the tumour ($P = .0009$). Human HOXD10 gene expression is altered in endometrial carcinoma and varies with the histologic grade of differentiation (Osborne J, et al.1998). Also, HOXC5 and HOXC8 expression are selectively turned on in human cervical cancer cells compared to normal keratinocytes (Alami Y, et al.1999).

1.6.2.1.2 The role of Hox genes in oncogenesis

The role of Hox genes in oncogenesis is still not very clear, but some lines of evidence have suggested that Hox genes may have a role in oncogenesis.

1), Constitutive expression of HoxB8 genes could be oncogenic in mice. HoxB8 gene rearrangement was first detected in WEHI-3B mouse myeloid leukaemic cells. The rearrangement is due to insertion of an intracisternal A particle 5' upstream to HoxB8 (Blatt C, et al.1988). Furthermore, that normal bone marrow cells were infected with retroviruses bearing the genes for IL-3 or IL-3 plus HoxB8 showed that there was a clear difference between bone marrow cells infected with IL-3 virus alone and cells infected with the IL-3/HoxB8, both in vitro and in vivo. Soft agar growth of IL-3-infected cells resulted in diffuse colonies due to migration of terminally differentiated granulocytes and macrophages, whereas the cells infected with the virus containing both genes formed large and compact colonies containing myeloid precursors and

only few mature cells. Many of these colonies could be established as lines in liquid culture. These results may indicate that HoxB8 expression impedes terminal differentiation of bone marrow cells. Infection of mice with the IL-3/HoxB8 virus resulted in aggressive leukaemia. All 16 animals transplanted with infected cells developed myeloid disease that was aggressively tumorigenic, whereas IL-3 virus provoked a non-transplantable myeloid hyperplasia (Perkins A, et al. 1990). The results suggest that homeobox genes can regulate key differentiation processes such as self-renewal capacity and that their inappropriate expression can be oncogenic.

2), NIH3T3 transfected with Hox B8 produced fibrosarcomas in nude mice. All 12 nude mice subcutaneously injected with the transfected cells developed fibrosarcomas leading to the death of the animals after 4 to 6 months. Injection of the same cells directly in the liver of mice led to more aggressive tumour, causing death within 6 weeks (Aberdam D, et al. 1991).

3), Blockade of HOX gene function using antisense oligonucleotides has revealed that several HOX genes appear to influence either myeloid or erythroid colony formation (Lawrence HJ, et al.1996).

4), Some HOX genes (HOXB2, HOXB4, HOXB7) that they are expressed in normal tissue were methylated in xenografted small-cell lung cancers (Flagiello D, et al.1996), suggesting a possible mechanism for gene silencing.

5), Antisense oligomers targeting HOXB7 mRNA markedly inhibited cancer cell lines proliferation and specifically abolished expression of basic fibroblast growth factor (bFGF) (Care A, et al.1996). Also, transfection of HOXB7 gene into SkBr3 cells, breast cancer cells line, induced bFGF expression and increased growth rate of cancer cell lines (Care A, et al.1998). These indicate a key role for constitutive HOXB7 expression in cancer cell proliferation via bFGF.

6), Hox D3 was involved in angiogenesis because it could mediate conversion of endothelium from the resting to angiogenic/invasive state (Boudreau N, et al.1997).

1.6.2.2 Divergent homeobox genes in cancer

1.6.2.2.1 The POU homeobox gene family in cancer

Brn3 has been found to be not only highly expressed but also strikingly elevated in the pituitary tumours and small cell lung cancer (Leblond-Francillard M, et al. 1997) and use of retinoic acid and antisense against Brn-2 can enhance differentiation of P19 mouse embryonal carcinoma cells into smooth muscle cells (Suzuki T, et al.1996), suggesting Brn genes may be oncogene.

OCT3 and OCT11 were expressed in both human breast cancer cell lines and human primary breast tumours, but not in normal human breast tissue (Jin T, et al.1999). Therefore, these genes could play role in mammary gland carcinogenesis, but their role is unknown.

Although several investigators have reported Pit-1 gene expression in human pituitary adenomas (Yamada S, et al.1996; Pellegrini I, et al.1994; Sanno N, et al.1996), there was no correlation between Pit-1 transcripts and biological behaviour or histological findings in pituitary adenoma (Yamada S, et al.1996).

1.6.2.2.2 The PAX homeobox genes family in cancer

PAX5 is misexpressed in glioblastoma multiforme and was increased in a range of astrocytomas (WHO grade II-IV) which originated in the forebrain. Expression of PAX5 was limited to those cells which also expressed the oncogenes myc, fos, or jun singularly or in combination. (Stuart ET, et al.1995) Its expression is inversely related to the expression of p53 in the same neoplasms because PAX5 inhibits p53-dependent transactivation. The human p53 gene harbours a PAX binding site within its untranslated first exon that is conserved throughout evolution. PAX5 is capable of inhibiting both the p53 promoter and transactivation of a p53-responsive reporter in cell culture. Mutation of the identified binding site eliminates PAX protein binding in vitro and renders the promoter inactive in cells (Stuart ET, et al.1995). PAX2 and PAX8 are also able to interact in a similar manner with p53. According to the critical

role played by p53 in the evolution of different types of cancer, the action of the PAX genes in oncogenesis is crucial. On other hand expression of PAX3 has been found in most of the specimens analysed of Ewing's sarcoma family of tumours, including cell lines and patient material (Schulte TW, et al.1997). Expression of PAX 2 and PAX8 has been detected in the Willm' Tumour and the Wilms' tumour-suppressor gene WT1 can down-regulate Pax-2 expression (Dressler GR.1996). PAX5 is also misexpressed in medulloblastoma (Kozmik Z, et al1995). Three established transitional cell carcinoma cell lines and 79% of primary transitional cell carcinoma of the bladder (TCCs) expressed PAX5 mRNA. There was a significantly higher proportion of PAX5 expression in malignant than in benign urothelium. A higher proportion of tumours with increasing de-differentiation expressed PAX5, which correlates well with the expression pattern of PAX5 in development. In well-differentiated tumours (grade 1), half expressed PAX5, compared with 84% of moderately to poorly differentiated tumours)(Adshead JM, et al.1999). Furthermore, the high incidence of PAX5 expression suggests its potential use as a diagnostic tool and therapeutic target in TCC.

1.6.2.2.3 The NK homeobox gene family in cancer

Human NKX3.1 is known to map to a prostate cancer hot spot and is proposed as a prostate-specific tumour suppressor gene (Bhatia-Gaur R, et al.1999). Northern blot analyses indicate that this gene is expressed at high levels in adult prostate and at a much lower level in testis, but is expressed little or not at all in several other tissues. The NKX3.1 gene maps to chromosome band 8p21, a region frequently reported to undergo a loss of heterozygosity associated with tissue differentiation and loss of androgen responsiveness during the progression of prostate cancer (He WW, et al.1997). Therefore, NKX3.1 is a candidate gene for playing a role in the opposing processes of androgen-driven differentiation of prostate tissue and loss of that differentiation during the progression of prostate cancer. However, single-strand conformational polymorphism analysis of 48 radical prostatectomy cancer specimens and 3 metastases for the entire coding region of NKX3.1 showed no tumour-specific

sequence alterations in 50 specimens and total absence of the gene in 1 specimen known to have a biallelic deletion of 8p21 (Voeller HJ, et al.1997).

1.6.2.2.4 Cdx homeobox genes in cancer

The expression of Cdx gene was found to be either reduced or absent in colon-cancer cell lines and colorectal cancer tissues (Mallo GV, et al.1997) but be expressed in normal epithelial cells of the small intestine and colon (Silberg DG, et al. 1997), suggesting that decrease in human Cdx1 and/or Cdx2 expression is associated with colorectal tumorigenesis. Meanwhile, The mutation analysis for 85 cases of colorectal tumours revealed that one with extensive microsatellite instability (RER+ phenotype) has mutations in both alleles of CDX2. Both mutations occur in coding regions which contain repetitive elements and are consistent with those found in RER + tumours (Wicking C, et al.1998). But, recently another survey reported that in 49 sporadic colorectal carcinomas and ten hereditary non-polyposis colorectal cancers (HNPCC) there was no microsatellite instability and mutation (Yagi DK, et al. 1999).

Introduction of Cdx1 and/or Cdx2 cDNA into colorectal cancer cell line with expression of Cdx1 and/or Cdx2 showed that; 1) Growth rate of cells overexpressing Cdx2 decreased by half, whereas overexpression of Cdx1 had no effect. However, cells overexpressing both Cdxs had a growth rate reduced to 20% of control. 2) In cells overexpressing Cdx1 or Cdx2, tumorigenicity and resistance to apoptosis induced by serum starvation, ceramide, or staurosporine were not changed compared with control cells; yet phorbol ester-stimulated cell migration was decreased by 50%. 3) In cells overexpressing both Cdx1 and Cdx2, tumorigenicity was decreased by 50%, and resistance to apoptosis was significantly lowered, and stimulated cell migration was further decreased to 15% of control compared with cells expressing Cdx1 or Cdx2. 4) cells overexpressing both Cdxs showed strongly decreased Bcl-2 expression, which could account for their increased sensitivity to apoptosis (Mallo GV, et al.1998). These findings further confirmed that loss of Cdx1 or Cdx2

expression is associated with tumour development and invasiveness in colorectal tumours.

1.6.2.2.5 The APC homeobox gene in cancer

The adenomatous polyposis coli (APC) gene, responsible for familial adenomatous polyposis coli, is one of the tumour suppressor genes involved in tumorigenesis of colorectal cancer.

There is quite a body of literature that demonstrated that APC gene has been mutated (including initially somatic and germ line mutations) during the development of sporadic colorectal cancers as well as in familial adenomatous polyposis (FAP). These studies confirmed that: 1) Clinical investigation revealed that mutation of the APC gene was associated with LOH at the APC locus (Miyoshi Y, et al. 1992). 2) The great majority of the mutations found would result in expression of COOH-terminally truncated protein (Nagase H, et al.1992). 3) The great majority of these mutations accumulate in the central region of the of the APC gene and occurred within the first half of the coding sequence (Miyoshi Y, et al.1992). 4) Most identified point mutations in the APC gene are transitions from cytosine to other nucleotides (Nakatsuru S, et al.1993). 5), The location of germ-line mutations tends to correlate with the number of colorectal polyps and age in FAP patients (Friedl W, et al.1996). 6), Inactivation of both alleles of the APC gene seems to be required as an early event for development of adenomas and carcinomas in the colon and rectum as well as some of those in the stomach (Polakis P. 1995; Levy DB, et al.1994)

The APC gene was also shown to regulate cell growth and cell death. Growth control by APC may relate to its ability to downregulate beta-catenin, which is essential for the function of cadherins, a family of Ca²⁺-dependent cell-cell adhesion molecules (in the assembly of adherents junctions), by linking them to (alpha)-catenin and the actin cytoskeleton (in nuclear signalling) (Barth AI, et al.1997). In cancer, mutations in APC ablate its ability to regulate beta-catenin, and mutations in beta-catenin prevent its downregulation by wild-type APC. Owing to its beta-catenin binding, APC can

modify the pool of beta-catenin, but truncated APC is unable to regulate this pool thereby altering adhesion and cell signalling. APC truncation was therefore hypothesized to alter cell multiplication and cells are no longer able to undergo apoptosis (Mahmoud NN, et al.1999). The protein products of mutant APC genes present in colorectal tumours were found to be defective in this activity. Furthermore, colorectal tumours with intact APC genes were found to contain activating mutations of beta-catenin that altered functionally significant phosphorylation sites (Morin DJ, et al.1997). These results indicate that regulation of beta-catenin is critical to APC's tumour suppressive effect and that this regulation can be circumvented by mutations in either APC or beta-catenin. Whereas, expression of wild-type APC caused a pronounced reduction in total beta-catenin levels by eliminating an excessive supply of cytoplasmic beta-catenin indigenous to the colorectal cancer cell line. This reduction was due to an enhanced rate of beta-catenin protein degradation. Truncated mutant APC proteins lacked this activity (Munemitsu S, et al.1995). On the other hand, clinical investigations also confirmed that the absence of wild type APC protein affects the subcellular localization and expression levels of beta-catenin in human colorectal cancer tissues (Inomata M, et al.1996).

1.6.2.2.6 The GBX genes in cancer

The human GBX2 homeobox gene is overexpressed in TSU-PR1, LNCaP, PC-3, and DU145 metastatic prostate cell lines relative to the normal prostate (Gao AC, et al. 1996). These results suggest that the homeobox gene GBX2 may participate in metastatic progression in prostatic cancer. Furthermore, GBX2-overexpressing TSU-pr1 and PC3 human prostatic cancer cells were transfected with an eukaryotic expression vector containing an antisense GBX2 homeobox domain cDNA. When tested in vitro, the clonogenic ability of the GBX2 antisense transfectants was reduced by approximately 50% in both cell lines. When implanted, the tumorigenicity of the antisense GBX2 transfectants from both human prostatic cancer cell lines was inhibited by more than 70% compared to the parental cells (Gao AC, et al.1998).

Thus, expression of GBX2 gene is required for malignant growth of human prostate cells.

The GBX2 gene was also identified as a target gene of the v-Myb oncoprotein encoded by the avian myeloblastosis virus (AMV). GBX2 activation by c-Myb requires signal transduction emanating from the cell surface while the leukemogenic AMV v-Myb constitutively induces the GBX2 gene. Mutations in the DNA binding domain of AMV-Myb render it independent of signalling events and concomitantly abrogate the collaboration between Myb and CCAAT Enhancer Binding Proteins (C/EBP), which are involved in granulocyte differentiation. Ectopic expression of GBX2 in growth factor-dependent myeloblasts induces monocytic features and independence from exogenous cytokines, reflecting distinct features of AMV-transformed cells (Kowenz-Leutz E, et al.1997).

1.7 The Barx2 Gene in Cancer

1.7.1 The molecular organization of the Barx2 gene

The Barx2 gene, a divergent homeobox gene, was first found and isolated during screening experiment of a mouse cDNA expression library using a concatamer of the sequence CCATTAGPyGA (Jones FS, et al. 1997), and was homologous with the class of invertebrate homeobox proteins of the Bar subfamily. Homeodomains of Bar subfamily all contain a glutamine residue at position 50 in the third helix of the homeodomain. These properties shared by several other homeodomain proteins including all members of the Antennapedia family confer a binding preference for target sites containing the core sequence CATTA. Barx class homeodomains are further distinguished from those of the Antennapedia family by threonine and tyrosine residues at positions 47 and 49 respectively (Scott MP, et al. 1989; Kappen C, et al.1993). Barx2 is most closely related to the Barx1 gene and encodes a homeodomain that is 87% identical. Barx2 gene shares a specific segment of 17 amino acid containing a number of basic residues with Barx1 designated the Barx basic region (BBR) located immediately downstream of the homeodomain.

Barx2 also exhibits several other notable features, including a putative leucine zipper, a polyalanine tract within the amino-terminal region (in mouse only) and an acidic domain within the carboxyl-terminal region (Jones FS, et al. 1997), which has been confirmed to function as a repressor domain in other studies (Hanna-Rose W and Hansen U. 1996).

The human Barx2 gene encodes a homeodomain protein of 254 amino acids, which has a glutamine residue at position 50, and binds optimally to the DNA consensus sequence YYTAATGRTTTTY (Kriasner A, et al. 2000), which is closely related to the Barx2 binding sequences in the N-CAM and L1-CAM (Jones FS, et al. 1997), but somewhat more divergent from the sequences in human calcitonin gene promoter (Kriasner A, et al. 2000).

The human Barx2 gene contains four exons with a CpG island consisting of two Mse I fragments, and three introns. The gene cover about 14 kb of genomic DNA and encodes an mRNA of 1.9-2 Kb. Figure 1-2 shows the structure of the Barx2. The exons and introns of the Barx2 are outlined in Table 1- 11. The Barx2 gene is located at chromosome 11q25 between the markers D11S912 and D11S1320 (Hjalt TA and Murray JC. 1999; Krasner A, et al.2000).

Figure1-2 The Genomic organization and Protein Structure of Barx2

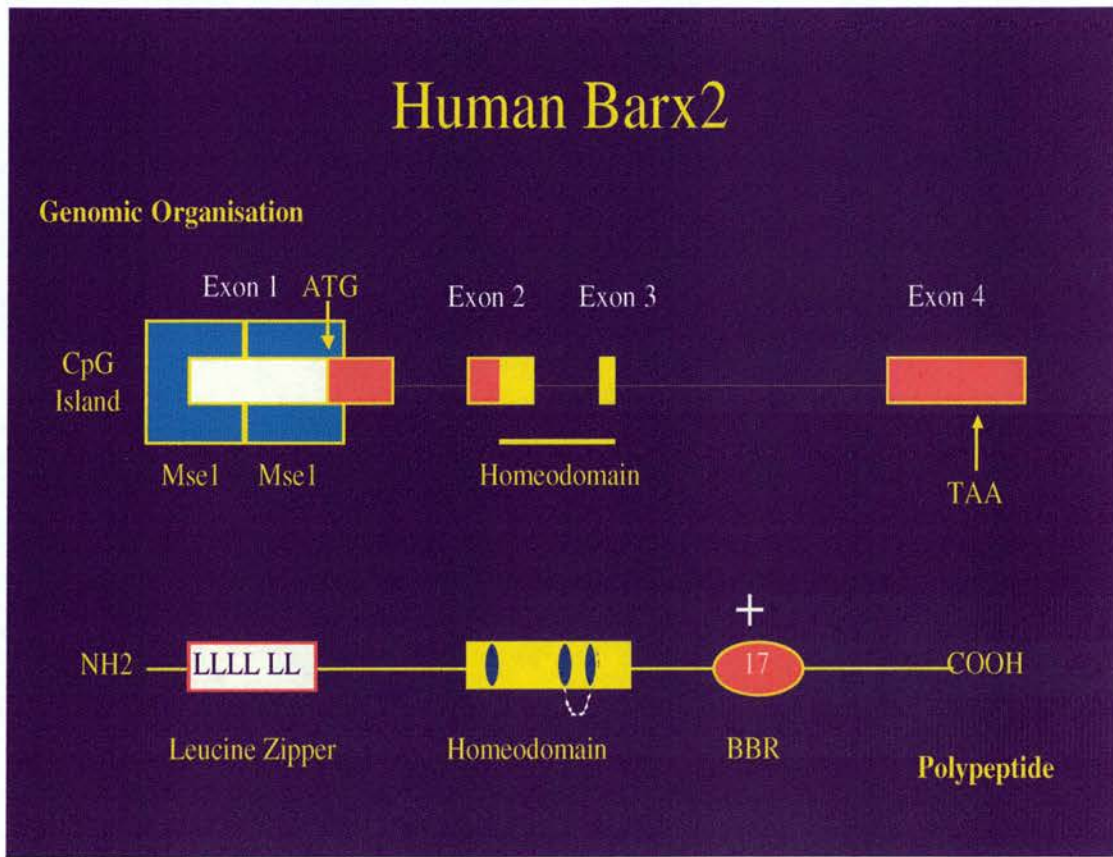


Table 1- 11 The Exon and Intron within Barx2 Gene

Exon no	Exon size (bp)	cDNA position	Intron no	Intron size (kp)
1	1099	1- 281	1	2.1
2	301	282- 582	2	2.7
3	85	583- 667	3	8.0
4	603	668-1270		

1.7.2 The normal function of the Barx2 gene

1.7.2.1 The Barx2 is involved in coordinated gene expression during embryonic development.

During development, Barx2 is expressed at the sites of epithelio-mesenchymal interactions. The expression of Barx1 and Barx2 also overlap in the nervous system, particularly in the telencephalon, spinal cord, and dorsal root ganglia. Barx2 was also prominently expressed in the floor plate of development midbrain and in Rathke's pouch. During craniofacial development, Barx1 and Barx2 showed complementary patterns of expression: whereas Barx1 appeared in the mesenchyme of the mandibular and maxillary processes, Barx2 was observed in the ectodermal lining of these tissues. Intense expression of Barx2 was observed in small groups of cells undergoing tissue remodelling, such as ectodermal cells within indentations surrounding the eye and maxillo-nasal groove and in the first branchial pouch, lung buds, precartilagenous condensations, and mesenchyme of the limb (Jones FS, et al. 1997). So the Barx2 gene is a co-ordinator of gene activity during embryonic development.

In adult, the Barx2 is expressed in salivary gland, mammary gland and placenta (Krasner A, et al. 2000). Initial work on the Barx2 in our lab has shown that the gene is expressed in normal human ovarian surface epithelium.

1.7.2.2 The Barx2 gene functions as transcription regulator of genes encoding several of celladhesion molecules (CAMs).

The Barx2 may have dual functions as regulator for the L1-CAM gene. In cellular co-transfection experiments, Barx2 activated L1-CAM regulator sequence constructs containing the CCATTGPyGA motif and repressed the activity of constructs lacking this sequence. These results suggest that Barx2 may regulate expression of L1-CAM gene. In cells that express the L1-CAM gene, it may function as an

activator, but in cells that do not express L1-CAM, it may function as a repressor. The L1-CAM gene is a transmembrane glycoprotein belonging to the immunoglobulin superfamily and is essential in the development of the nervous system as well as being expressed on neurons and Schwann cells and plays a key role in axon outgrowth and pathfinding through interactions with various extracellular ligands and intracellular second messenger systems (Fransen E, et al. 1997; Meech R, et al. 1999). Mutations in human L1-CAM gene are responsible for a variety of congenital neurological disorders, including hydrocephalus, mental retardation, and agenesis of the corpus callosum (Wong EV, et al. 1995).

The Barx2 homeodomain is also sufficient for binding homeodomain binding sites sufficient (HBS) from neuron-glia CAM (Ng-CAM) and neural CAM (N-CAM) and the Barx2 may regulate these genes. The presence of a 17-amino acid Barx basic region resulted in decrease in binding to HBS sequences from the Ng-CAM, whereas it led to a increase in binding to HBS from the N-CAM promoter. In co-transfection experiments, Barx2 repressed N-CAM promoter activity. When a 24-residue N-terminal region of Barx2 was absent, Barx2 activated the N-CAM promoter (Edelman DB, et al. 2000).

A recent study of sheep wool follicle development has demonstrated that the Barx2 is expressed in the epithelial component of the developing follicle and in the outer root sheath of the adult follicle in pattern that is similar to E-cadherin. This suggests that the Barx2 may have a general function in controlling adhesive processes in keratinising epithelia (Sander G, et al. 2000).

1.7. 2. 3 The Barx2 gene plays a role in regulateing CREB family.

The Barx2 gene contains leucine zipper motifs. It has now been demonstrated that the Barx2 gene can interact with members of the cyclic AMP response element (CRE)-binding protein (CREB) family, CREB1 and ATF2, potentially via leucine zipper motifs, and play a role in dual modulation of the function of transcription factors of the CREB family (Edelman DB, et al. 2000). CREB was initially isolated

from rat brain tissue (Montminy MR, et al. 1987; Yamamoto KK, et al.1988) and found to be ubiquitously expressed. CREB belongs to the leucine zipper class of protein (Borrelli E, et al. 1992) and can form heterodimers in specific combinations. CREB might be responsible for programmed nerve-cell survival (Walton MR, et al.2000) and is involved in the formation of memory in organisms with diverse phylogenetic background from mollusks to mammals (Lamprecht R. 1999).

1.7. 2. 4 The Barx2 gene is identified as a regulatory element in the ras/raf pathway

Within the calcitonin gene promoter, gene involved in increased transcription via the ras/raf pathway, a ras/raf responsive transcriptional element (RRE) has been identified (Thiagalingam A, et al.1996). The Barx2 protein-binding domain was identified as being sufficient to confer responsiveness of a reporter gene to ras/raf activation (Krasner A, et al. 2000) and the Barx2 therefore may take part in regulation of the ras/raf pathway.

The ras/raf pathway activates several protein kinase cascades, which can result in the subsequent activation of transcription factors; these, in turn, can alter gene expression and cell phenotype in terms of the regulation of cell proliferation, differentiation, and morphology (Yamamoto T, et al.1999).

1.7.3 The Barx2 gene in cancer

One of the hypothesis underlying this thesis is that the Barx2 gene may be a candidate tumour suppressor gene because it is located within minimal critical region LOH region, is a regulator of CAM expression and is expressed developmentally at the site of epithelio-mesenchymal interaction.

The barx2 gene is located at chromosome 11q25 between the markers D11S912 and D11S1320, within a minimal region associated with frequent LOH in ovarian cancer, breast cancer and colorectal cancer (Habra H, et al.1996; Negrini et al.1995; Davis M, et al. 1996; Connolly KC, et al.1999). It has also been demonstrated that disruption

of this region is associated with poor prognosis in ovarian cancer patients (Habar H, et al.1996; Launonen V, et al. 1998; Launonen V, et al. 2000).

As mention above, the Barx2 gene functions as a transcriptional regulator CAMs. More evidence is now appearing to suggest that disturbance in protein-protein interaction in the CAMs complex, especially in the E-cadherin-catenin adhesion complex, is one of the main events in the early and late steps of cancer development. It has been found that the expression of CAMs was associated with the invasive and metastatic behaviour of tumour cells. For example, loss of expression or function of CAMs, intracellular molecules that interact with CAMs, can result in the loss of CAMs mediated adhesion and a more invasive phenotype (Debruyne P, et al. 1999; Rowlands TM, et al. 2000; Sommers CL. 1996). Besides their role in establishing tight cell-cell adhesion, CAMs also play a role in cell signalling and promotion of neoplastic growth (Wijnhoven BP, et al. 2000), suggesting its dual role in human cancer. Therefore, alteration of the function of Barx2 may influence CAMs functions.

The Barx2 gene is involved in regulating CREB family. Previous studies have showed that the expression of CREB correlated directly with the metastatic potential of melanoma cells (Rutberg SE, et al. 1994). Furthermore, transfection of a dominant negative KCREB into melanoma cells showed that the expression of the KCREB construct in metastatic melanoma cells decreased their tumorigenicity and metastatic potential in nude mice. The KCREB-transfected cells displayed a significant decrease in MMP-2 (collagenase type IV) mRNA and activity that resulted in a decrease in invasiveness through the basement membrane, an important component of tumour invasion and metastasis. In addition, the cell surface adhesion molecule that was involved in the metastasis of human melanoma (Luca M, et al. 1993; Xie S, et al. 1997) was down-regulated in the KCREB-transfected cells (Xie S, et al. 1997). Transfection of KCREB into the melanoma cells also increases their sensitivity to UV and ionising radiation and renders them susceptible to thapsigargin induced apoptosis (Jean D, et al. 1998).

The *barx2* was also involved in mediating *ras/raf* dependent transcription of the calcitonin gene via a *ras/raf* responsive transcriptional element within the calcitonin gene promoter (Krasner A, et al.2000). The clinical investigation showed that down-regulation of the calcitonin gene was associated with the poor differentiation of thyroid carcinoma cells and also with adverse patient survival (Ball DW. 1996). Hypermethylation of the calcitonin gene has been described in various hematologic malignancies (Thomas X, et al. 1999; Ihalainen J, et al. 1993).

Although some lines of evidence have suggested that the *Barx2* gene may have a role in oncogenesis, until now, there has not been direct evidence that the *Barx2* gene is involved in cancer. So the objectives of this project were the further analysis of the LOH region to refine the LOH minimal region and , if confirmed to remain in the region, the formal structural and functional analysis of *Barx2* as a candidate tumour suppressor gene.

2. MATERIALS AND METHODS

2.1 Refinement of the Region of Frequent Loss of Heterozygosity of Chromosome 11q D11S1894-D11S11309 Region in Colorectal Cancer.

In order to further refine the TSG region at chromosome 11q between D11S1894 and D11S1390 (4.5Mb) and also confirm location of Barx2, 14 polymorphic loci in this region were investigated using microsatellite analysis in colorectal cancer tumour/blood pairs. It is due to previous LOH studies have demonstrated that LOH at chromosome 11q between D11S1894 and D11S1309 occurs frequently in ovarian and colorectal cancer (using same polymorphic markers and got same results in ovarian and colorectal cancer in our lab) and there was shortage of ovarian tumour/blood pairs samples in that time,

2.1.1 DNA preparation

2.1.1.1 Clinical Material

Matched normal/tumour samples from 50 patients with colorectal cancer were used. The patient characteristics are outlined in Table 2- 1 Patients with colorectal cancer were on continuing follow-up, which ranged up to 2491 days.

Table 2-1 **Clinicopathological Characteristics of Colorectal Cancer Patients**

Clinicopathological Character	No
<u>Patients' age</u>	
mean age	67.3 years
median	68 years
SD	10.31
<u>Sex</u>	
female	23
male	24
unknown	3
<u>Anatomical location</u>	
ascending	9
transverse	4

descending	2
sigmoid	19
rectum	12
unknown	4
<u>Dukes' stage</u>	
A	1
B	26
C1	19
unknown	4
<u>Differentiation</u>	
well	3
moderate	33
poor	9
unknown	5
<u>Vascular invasion</u>	
no	41
yes	3
unknown	6
<u>Perineural infiltration</u>	
no	42
yes	2
unknown	6
<u>Mucin production</u>	
no	35
yes	9
unknown	6

2.1.1.2 Extraction of high molecular weight DNA of cancer tissue and rapid miniprep extraction of DNA of lymphocytes.

These extractions were previously done in our laboratory and all of DNA sample was stored at 4 °C.

2.1.2 Automated laser fluorescence (ALF) analysis of microsatellites

2.1.2.1 ALF- Polymerase Chain Reaction (PCR)

2.1.2.1.1 The design of primer

The primers were designed on the basis of recently generated microsatellite index maps for locus, informativeness and spacing.

All oligonucleotides were obtained from the ICRF oligonucleotide laboratory, and were fluorescently labelled.

2.1.2.1.2 The treatment of oligonucleotides

20 μl of NaOAc (3 M, pH 5.6), 80 μl of 25 mM MgCl₂, 100 μl of sterile distilled water and 600 μl of 100% cold ethanol were added to each primer and then incubated in -70° C for 30 minutes or incubated in -20° C for overnight.

The sample was centrifuged at 1300 g for 20 minutes at 4 °C then removed all of the liquid. Added 1 ml of 70 cold ethanol and then centrifuged at 1300 g for 15 minutes at 4 °C then removed ethanol.

The primer was dried at room temperature for 10 minutes and then added 100 μl of sterile distilled water to resuspend it.

10 μl of the primer solution was added to 990 μl distilled water. The concentration of primer was estimated by Spectrophotometric reading of absorbance at 260 nm (O.D. 260).

2.1.2.1.3 Optimization of reaction condition of PCR

PCR was performed in a reaction volume of 25 μl . The reaction condition of PCR for different primers was first optimized with different concentrations of MgCl₂ and then different quantities of genomic DNA were tested at that reaction condition for PCR. Figure2-1 shows the result of optimization PCR with different concentrations of MgCl₂ by using primer 969

2.1.2.1.4 PCR programme for genomic PCR

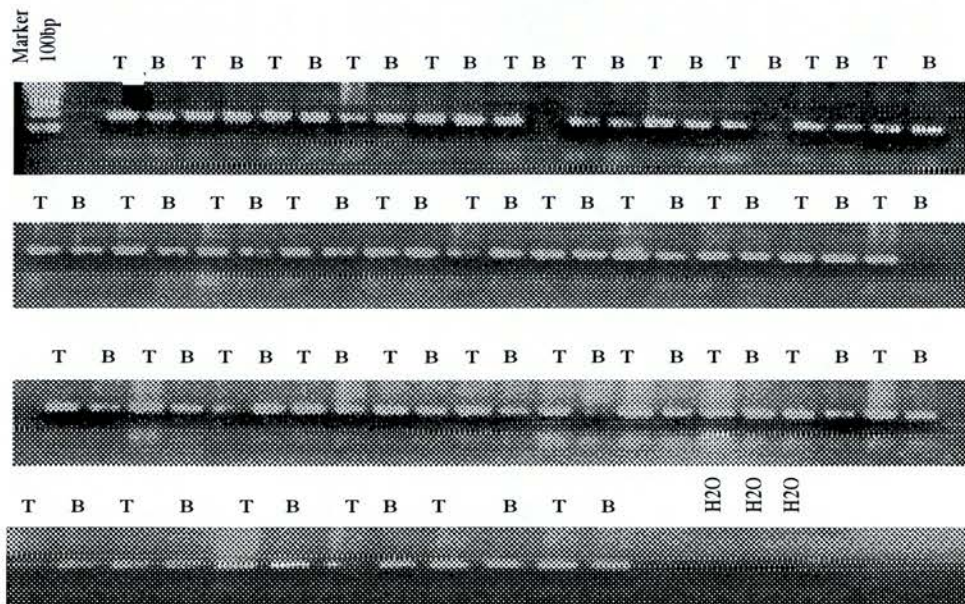
A touchdown PCR programme was used as follows:

94⁰ C for 5 minutes 1 cycle; 94⁰C for 30 sec/67⁰ C for 30 sec/72⁰ C for 42 sec 2cycle; 94⁰C for 30 sec/64⁰ C for 30 sec/72⁰ C for 42 sec 2cycle; 94⁰C for 30 sec/61⁰C for 30 sec/72⁰C for 42 sec 2cycle; 94⁰C for 30 sec/58⁰C for 30 sec/72⁰C for 42 sec 2 cycle; 94⁰C for 30 sec/55⁰C for 30 sec/72⁰C for 42 sec 30 cycle; then final 72⁰C for 5 minutes.

2.1.2.1.5 Checking of PCR products by Agarose gel electrophoresis

4 µl of reaction product with 1 µl of electrophored buffer was checked on standard 2% agarose run at 100-120 volts in 1x TAE and a 100 bp DNA ladder was used as a convenient marker for size estimates of the products, stained with ethidium bromide and visualised under UV light. Figure 2-2 shows the result of products of ALF-PCR for colorectal cancer samples blood/tumour pair.

Figure 2-2 the Result of Products of ALF-PCR (D11S1309) For Colorectal Cancer Blood/Tumour Pair.



T= Tumour DNA

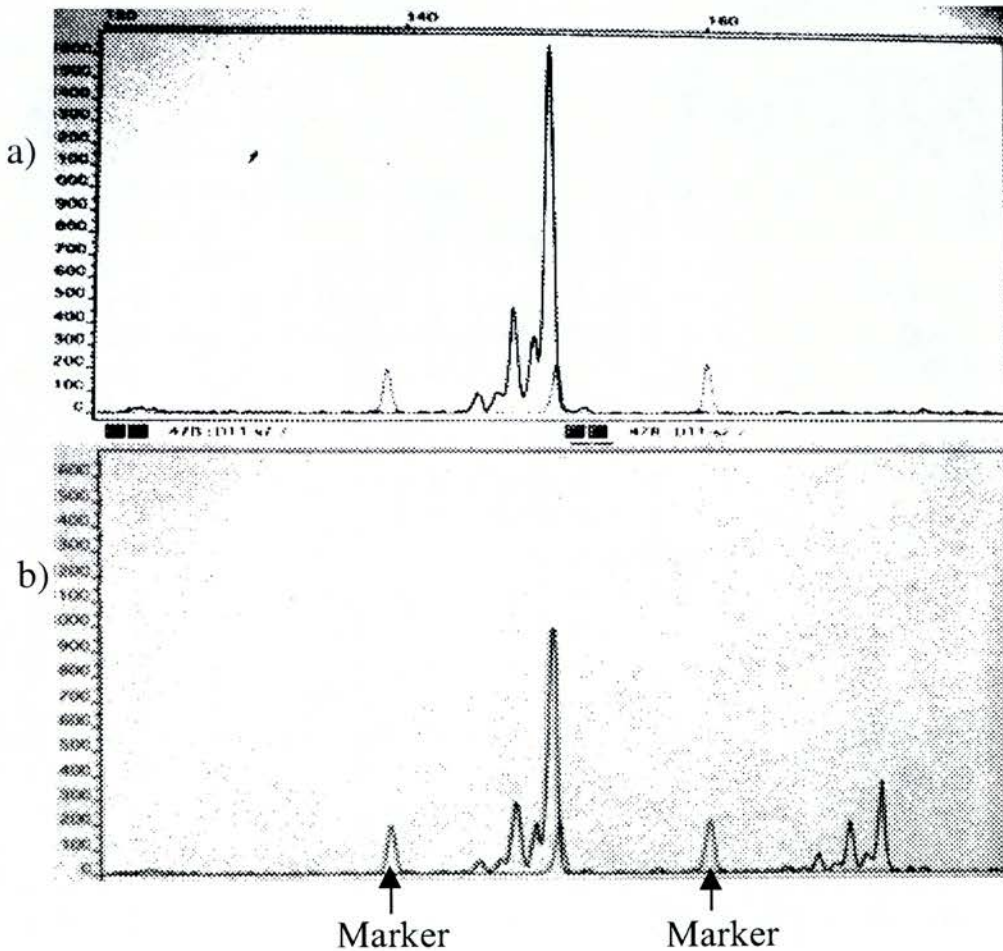
B= Blood DNA

2.1.2.2 The PCR products were run on the ABI Prism (MRC).

2.1.2.3 The method of analysis for result of microsatellites

Allelotype analysis was performed using Genescan software. Each pair of samples was assigned to one of three groups; heterozygous, uninformative (homozygous) or nondeterminable. For the heterozygotes the relative ratios of alleles was determined. LOH was assigned where the tumour allele ratio differed from the normal allele ratio by >30 % ($r < 0.7$) (see Figure 2-3)

Figure 2-3 the Example of ABI Prism for PCR Products of DNA of Colorectal Cancer



This example is result of ABI prism for PCR (D11S4126) products of DNA of colorectal Cancer tumour/blood pairs. Figure a) showed ABI of tumour DNA, there is

only one peak. Figure b) showed ABI prism of blood cell DNA and there are two peaks. Comparably tumour DNA with blood cell DNA, tumour DNA had completely lost one allele. This demonstrates loss of heterozygosity (LOH). If there are two allele in tumour tissue and where the tumour allele ratio differed from the normal allele ratio by >30 % ($r < 0.7$). It also suggests LOH. If this ratio is <30% ($r > 0.7$), It is assumed that there no LOH (retention of heterozygosity). If there is only one peak in tumour DNA and blood cell DNA, the locus is uninformative (homozygous) for that sample.

2.2 Expression Of Barx2 Gene In Ovarian Cancer Cell Lines and Tissue

2.2.1 Reverse transcription PCR

2.2.1.1 Clinical Material

Fresh primary ovarian cancer tissues from 28 patients were obtained. The patient characteristics are outlined in Table 2-3. Patients were on continuing follow-up, which ranged up to 2780 days.

Table 2-3 Clinicopathological Characteristics of the Study Cohort of Ovarian Cancer

Clinicopathological Character	No
<u>Pathology</u>	
Serous	15
Endo	7
MMT	3
Clear cell	2
Steroid cell	1
<u>Stage</u>	
I + II	10
III+IV	16
Unknown	2
<u>Grade</u>	
Poor	13
Moderate	8
Moderate/ Poor	2
Borderline	1
Unknown	4

2.2.1.2 Cell lines or tissue RAN preparation

2.2.1.2.1 Extraction of RNA by TRI REAGENT

Cells were Lysed directly on the culture flasks, aspirating the medium and washing cells with PBS once then adding the TRI REAGENT (1 ml per 10 cm of culture flask). Sample was incubated at room temperature for about 5 minutes then the cell lysate was passed several times through a pipette to form a homogenous lysate.

0.2 ml of chloroform per ml of TRI REAGENT was added. The sample was covered tightly then shakes vigorously for 15 seconds and allowed to stand for 10 minutes at room temperature. The resulting mixture was centrifuged at 12,000 g for 15 minutes at 4 °C.

Transferring the aqueous phase to a fresh tube and added 0.5 ml of isopropanol per ml of TRI REAGENT used in sample preparation and mixed. The samples were allowed to stand for 10 minutes at room temperature, then centrifuged at 12,000 g for 10 minutes at 4 °C.

The supernatant was removed and the RNA pellet was washed by adding 1ml of 75% ethanol in DEPC water per 1 ml of TRI REAGENT used in sample preparation, then vortexed and centrifuged at 12,000 g for 10 minutes at 4 °C.

The supernatant was removed again. The RNA pellet was dried for 10 minute and then added an appropriate volume (50 - 100 µl) of DEPC water. To facilitate dissolution, the pellet was mixed by repeated pipetting with a micropipette at 55°C for 10 minutes.

The ovarian cancer tissue total RNAs for this study were kindly provided by Kenny MacLeod.

2.2.1.2.2 Quantitation of RNA by spectrophotometer

5µl of the RNA solution were added to 1295 µl of distilled DEPC water. Spectrophotometric reading of absorbency at 260 nm (O.D. 260) is proportional to the concentration of RNA in the sample. Absorbance at 280 nm divided by

absorbance at 260 nm indicates the purity of the sample. A value of 1.6 to 1.8 is optimal and ratios below or above this indicate contamination of the sample. An O.D. 260 of 1.0 is equivalent to an RNA concentration of 40 $\mu\text{g}/\text{ml}$, allowing simple proportional calculations to determine RNA concentration of any sample.

2.2.1.3 Treating RNA with DNase

Samples were prepared in the hood.

Each RNA sample was diluted in a volume of 50 μl and the following was added:

<u>Reagent</u>	<u>Quantity</u>
DNase I (RNase free)(10u/ μl)	2 μl
RNasin (40u/ μl)	1 μl
<u>10x Buffer</u>	<u>5.8 μl</u>

The total volume was 58 μl . The samples were incubated at 37 $^{\circ}\text{C}$ for 1 hour.

60 μl of Phenol:Chloroform (1:1) were added, vortexed for 5 seconds then spun in 13000g for 15 minutes at 4 $^{\circ}\text{C}$. 55 μl of the top layer was removed and put into new tube on ice.

55 μl of Chloroform was added, vortexed for 5 second then spun in 13000g for 15 minutes at 4 $^{\circ}\text{C}$. 55 μl of the top layer was removed and put into new tube on ice and precipitated with 5.5 μl of NaOAC pH5.6 and 120 μl of Ethanol and incubated at -70 $^{\circ}\text{C}$ for 30 minutes.

The samples were centrifuged at 4 $^{\circ}\text{C}$ with 13000g for 15 minutes the put them straight on ice.

The supernatant was removed and washed PNA pellet by adding 1 ml of 75% ethanol and vortexing, and removed 75% ethanol as much liquid off as possible.

The pellet was resuspended in 20 μl of DEPC treated water. Checked quantification of RNA by spectrophotometer as described above.

2.2.1.4 First stand cDNA syntheses

This was performed using the 1 st Strand cDNA Syntheses Kit (AMV)

For each sample, prepared two sterile microfuge tubes.

2 μ l of total RNA (0.5 μ g/ μ l) was added to each sterile microfuge tube then 5.8 μ l of DEPC treated water was added. The samples were incubated at 65 $^{\circ}$ C for 15 minutes then were placed on ice immediately.

Two mixtures were prepared as following in sterile microfuge tube respectively.

Mixture 1

<u>Reagent</u>	<u>Quantity</u>
10x reaction buffer	2 μ l
25 mM Mgcl ₂	4 μ l
10mM dNTP	2 μ l
Oligo-p(dT) primer (0.8 μ g/ μ l)	2 μ l
RNase inhibitor (50u/ μ l)	1 μ l
AMV Reverse Transcriptase(u/ μ l)	0.8 μ l
<u>Gelatin (0.5mg/ml)</u>	<u>0.4 μl</u>

Mixture 2

<u>Reagent</u>	<u>Quantity</u>
10x reaction buffer	2 μ l
25 mM MgCl ₂	4 μ l
<u>distilled DEPC water</u>	<u>6.2 μl</u>

The mixture 1 was added into the sterile tube containing the sample on ice then mixture 2 was added into the other sterile tube which also contained the sample on ice as RT-negative control.

The sample was placed on Techne PHC-3 Thermal Cycler and then incubated as following program;

25 °C for 10 minutes, 42 °C for 60 minutes and 95 °C for 10 minutes.

After incubating, the samples were placed on ice immediately then centrifuged briefly.

2.2.1.5 PCR

2.2.1.5.1 The design and treatment of RT-PCR oligonucleotides primers for Barx2.

A total of 4 pair of primer were used in this project. According to the cDNA sequence of Barx2 gene, we designed three pair of primer for each exon of Barx2 (see Figure 2-4) in order to analyse expression for each exon as well as full length cDNA of Barx2 in ovarian cancer cell lines and tissue. Table 2-4 shows these primers sizes, sequences, and exons.

Figure 2-4 Human Barx2 RT-PCR Primers

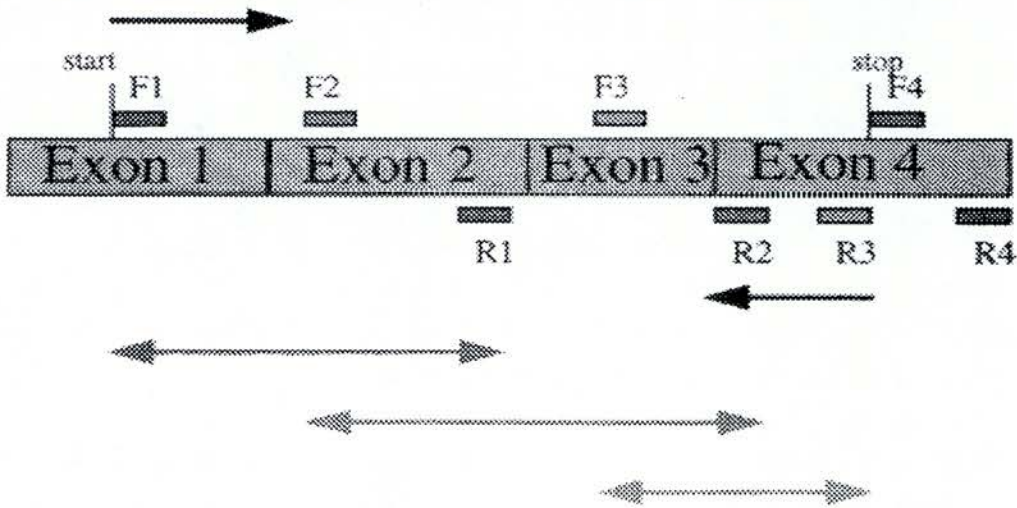


Table 2-4 the Primers of Barx2 for RT-PCR of cDNA

Primer	Sequence	Size (bp)	Exon
F1/R1	5' -ATGATCGACGAGATCCTCTC-3' 5' -GTGTTCCGTCTCTGACTCGC-3'	300	1- 2
F2/R2	5' -CACCGAGGCGGTCTCTGCTG-3' 5' -GCTTCCTGTCCACCTTTAAG-3'	291	2- 3
F3/R3	5' -TGGTATCAGAATCGCAGGAT-3' 5' -GCTTAATGGTGGGGGTTCG-3'	300	3- 4
F1/R3	5' -ATGATCGACGAGATCCTCTC-3' 5' -GCTTAATGGTGGGGGTTCG-3'	780	1- 4

All of oligonucleotides were obtained from the ICRF oligonucleotide laboratory and were treated as described above, except that distilled water was also treated with DEPC.

2.2.1.5.2 PCR reaction of the Barx2

PCR was performed in a reaction volume of 25 μ l. The PCR reaction mixture contained 2.5 μ l of 10x buffer, 1.5 μ l of 25 mM MgCl₂, 0.5 μ l of 10mM dNTP, 0.2 μ l of Taq polymerase (5u/ μ l), 12.5 pmol of each primer, 2.5 μ l of first stand cDNA and 17.3 μ l of distilled DEPC water. Meantime, other reactions were set up in which the water and RT-negative product replaced first stand cDNA as negative control respectively.

PCR was performed under the following condition; 94 °C for 5 minutes in 1cycle. 94 °C for 30 seconds. 55 °C for 30 seconds. 72°C for 42 seconds, repeated for 35 cycles, then 72°C for 5 minutes in 1cycle.

2.2.1.5.3 PCR reaction of γ -actin

In order to identify the quantity and quality of cDNA, the RT-PCR of γ -actin was also performed for the same sample. A reaction volume for γ -actin was the same as above.

PCR was performed under the following condition; 94 °C for 2 minutes in 1 cycle. 94 °C for 30 seconds. 57 °C for 45 seconds. 72°C for 45 seconds, repeated for 35 cycles, then 72°C for 5 minutes in 1 cycle.

2.2.1.5.4; Checking of PCR products by Agarose gel electrophoresis

5 μ l of reaction product with 1 μ l of electrophoresis buffer were checked on standard 2% agarose run at 100-120 volts in 1x TAE and used the 100 bp DNA ladder as a convenient marker for size estimates of the products, and stained with ethidium bromide and visualised under UV light.

2.2.2 Northern blot

2.2.2.1 The treatment of plastics and glassware

Glassware was filled with 0.1% DEPC in distilled water and incubated overnight. DEPC water was poured off into a sterilised bottle and then baked for 4 hours at 200 °C and stored them in a sterile manner. Washed the gel tank with mild detergent and rinsed well with distilled-DEPC water. Treated the gel tank and comb with RNase.

2.2.2.2 Blotting

1.8 % of agarose gel was prepared in distilled-DEPC water with 12 % of 10x MOPS/EDTA and 6.1% of formaldehyde (37%) and allowed the gel to set for 1 hour at room temperature in fume hood.

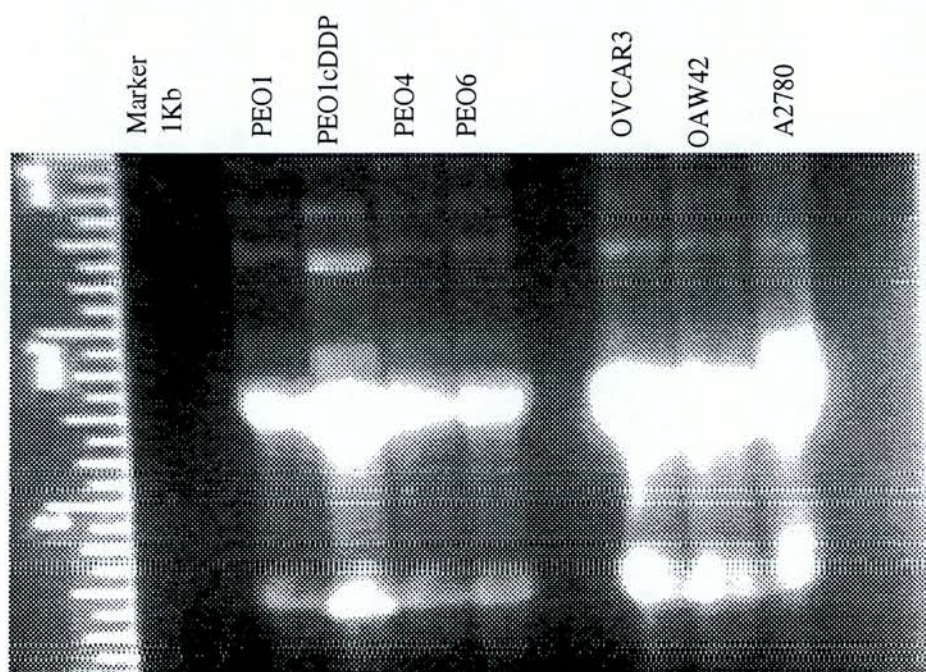
RNA was thawed on ice and then placed 10-20 μ g of total RNA in 5 μ l of distilled-DEPC water. 10-20 μ g of RNA ladder was placed. 25 μ l of electrophoresis sample buffer was added to each sample and the RNA ladder and then heated to 65 °C for 15 minutes and then the samples were placed on to ice. 1 μ l of 1mg/ ml ethidium was

added bromide per tube then centrifuged the sample briefly. The samples were loaded into the gel immediately.

The gel was run overnight at 30 volts in 1x MOPS/EDTA and then the voltage was increased to 70 volts the next day and run until the dye front was 4/5 down the gel.

The gel was photographed under UV light and the RNA marker position was measured by using a ruler. Figure 2-5 showed an example of a northern blot gel

Figure 2-5 an Example of A Northern Blot Gel



A corner of the gel was removed for orientation. The gel was soaked in 10x SSC for 20 minutes two time in a pyrex dish with shaking platform at room temperature.

The Hybond-N membrane and five pieces of 3MM filter paper were cut to the size of the gel. The membrane was soaked in distilled water for 5 minutes then soaked in 10x SSC for other 5 minutes.

The gel was transfered overnight in 10xSSC. The membrane was removed after marking the positions of the lanes in the gel. The membrane was then washed briefly in 2xSSC. The membrane was dried at room temperature and then was UV irradiated

by using GS GENE LINKER UV chamber (program C3). The membrane was stoked at room temperature.

From the position of the RNA marker on the gel, a plot of log₁₀ of the RNA fragment size (y- axis) vs distance the RNA fragment migrated (cm)(x-axis) was generated.

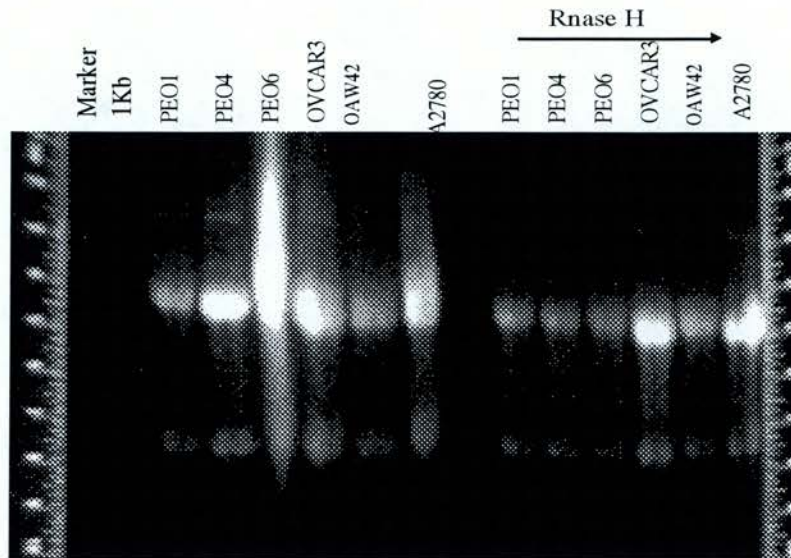
Kb	log 10
9.49	0.977
7.46	0.873
4.40	0.643
2.37	0.375
1.35	0.130
0.24	0.620

2.2.2.3; The hybridisation and development of the X-ray film for Northern blot as described as below.

2.2.3 RNaseH Assay of ovarian cancer cell lines total RNA

10 µl of total RNA was dried down in speed oven. 10 µl of oligo(dT) was added to 15 µl of buffer A (100 mM KCl and 10mM MgCl₂) then incubated it at 65 °C for 2 minutes and then annealed at room temperature in the dark for 30 minutes . Annealed samples were digested with 2 units of RNase H in 20 µl of buffer B (50 mM KCl, 11.25 mM MgCl₂, 62.5 mM Tris, pH 7.8 and 1.25 mM dithiothreitol) for 30 minutes at 37 °C, after which the reactions were stopped by the addition of 50 µl of buffer C (20 mM EDTA and 0.3 M NaOAc, pH 5.5). Following a single phenol/chloroform extraction, the digested RNA was ethanol-precipitated overnight at -20 °C and subjected to Northern blot analysis by using the Barx2 probe as described above. Figure 2-6 showed the results of a running gel for RNaseH blot

Figure 2-6 Running Gel for RNase Blot



2.3 The *Structure Analysis of Barx2 Gene in Cancer*

2.3.1 *The Southern blot*

2.3.1.1 Extraction of high molecular weight DNA of cancer cell lines

Cell lines was harvested, aspirating the medium and washing cells with PBS twice then adding trypsin solution. After the cells became detached from the flask, Up to 1×10^7 cells were collected by centrifugation at 600 g for 5 minutes at 4 °C. The supernatant was discard without disturbing the pellet. The cells were resuspend in 0.1ml reagent A and left on ice for 5 minute. Centrifuge at 1300 g for 5 minutes at 4 °C and discard the supernatant.

2 ml of reagent B was added to the pellet and vortexed briefly to resuspend the pellet, then the samples was incubated at 37 °C for 10 mines. Cell suspension was transferred to a 5 ml screw-capped polypropylene centrifuge tube. 15 μ l of a 50 μ g/ml

RNase A solution was added and incubated at 37 °C for 30 minutes. 500 µl of sodium perchlorate was added and then mixed by inverting 7 times.

2 ml of chloroform was added and mixed by inverting 7 times to emulsify the phases. 300 µl of Nucleon resin was added and without re-mixing the phases, centrifuges at 1300 g for 3 minutes.

Holding the tube vertically, without disturbing the Nucleon resin layer, The upper phase was transferred to a fresh tube of minimum volume 7.5 ml. Two volumes of cold absolute ethanol were added until the DNA was precipitated.

The DNA was pelleted, resuspended and washed in 70% cold ethanol, then the DNA pellet was air-dried, resuspended in 1xTE (pH 7.4) and finally the concentration was estimated by spectrophotometer.

2.3.1.2 Quantification of DNA by spectrophotometer

5 or 10 µl of the DNA solution were added to 995 or 990 µl distilled water. Spectrophotometric reading of absorbance at 260 nm (O.D. 260) is proportional to the concentration of DNA in the sample. Absorbency at 280 nm divided by absorbance at 260 nm indicates the purity of the sample. A value of 1.8 is optimal and ratios below this indicate contamination of the sample. An O.D. 260 of 1.0 is equivalent to a DNA concentration of 50 µg/ ml, allowing simple proportional calculations to determine DNA concentration of any sample.

2.3.1.3 Digestion of DNA

10 µg genomic DNA was dissolved in sterile distilled water using bore pipette tip to measure out the DNA and then standed the sample for overnight at 4 °C in order to fully solubilise. 10x reaction buffer was added to give a 1x solution and spermidine and BSA to final concentrations of 5mM and 0.1 mg per ml respectively. The sample was mixed by tapping the tube and then allowed to stand for 5 minutes on ice to equilibrate. Finally the enzyme was added (EcoR I and BamH I respectively), 1 unit

per 1µg of DNA and then gently mixed with the sample by tapping the tube and spun briefly. The samples were incubated for 1 or 1.5 hour at 37 °C. Taking out 1 µl of digested DNA (mixing with 2 µl of DNA loading buffer and 7 µl of distilled water) to run a mini gel (1% agarose gel) in order to check if the sample was fully digested. If the DNA was completely digested then the reaction was stopped by adding 0.5 M EDTA (pH 7.5) to a final concentration of 10mM.

2.3.1.4 DNA precipitation

3 volumes of cold ethanol (100%) was added into the sample and then allowed to stand for overnight at -20 C. The sample was centrifuged at 13000 g for 15 minutes at 4 C. The ethanol mixture was removed and discard and then the sample was washed with 1 ml of 75% cold ethanol then spun at 13000g for 15 minutes at 4 C. Removed the ethanol and discarded it and then the sample was dried in vacuum jar. The DNA was resuspended in 20 µl of 1xTE (pH 7.6) then incubated for 1 hour at 37 C. The sample was cooled to 4 C then stored frozen at -20 C.

2.3.1.5 Blotting

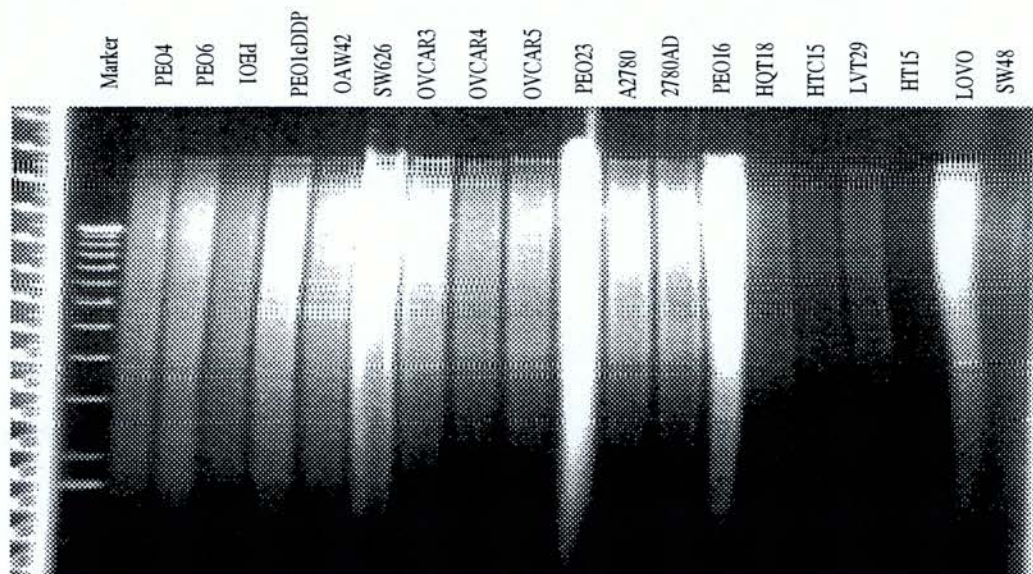
0.7% of the agarose gel was prepared, then thawed and heated to 56⁰C for 2 minutes. 0.2 volume of electrophoresis sample buffer was added. The samples were spun briefly then loaded slowly into the gel and 1 kb of DNA ladder was also loaded at each side of the gel as markers.

The samples was allowed to sit in the gel for a few minutes after loading them. The gel was run at 30 volts overnight in 1xTAE buffer. The next day electrophoresis continued at 70 volts until the dye front was 4/5 down the gel. The gel was removed from the tank and placed in a pyrex dish on a shaking platform.

The gel was soaked in 0.5µg/ml ethidium bromide in 1xTAE in the shaking platform for 30 minutes and then destained for 10 minutes in distilled water.

The gel was photographed under UV light with a ruler at the side of the marker lane. The position of the ladder DNA fragments was accurately measured on the gel. Figure 2-7 showed the blot of DNA digested with EcoR I

Figure 2-7 the Blot of DNA Digested with EcoR I



The gel was soaked in 0.2 N HCL for 10 minutes in the shaking platform and then rinsed gel briefly in distilled water. The gel was soaked in denaturation solution (1.5M NaCL, 0.5M NaOH) with shaking platform for 45 minutes and then rinsed briefly in distilled water. Neutralisation solution (1M Tris pH 7.4, 1.5M Nacl) was added for 30 minutes on the shaking platform and the solution was changed at 15 minutes and then gel was briefly rinsed in distilled water.

Hybond N membrane and 3MM filter paper were made for transfer. Wet Hybond N membrane was incubated in 2xSSC for 5 minutes. Transfer was performed overnight in 20xSSC.

After transfer, the membrane was soaked in 6xSSC for 5 minute and dried for 30 minutes at room temperature. The membrane was UV irradiated by using GS GENE LINKER UV chamber with program C2.

2.3.1.6 The preparation of probe of Barx2

Barx2 plasmid was used as template generate the Barx2 probe. The plasmid used was construct containing full length Barx2 fused to thioredoxin in pET32a (Novagen), with the 8 amino acid FLAG epitope added at the beginning of the Barx2 moiety. Barx2 primer (F1/R3) was used to amplify the full length coding region sequence of Barx2 gene. The PCR product of Barx2 was purified and used as probe. The primer sequence and PCR condition are described as below.

Dr Barry D. Nelkin kindly donated pET32a-Barx2

2.3.1.7 The hybridisation of Southern blots

2.3.1.7.1 Pre-hybridisation

20 ml of Hybond hybridisation mixture was placed in a universal tube and heated to 68 °C in the hybridisation oven.

200 ml of 0.1% of SDS was placed in a pyrex dish then heated to boiling point. Gauzes (two pieces per blot) were added and incubated for 5 minutes after turned off the heat.

120 µl of sonicated salmon sperm DNA (10 mg /ml) was placed in a sterile 1.5 ml eppendorf tube and then boiled for 5 minutes then immediately quenched on ice.

30 ml of 6x SSC was placed in a pyrex dish. Gauze was added and the blot to be hybridised was placed on top with second a piece of gauze to cover the blot. The blot and gauzes were rolled up into a cigar shape then placed into hybridisation bottle. The bottle was unrolled then the 6xSSC was poured out of the bottle. 100 µl of sonicated salmon sperm DNA with 20 ml of hybridisation mixture was then added in the hybridisation bottle.

The blot was incubated for 24 hours at 68 °C in the hybridisation oven.

2.3.1.7.2 The hybridisation.

100µl of sonicated salmon sperm DNA was placed into a sterile eppendorf tube then boiled for 5 minutes and cooled on ice immediately.

A solution containing 50-75 ng of probe was prepared in 13 µl of sterile distilled water then boiled for 5 minutes and cooled on ice immediately. 4µl of High Primer was added with 3 µl of ³²P dCTP, 300µCi/mmol into the solution containing the probe. The solution was incubated for 15 minutes at 37 °C then quenched the reaction on ice.

A Whatman glass microfibre filter was placed on sheet of plastic inside a perspex box. 0.5 µl of probe was placed on the filter. The filter was placed into a Manifold pump apparatus. The upper reservoir was filled with 5% TCA through under vacuum and repeated. The filter was removed from the manifold and the ³²P level was monitored. If the level of radioactivity left on the filter after TCA extraction was low then it was not worth carrying on and a fresh labelling reaction was set up.

A NICK column was filled with 1x TE and allowed to drip through. 80 µl of 1xTE was added to the solution containing probe and then added to the centre of the column following by 300 µl of 1x TE to the column and again allowed to drip through the column. An eppendorf tube with 100 µl SSC DNA was placed under the column and 400 µl of 1x TE was added to the column then allowed drip through the column. This should contain a high level of ³²P. The solution was incubated in the 100 °C hot block, covered with a lead pot for 10 minutes to denature the DNA, then quenched the on ice immediately.

500 µl of probe with solution was added in the hybridisation bottle and blot was incubated for another 24 hours at 68 °C in the hybridisation oven.

2.3.1.7.3 Washing membrane

Hybridisation wash buffer A (2x SSC, 0.1% SDS) and B (0.2xSSC, 0.1% SDS) were heated to 68 °C. The hybridisation liquid was poured off. Hybridisation wash buffer

A was added and incubated for 20 minutes at 68 °C in the hybridisation oven then discarded. Hybridisation wash buffer B was added until the bottle was half full then incubated for 20 minutes at 68 °C in the hybridisation oven then discarded. This was repeated.

The blot from the hybridisation bottle was placed in a plastic box with the hybridisation wash buffer B and incubated for 20 minutes at 68 °C in the hybridisation oven with the shaking platform.

The blot was placed it in plastic bag then exposed to X-ray film in a film cassette at -70°C. The X-ray film was developed in a GURIX 60-AGFA developer.

200 ml of 0.1% SDS was placed into a pyrex dish and heated to boiling point. Once the solution was boiling, added the blot into the solution and turned off the heat source and the blot was incubated for 5-10 minutes to stop it. The blot was removed and rinsed in 6xSSC. The blot was sealed into a plastic bag and was exposed to X-ray film for 5-7 day to check that no radioactivity was detected.

2.3.2 Pulsed-Field Gel Electrophoresis (PFGE)

2.3.2.1 Method

PFGE allows the separation of DNA ranging in size from a few kb pairs to 10 µb pairs. Because of the large size of these molecules, simple pipetting mechanically shears the DNA resulting in unacceptable quality for PFGE separations. This has necessitated procedures for lysis of whole cells embedded in agarose, allowing purification of chromosome-size DNA without shearing.

2.3.2.2 Preparation of agarose embedded mammalian DNA

A Cell suspension was prepared in tissue culture medium (OAW42. A2780. PEO4 and FATU cell lines in RPMI-1640; OVCAR3 in MOD) without fetal bovine

serum. The cells were counted and removed 5×10^7 cells for 500 μl of agarose plugs to be made (100 μl /plug for disposable mold) then placed on ice.

The 2% clean-cut agars solution was melted by using a microwave and equilibrated to 50 $^{\circ}\text{C}$ in a water bath.

The amount of Cell Suspension Buffer calculated. For a final concentration of 0.7% agar 0.63 ml of Cell Suspension Buffer per 1 ml of agar plugs was used. 0.37 ml of 2% CleanCut agarose per ml of agarose plugs was used.

The cell suspension was centrifuged at 1,000x g for 5 minutes at room temperature. The cells were resuspend in the volume of Cell Suspension Buffer calculated above and equilibrated the cell suspension to 50 $^{\circ}\text{C}$.

The calculated volume of 2% CleanCut agarose was combined with the cell suspension and mixed gently, and kept at 50 $^{\circ}\text{C}$, the transferred to plug molds by using sterile pipettes and incubated for 15 minutes at 4 $^{\circ}\text{C}$ in order to allow the agarose to solidify.

A 50 ml conical centrifuge tube was used to which was added 200 μl of Proteinase K stock to 5 ml of Proteinase K Reaction Buffer for each ml of agarose plugs. the solidified agarose plugs were pushed into 50 ml centrifuge tube containing the Proteinase K solution, and incubated overnight at 50 $^{\circ}\text{C}$ without agitation.

2.3.2.3 Restriction enzyme digestion of pluges

A plug was placed for each digest in a sterile 1.5 ml microcentrifuge tube, and wash once for 1 hour in 1 ml 0.1x Wash Buffer, decanted and resuspend in a sufficient amount of fresh 0.1x Wash Buffer to cover the plugs. This last wash reduces the EDTA concentration, allowing faster buffer equilibration with restriction enzyme buffers

Wash buffer was aspirated and 1 ml of the appropriate 1x restriction enzyme buffer was added and incubated for about 1 hour with gentle agitation at room temperature. The buffer was aspirated off and 0.3 ml of fresh 1x enzyme buffer and the restriction enzyme Hind III and Sac I were added respectively (50 u per 100µl plug) and incubated overnight at appropriate temperature. The southern blot was performed for Pulsed-field gel described as above

2.4 Mutation Analysis of Barx2 Gene.

2.4.1 Polymerase Chain Reaction (PCR) of genomic DNA for amplifying Barx2 exon

2.4.1.1 The design and treatment of primer

The primers were designed on the basis of encoding sequences of human Barx2 gene. There are 4 pair of primer in total. The table 2- 5 shows primer sizes, sequences in relation to exons.

Table 2 - 5 The Primers of Barx2 for PCR of Genomic DNA

Primer	Sequence	Size (bp)	Exon
F11/R5	5' -TACACGGACGTGAAAGCTAC-3' 5' -TCACCATGCACTGCCACG-3'	213	1
F6/R6	5' -CAGGTCCTGGCCTGCTTCCC-3' 5'' -CCCACAATGGGAGCAAGTCT-3''	426	2
F9/R9	5'-CCAGGCTCCCCTTCCCTGCGGGCA-3' 5'-TCACCTGTCTGGGGTTGACAAATA-3'	309	2
F10/R1	5'-TCCTGCTGCCTCCCATTCTG-3' 5'-CAACAGCTTCCCCGCAAGCC-3'	300	3
F8/R8	5' -TGGAGGGAAGGAATTATTC-3' 5' -AGTCTCCCTCTTCCCTCAAA-3'	349	4

Oligonucleotides were synthesised at the ICRF oligonucleotide laboratory and were treated described above.

2.4.1.2 Optimization of reaction condition of PCR

PCR was performed in a reaction volume of 25 μ l. Reaction conditions for PCR optimization were performed for $MgCl_2$ and template quantity. Figure 2-8 shows results of optimization PCR with different concentration of $MgCl_2$ by using primer F6/R6.

Figure 2-8 the Result of Optimization PCR with Different Concentration of $Mgcl_2$ by Using Primer F10/R10.

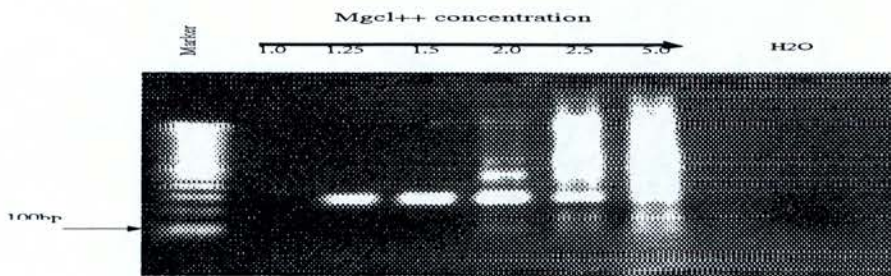


Table 2 –6 shows all optimised reaction conditions for genomic of Barx2 exon amplification

Table 2-6 Optimization Reaction Condition for PCR of Genomic DNA for Amplification Exon of Barx2 (μ l)

Primer	10x Buffer	dNTP (10mM)	$Mgcl_2$ (25mM)	Taq Gold Mix (u/μ l)	Primer (50pomal/ μ l)	DNA (0.5 mg/ μ l)	H ₂ O
F11/R5	2.5	0.5	1.0	0.2	0.25	2.5	17.8
F6/R6	2.5	0.5	1.0	0.2	0.25	2.5	17.8
F9/R9	2.5	0.5	1.5	0.2	0.25	2.5	17.3
F10/R10	2.5	0.5	1.5	0.2	0.25	2.5	17.3
F8/R8	2.5	0.5	1.5	0.2	0.25	2.5	17.3

2.4.1.3 PCR programme

Two touchdown PCR programmes were used as follow.

One was used for amplifying exon 1, 2 and 3 as follow;

95⁰ C for 10 minutes 1 cycle; 94⁰C for 30 sec/63 °C for 30 sec/72°C for 42 sec 2cycle; 94⁰C for 30 sec/61 °C for 30 sec/72 °C for 42 sec 2cycle; 94⁰C for 30 sec/59 °C for 30 sec/72 °C for 42 sec 2cycle; 94⁰C for 30 sec/57 °C for 30 sec/72 °C for 42 sec 2cycle; 94⁰C for 30 sec/55 °C for 30 sec/72 °C for 42 sec 30cycle; then final 72 ° C for 5 minutes.

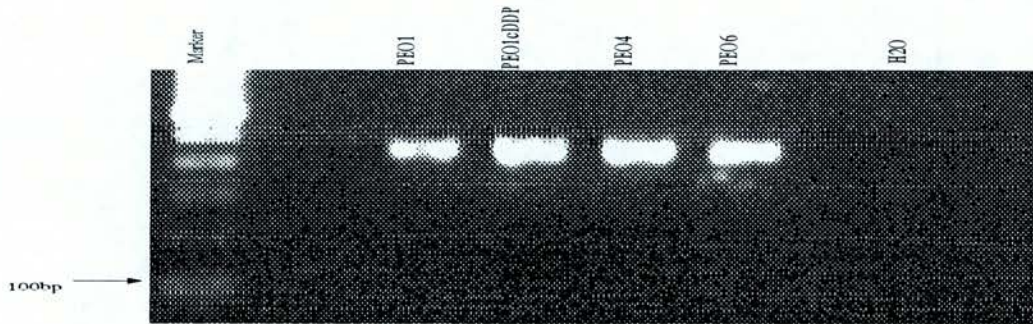
The second was used for amplifying exon 4 as follow.

95⁰ C for 10 minutes 1 cycle; 94⁰C for 30 sec/61°C for 30 sec/72°C for 42 sec 2cycle; 94⁰C for 30 sec/59°C for 30 sec/72 °C for 42 sec 2cycle; 94⁰C for 30 sec/57 °C for 30 sec/72 °C for 42 sec 2cycle; 94⁰C for 30 sec/55 °C for 30 sec/72 °C for 42 sec 2cycle; 94⁰C for 30 sec/53 °C for 30 sec/72 °C for 42 sec 30cycle; then final 72 ° C for 5 minutes.

2.4.1.4 Checking of PCR products by Agarose gel electrophoresis

5 µl of reaction product with 1 µl of electrophoresis buffer were checked on standard 2% agarose run at 100-120 volts in 1x TAE. The 100 bp DNA ladder was used as a convenient marker for size estimates of the products. The gel was stained with ethidium bromide and visualised under UV light. Figure 2-9 shows the results of products of genomic PCR for primer F6/R6 of barx2 in cancer cell lines.

Figure 2-9 the Result of Products of Genomic PCR in Cancer Cell Lines by Using Primer F6/R6 of Barx2.



2.4.2 *Single stranded conformation polymorphism analysis (SSCP)/Heteroduplex mutation analysis*

2.4.2.1 Electrophoresis

A non-denaturing 10% acrylamide, 3% bis-acrylamide gel was prepared. The buffer concentration in the gel was 1x TBE. 5 microlitres of PCR product were mixed with 2 microlitres of denaturing dye (95% formamide, 20mM EDTA, 0.25% Bromophenol blue and 0.25% xylene cyanol), denatured for 3 minutes at 98^oC and then placed immediately on ice. 7 μ l of mixture was loaded on to the gel with duck billed tips and run in the cold room (4 ^oC) with 1x TBE buffer in gel apparatus (PROTEAN TM II). The gel was run at 25 mA for 17 hours.

2.4.2.2 Silver staining

Following electrophoresis the gel was transferred on the back-plate to a dish for silver staining.

The gel was soaked in 10% ethanol for 5 minutes, 1% nitric acid for 3 minutes, then rinsed briefly in distilled water.

The gel was then soaked for 20 minutes in a freshly prepared solution of 1mg/ml silver nitrate in distilled water, ensuring that silver nitrate was completely dissolved before being added to the gel. After that, the developer buffer, a solution of 15 g

anhydrous sodium carbonate in 500 ml of distilled water and 250 µl of the formaldehyde, was prepared.

The gel was rinsed in distilled water to remove the silver nitrate and 100 ml of the developer buffer was added until the buffer turned brown and was then discarded. The remaining 400 ml of developer buffer was added; at this stage the nucleic acid bands would begin to become visible in the gel. When the desired intensity had been reached the developer buffer was discarded.

The gel was rinsed twice in the water and the reaction was stopped with 10% glacial acetic acid for 2 minutes.

The gel was photographed and then dried on the gel drier at 80 °C for 2 hours to make a permanent record.

2.4.3. Denaturing High Pressure Liquid Chromatograph(DHPLC)) for mutation screening of the Barx2 in cancer cell lines and tumour tissues

2.4.3.1 The genomic PCR of Barx2

Genomic PCR of Barx2 (exon 1 to exon 4) was performed in total 64 cancer cell lines and 47 cases of colorectal cancer tissues for mutation screening of the Barx2. The method of genomic PCR for the Barx2 in cancer cell lines or tumour tissues was the same as description above.

2.4.3.2 The mutation analysis of the Barx2 using DHPLC (WAVE)

Mutation analysis was performed at the Imperial Cancer Research Fund Mutation Detection Facility at St James' Hospital, Leeds, UK. DHPLC was performed using a Transgenomic Wave machine.

2.4.4 Subcloning of PCR Product

2.4.4.1 Purification of PCR product

The PCR products were gel-purified by using the Wizard PCR Preps DNA Purification System.

A 2% low-melting temperature agarose was prepared and samples were run on low-melting temperature agarose gel, ethidium bromide stained and visualised under UV light. The correct slice was cut out from the gel.

The slice (about 300mg) was transferred to a microcentrifuge tube and incubated at 70 °C until the agarose was completely melted.

1 ml of resin was added to the melted agarose slice and then mixed thoroughly for 20 second. One Wizard Minicolumn was prepared for each sample. The syringe barrel was attached to the Luer-Lok extension of each Minicolumn.

The Resin/DNA mix was pipetted into the syringe barrel and then the syringe plunger was inserted slowly, and the slurry was gently pushed into the Minicolumn with the syringe plunger.

The syringe was detached from the Minicolumn, and the plunger was removed from the syringe. 2 ml of 80% isopropanol was pipetted into the syringe to wash the column and the syringe plunger was used to gently push the isopropanol through the Minicolumn.

The syringe was removed and the Minicolumn was transferred to a 1.5 ml microcentrifuge tube and centrifuged for 1 minute at 13000g to dry the resin.

The Minicolumn was transferred to a new microcentrifuge tube. 50 µl of distilled water was applied to Minicolumn and after 2 minutes, it was centrifuged at 13000g for 1 minute to elute the bound DNA fragment. The concentration of post-purification DNA was estimated by spectrophotometric reading of absorbency at 260 nm (O.D. 260).

2.4.4.2 Ligation using the pGEM-T Easy Vectors

Ligation reactions were set up as described below

<u>Reagent</u>	<u>Quantity</u>
T4 DNA ligase 10x Buffer	1 μ l
pGEM-T Easy Vector	1 μ l (50 ng)
PCR product	5 μ l (about 25 - 75 ng)
T4 DNA Ligase	1 μ l (3 Weiss units)
<u>deionized water</u>	<u>a final volume of 10 μl</u>

The reaction was mixed by pipetting then incubated overnight at 4 °C.

2.4.4.3 Transformations

For each ligation reaction, two agar/ampicillin/PTG/X-Gal plates were prepared and equilibrated the plates to room temperature prior to plating

The tubes containing the ligation reaction were centrifuged to collect contents at the bottom of the tubes. 2 μ l of each ligation reaction was added to sterile 1.5 ml tube on the ice.

Frozen (-70 °C) JM 109 High Efficiency Competent cells were placed in ice until thawed. Cells were mixed by gently flicking the tube. 50 μ l of cells was incubated with ligation mixture on ice for 20 minutes.

The cells were heat-shocked for 45 seconds in a water bath at exactly 42 °C, and immediately returned on to ice for 2 minutes.

950 μ l of room-temperature SOC medium was added to the tubes containing transformed cells and incubated for 1.5 hours at 37 °C with shaking.

100 μ l of transformation culture was plated onto an agar/ampicillin/PTG/X-Gal plate and another 900 μ l of transformation culture was centrifuged and resuspended in 100 μ l of SOC medium then plated onto a second agar/ampicillin/PTG/X-Gal plate.

The plates were incubated overnight at 37 °C. After overnight incubation, the blue/white colonies could be seen on the plates. White colonies generally contained insert.

2.4.4.4 Bacterial culture

2.4.4.4.1, Media and additives

All media was sterilised by autoclaving prior to use.

LB media was provided by the MRC Human Genetics Unit.

Ampicillin;

Ampicillin was added to the above at a final concentration of 50 µg/ml to select for bacteria carrying the *amp* antibiotic resistance marker conferred by the plasmid transformed into the bacteria. Ampicillin (Sigma) stock solution was prepared sterile at a final concentration of 50 mg/ml and stored at -20 °C.

2.4.4.4.2, Bacterial culture

5-10 ml of LB containing ampicillin was added into sterile tubes. A single whole colony was picked up from the plate and transferred to a tube, and incubated at overnight at 37 °C with shaking. Next morning the cells were pelleted by centrifugation (1300 rpm at room temperature for 10 minutes)

2,4.4.5 Identification of bacterial colonies that contain Barx2 plasmid.

PCR was used to identify the bacterial colonies that contain Barx2 plasmid. Barx2 F1/R3 oligonucleotides were used as primer for PCR.

Each PCR reaction mixture contained 2.5 µl 10x PCR buffer, 1.5 µl 10mM MgCl₂, 0.5 µl of 10 mM dNTP, 0.2 µl of Taq polymerase (5u/µl), 12.5 pmol of each primer and 1µl suspension bacterial colonies. The total reaction volume was 25 µl.

PCR was performed under the following condition; 94 °C for 5 minutes in cycle. 94 °C for 30 seconds. 55 °C for 30 seconds. 72°C for 42 seconds, repeated for 35 cycles, then 72°C for 5 minutes in cycle.

5 µl of reaction product with 1 µl of electrophoresis buffer were checked on standard 2% agarose run at 100-120 volts in 1x TAE and used the 100 bp DNA ladder as a convenient marker for size estimates of the products (the correct size of Barx2 is 780 bp). The gel was stained with ethidium bromide and visualised under UV light.

2.4.4.6 Plasmid DNA preparation

QIA Spin Plasmid Kit was used to extract the plasmid DNA.

Pelleted bacterial cells were resuspended in 250 µl of Buffer P1 and transferred to a microfuge tube. 250 µl of Buffer P2 was added and gently inverted the tube 4-6 times to mix. 350 µl of Buffer N3 was added and inverted the tube immediately but gently 4-6 times, and were centrifuged at 1300rpm at room temperature for 10 minutes.

QIAprep spin columns were placed in 2 ml collection tubes, then the supernatant which had been centrifuged was transferred to a QIAprep spin column.

QIAprep spin columns were centrifuged with 1300rpm at room temperature for 1 minute. The flow-through was discarded.

QIAprep spin columns were washed by adding 0.5 ml of Buffer PB and centrifuged at 1300rpm at room temperature for 1 minute. The flow-through were discarded.

QIAprep spin columns were washed again by adding 0.75 ml of Buffer PE and centrifuged at 1300rpm at room temperature for 1 minute. The flow-through were discarded, and the QIAprep spin columns were centrifuged for an additional 1 minute to remove residual wash buffer.

QIAprep spin columns were placed in a sterilised 1.5 ml microfuge tubes. To elute DNA, 50 µl of 10 mM Tris-HCL, pH 8.5 was added to center of each QIAprep spin

columns, let stand for 1 minute at room temperature and then centrifuged it for 1 minutes.

The concentration of plasmid DNA was estimated by spectrophotometric reading of absorbency at 260 nm (O.D. 260).

The plasmid DNA was stored at 4⁰ C.

2.4.5 *The sequencing of the PCR product directly or the plasmid DNA of PCR product subclone of the Barx2 gene*

2.4.5.1 Reaction mixture was prepared (using ABI PRISM™ dRhodamine Terminator C4cle Sequencing Ready Reaction Kit).

For each reaction, the following reagents was added to a tube and made the total volume in 20 µl

<u>Reagent</u>	<u>Quantity</u>
Terminator Ready Reaction Mix	8 µl
PCR product or plasmid DNA	30-90 ng (about 2-4 µl)
Primer	3.2 pmol (1 µl)
<u>Distilled water</u>	<u>q.s (about 9-7 µl)</u>

Then mixed well and spun briefly.

2.4.5.2 Sequencing.

The tubes were placed in the HYBAID thermocycler with the following parameters:

Rapid thermal ramp was performed to 96 °C for 1 minute in 1 cycle, then 96 °C for 30 seconds. Rapid thermal ramp to 50 °C was performed and samples were left for 15 seconds. The rapid thermal ramp to 60 °C was performed and samples were stood for 4 minutes, and this was repeated for 25 cycles.

Rapid thermal ramp down to 4 °C was then performed and held until ready to DNA precipitate.

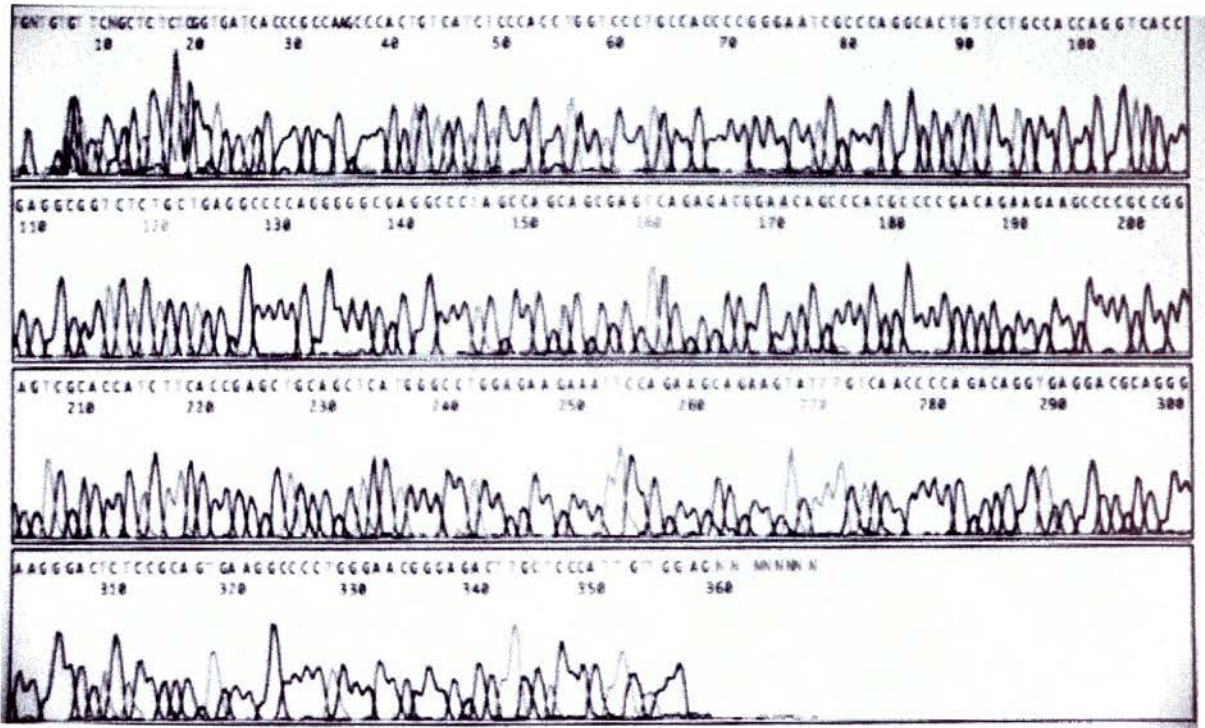
2.4.5.3 DNA precipitation

50 µl of 100% cold ethanol and 2 µl of 3 M sodium acetate (pH 5.6) were added to each tube then mixed thoroughly and incubated at room temperature for 1 hour. The samples were centrifuged at 13000 g for 20 minutes at 4 °C and then ethanol was removed. 0.5 ml of 70% cold ethanol was added to each tube and then centrifuged at 13000 g for 15 minutes at 4 °C. Ethanol was removed and the samples were dried in a Universal Vacuum System for 2-5 minutes.

2.4.5.4 Analysis of sequence result

The samples were run on an ABI 377 DNA sequencer (Agnes Gallagher MRC Human Genetics Unit), and reactions were analysed using ABI analysis software. The Figure 2- 10 shows an example of sequence analysis by using ABI analysis software.

Figure 2- 10 The Example of Sequence Result in ABI Analysis Software for the Barx 2 F6/R6 in ovarian cancer cell line



This is an example of sequence analysis using ABI analysis software, showing direct sequencing of the genomic PCR product of Barx2 (F6/R6) in the ovarian cancer cell line PEO4. Each peak represents an individual deoxyribonucleotide base (for example; cytidine, thymidine etc.).

2.4.6 DNA methylation investigation

The methylation status of Barx2 locus segments was studied using methylation sensitive-insensitive enzymes HpaII/MspI. HpaII is a methylation restriction sensitive enzyme that recognises the sequence CCGG, an isoschizomer of MspI, and blocked by methylation C of at CpG residue. MspI is a methylation insensitive enzyme, an isoschizomer of HpaII, and it can cleave the sequence even when the internal C residue is methylated.

10 µg of cancer cell line DNA was digested with 10 units of the enzymes overnight at 37 °C and run on a 0.7% agarose gel.

Southern blots were hybridized with a-³²P-dCTP nick-translated DNA probe in previously described conditions. The 5' region of the Barx2 used as probe was derived from a PCR product using F11/R1 to amplify exon 1 of Barx2 gene using plasmid DNA of Barx2 as template.

2.5 The Functional Analysis Of Barx2 Gene

2.5.1 The transfection of ovarian cancer cell lines with Barx2 gene

2.5.1.1 Plasmid preparation

2.5.1.1.1 Plasmid

PBabe-Hygro, a 5.2 kb plasmid, was kindly donated by Barry D. Nelkin. This plasma confers resistance to Hygromycin (hmB) under the control of a retroviral promoter.

PBabe-Hygro-Barx2 that the full length human Barx2 cDNA was cloned into PBabe-Hygro expression vector was kindly donated by Dr Barry D. Nelkin.

The methods of bacterial transformation and extraction of plasmid DNA were as described above, expect that culture plate used just agar/ampicillin plate.

2.5.1.1.2 Linearisation and purification of plasmid DNA

The plasmids were linearised by using the restriction enzyme Not I which cuts out with the resistance marker or the resistance marker and Barx2 gene sequence in each of the plasmids. This was done to improve the transfection efficiency of the plasmid.

The DNA was purified by using the WizardTM DNA Clear-Up Resin.

2.5.1.2 The transfection of ovarian cancer cell lines with Barx2 gene

2.5.1.2.1 Ovarian cell lines culture

Sterile condition were maintained routinely. Cell lines were maintained on RPMI or DMEM/10% FCS/P+S. Cells were routinely cultured in humidified incubators at 37 °C, 5% CO₂. Sterile culture flasks, universal tubes, freezing vials, glassware, and solution were used throughout. All tissue culture was carried out in a laminar flow hood. Cells were harvested by washing twice in PBS and incubating with a minimal amount of trypsin/versene. Cells were resuspended in 10% FCS media and pelleted at 1200 rpm for 5 minutes for further manipulation.

Cells were frozen in liquid nitrogen, after initial storage for 24 hours at - 70 °C, preserved in freezing mix composed of 10 % RPMI in FCS. Recovery of the cells was effected by rapid thawing of the vial in a beaker of water at 37 °C, followed by washing twice in media, and plating into flasks.

OAW42, PEO1, PEO1cDDP PEO4, PEO6, A2780, ON3 and OVCAR3, which are ovarian cancer cell lines, were used as recipient cell lines in this project. OAW42 PEO4, PEO6, A2780 and PEO1 are maintained on RPMI/10% FCS/P+S, OVCAR3 and ON3 is maintained on DMEM /10% FCS/P+S. PEO1 cDDP is a cell line derived from PEO1 but is resistant to Cisplatin about 2-3 fold and also maintained on RPMI/10% FCS/P+S.

2.5.1.2.2 Effectene transfection of plasmid DNA into OAW42, PEO1 PEO4, PEO6, A2780 and PEO1 cDDP cell lines.

Two to three days before transfection, 2 x10⁵ cells of OAW42 cells or 3 x10⁵ cells of PEO1 or PEO1 cDDP were seeded per 60-mm dish in 5ml of RPMI/10% FCS/P+S.

Cells were incubated at 37 °C and 5% CO₂ in an incubator. The dishes should be 80% confluent on the day of transfection.

The Effectene volume was first optimised for each cell line, keeping the amount of DNA and time for transfection constant. After this, transfection was routinely performed, under the following condition:

1 µg of plasmid DNA (2 µl) was diluted in TE, pH 7.4 with the DNA-condensation buffer to a total volume of 150 µl. 8 µl of Enhancer was added and mixed by vortexing for 1 second.

The sample was incubated at room temperature (20-25 °C) for 2-5 minutes and then the mixture was spun down for a few seconds to remove drops from the top of the tube.

25 µl of Effectene Transfection Reagent was added to the DNA-Enhancer mixture. Then the sample was vortexed for 10 second.

The sample was incubated for 10 minutes at room temperature (20-25 °C).

While incubating the sample, the media was gently aspirated from the plate, and cells was washed once with PBS. 4 ml of fresh RPMI/10% FCS/P+S was added to the cells.

1 ml of RPMI/10% FCS/P+S was added to the reaction tube containing the transfection complexes then mixed by pipetting and immediately added drop-wise onto the cells in 60-mm dishes. The dish was gently swirled to ensure uniform distribution of the complexes.

The cells were incubated with the transfection complexes at 37 °C and 5% CO₂ to allow for gene expression.

2.5.1.2.3 Lipofectin transfection of plasmid DNA into OVCAR3, A2780 and ON3 cell lines.

2x 10⁵ cells/60-mm dish were seeded in 4 ml of DMEM /10% FCS/P+S.

Cells were incubated at 37 °C and 5% CO₂ for 24-48 hours. Cells were 30-50% confluent.

The following mixtures were prepared in sterile tubes respectively;

Mixture 1: For each transfection dilute 2 µg (4µl) DNA into 100 µl of DMEM.

Mixture 2: For each transfection dilute 10 µl of Lipofectin into 100 µl of DMEM, and allowed to stand at room temperature for 40 minutes.

Combined the two mixtures and mixed gently then incubated at room temperature for 15 minutes.

The cells were washed once with 2 ml of DMEM.

1.8 ml of DMEM was added to each tube containing Lipofectin reagent-DNA complexes. Mixed gently then overlaid onto cells.

The cells were incubated at 37 °C and 5% CO₂, A2780 for 6 hours and each of OVCAR3, ON3 for 12 hours.

The medium containing Lipofectin reagent-DNA was replaced with 4 ml of DMEM /10% FCS/P+S and incubated the cells at 37 °C and 5% CO₂ for other 48 hours.

2.5.1.2.4 Selection conditions

One exogenous dominant selectable marker was used for positive selection.

The bacterial gene Hygromycin B phosphotransferase is a gene isolated from E.Coli which inactivates Hygromycin B (hmB), an aminocyclitol antibiotic produced by streptomyces hygrosopicus which inhibits protein synthesis by interfering with ribosomal translocation and with aminoacyl-tRNA recognition. Hygromycin B resistance was mediated by the expressing plasmid.

To determine the optimal geneticin concentration of Hygromycin B in selection medium, kill curves for Hygromycin B were performed for each of the parent un-transfected cell lines prior to introduction of the selectable markers by either Lipofectin or Effectene transfections.

2.5.1.2.5 Picking of resistant clones

After selection of cells using the Hygromycin B and conditions that completely kill parallel controls, resistant clones arising from single cells were picked at 3 weeks and 7 weeks after imposition of selection. When colonies reached about or over 1 mm in size, the flask or dish was washed with PBS. The tip of a fine tipped pastetette was half filled with trypsin/versene and applied to the clone, pipetting gently up and down until the cells were dislodged and then transferred to 24 well plates containing 500 μ l of selection media. The next day morning, the media was replaced by 1-2 ml media containing Hygromycin B at the appropriate concentration for that cell line.

2.5.2 Identification of transfection result of Barx2 gene in ovarian cancer cell lines

2.5.2.1 PCR of genomic DNA to identify Barx2 cDNA and Hygromycin B in ovarian cancer cell lines transfected with Barx2 gene.

2.5.2.1.1 Rapid miniprep extraction of DNA of cell lines.

Cell lines were harvested, the medium was aspirated and cells were washed with PBS twice. Trypsin solution was added. After the cells detached from the flask, they were collected in medium. Using the QIAamp Kit carried out the extraction of DNA of cell lines.

20 μ l of proteinase K was pipetted into the bottom of a 1.5-ml microcentrifuge tube.

The cell sample (up to 10^7 in 200 μ l PBS) was added to 1.5-ml microcentrifuge tube, adding 200 μ l of buffer AL to sample, mixing by vortexing for 15 sec.

The sample was incubated at 56 $^{\circ}$ C for 10 minutes, briefly centrifuged to remove drops from the inside of the lid.

200 μ l of absolute ethanol was added to the sample, and mixed again by vortexing for 15 sec, and briefly centrifuged again.

The lysate was applied to the QIAamp spin column and centrifuged at 6000g for 1 minute. Placing the QIAamp spin column in a clean 2 ml collection tube, the filtrate was discarded.

500 µl of Buffer AW1 was added and centrifuged at 6000 g for 1 minutes. The QIAamp spin column was then placed in a clean 2 ml collecting tube and discarded the collected tube containing the filtrate.

500 µl of Buffer AW2 was added and centrifuged at full speed for 3 minutes. The QIAamp spin column was then placed in a clean 2 ml collection tube and the collected tube containing the filtrate was discarded then centrifuged at full speed for a further 1 minute.

The QIAamp spin column was placed in a clean 1.5 ml collecting tube and the collected tube containing the filtrate was discarded. 100 to 200 µl of Buffer AE was added then incubated at room temperature for 2 minutes, and centrifuged at 6000 g for 2 or 3 minutes to elute the DNA, and was stored at 4 °C after the concentration was estimated by spectrophotometer.

2.5.2.1.2 Genomic PCR for testing the Barx2 insert and Hygromycin B marker in the Ovarian cancer cell lines transfected with Barx2 gene or Hygromycin B

The Barx2 gene primer was used F1/R3 (see above).

The Hygromycin primer was used as following;

5' -ATTTCGGCTCCAACAATGTC-3'

5'-GATGTTGGCGACCTCGTAT-3'

PCR mixture and condition were the same as those described above.

2.5.2.2 Northern blot and RT-PCR for detecting expression of Barx2 gene

in ovarian cancer cell lines transfected with Barx2 gene.

The Barx2 gene primer used was F1/R3. The PCR product of full length coding region sequence of Barx2 was used as probe. The method of Northern blotting and RT-PCR was the same as those described above.

2.5.3 *Growth Curves*

2.5.3.1 Setting up

Log phase cultures were harvested and 5×10^4 cells were seeded in 24 well trays. The media was changed 24 hours prior to first harvest and every 3 days thereafter. Cell counts were harvested every 2 or 3 days depending on the cell line up to 21 days in some cases.

2.5.3.2 Harvesting cells

The medium from the wells to be counted was removed, and the cells were washed twice with 250 μ l PBS. Then 250 μ l trypsin/versene was added and the cells were left for 15 minutes to completely detach. The cells in each well were syringed 3 times and 200 μ l of the cell suspension were transferred to one counting pot.

2.5.3.3 Use of the coulter counter

The coulter counter was allowed to warm up for 15 minutes. The orifice tube was rinsed with saline. A background count was taken to ensure that counter is working properly (background counts >20 suggest caution). The sample was then counted by aliquoting 9.8 ml saline to a counting pot and 200 μ l trypsinised syringed single cell suspension was added, mixing and sampling counts from each pot three times. After sampling, the coulter counter was rinsed with saline.

2.5.3.4 Analysis

The cell counter were plotted against time either on logarithmic or linear plots allowing comparisons between parent/control cell lines and cell lines transfected with the Barx2 gene

2.5.4 *MTT assay*

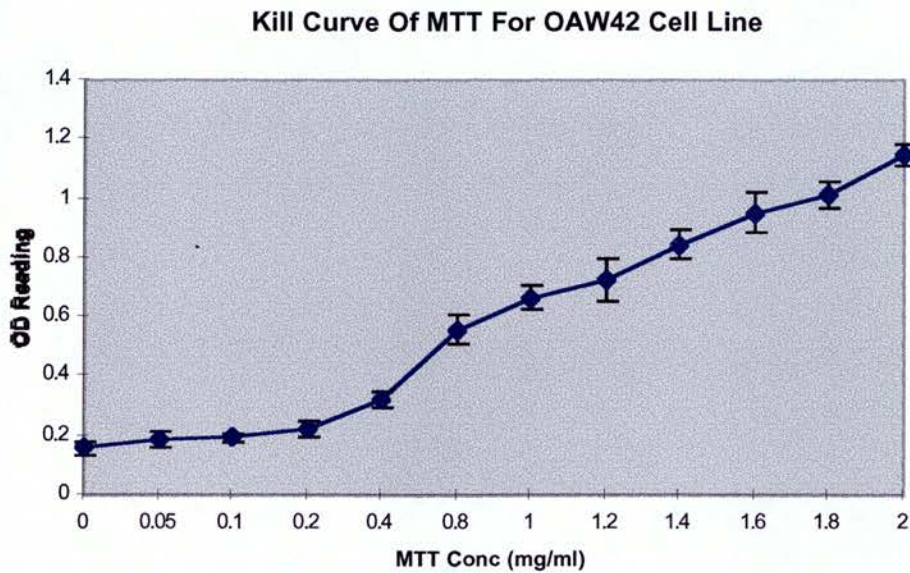
The matrigel invasion, migration and adhesion assays outlined below both depended on the MTT assay in order to quantitate the differences between cell lines transfected with Barx2 gene and control cell lines. Development of the MTT assay is outlined in this section.

An initial MTT toxicity curve was generated to determine the optimal MTT concentration for the 3 hour incubation. 5×10^3 cell were plated in each well of a 96 well plate. MTT was made up as a filter sterilised solution in BPS (2mg/ml) and wrapped in foil (MTT is light sensitive). 24 hours later MTT at a range of concentration between 0.005 to 1.4 mg/ml was added to the 96 wells and then incubated for 3 hours after wrapping the plate in foil.

A computerised plate reader to read absorbance at 570 nm was allowed to warm up for 15 minutes prior to use.

The 96 well plates were unwrapped and MTT was aspirated to a burnable waste container. 200 μ l of spectroscopic grade DMSO was added and the content of the well was mixed to solubilise MTT. The OD at 570 nm was then read immediately and the data was to a simple output data display program. Data was printed out and the peak concentration indicating activity at that cell number was observed from a graph plotting concentration of MTT versus OD at 570 nm. Figure 2 – 11 shows kill curve of OAW42 for MTT. The plate was disposed of in sawdust filled bag to burnable waste bin.

Figure 2 – 11 Kill curve of OAW42 for MTT.



2.5.5 Adhesion assays

This assays quantifies the ability of cells to attach to components of extra-cellular matrix. It was done by using Collagen-Coated Cell Wells™ (24 well tray).

Late log-phase cells were harvested and syringed to form a single cell suspension. The cells were resuspended with RPMI (serum free). 5×10^4 cells/ml/well were placed in each Collagen-Coated Cell Well. At same time, 5×10^4 cells/ml/well were placed in each well of 24 well tissue culture tray as a control. The sample was incubated at 37 °C and 5% CO₂ for 24 hours. After that, the media was removed very gently. The cells were gently washed 3 times in serum free media. The number of cells on the well was assessed using the MTT assay. 100 µl of 2mg/ml MTT was added into each well and the sample was incubated at 37 °C and 5% CO₂ for 3 hours with wrapped in foil. The media was removed and 1 ml of spectroscopic grade DMSO was added. The sample was incubated for 15 minutes at room temperature with the shaking platform and then 200 µl aliquots of solubilised crystals were transferred to 96 well tray and read at 570 nm on the spectrophotometer.

2.5.6 Matrigel invasion assays

This assay is based on the principle that basement membrane plays an important part as a barrier against tumour cell invasion. The invasion assay measures the ability for cells to invade through a matrigel layer in vitro. It is necessary to inhibit protease inhibitors present in serum in order to allow invasion to occur. Therefore FCS was pre-treated by lowering the pH to 3.0 for 2 hours with HCl and then neutralised to pH7.3 with NaOH.

Pre-aliquoted Matrigel (Beckton Dickinson) was thawed on ice and diluted 1:5 in ice cold pre-treated culture medium at a final concentration of 1ml.

The matrigel layer was formed in Transwell cell culture chambers (Costar) which consist of 12 well plates with inserts containing a porous Nuclepore 12um polycarbonate membrane. The chamber's margin at the interface with the filter was treated by rubbing a fine layer of paraffin was to provide a water-repellent treatment and prevent meniscus formation thereby ensuring even matrigel thickness over the surface of the polycarbonate membrane. The membrane was wetted with cold culture medium and re-aspirated with a fine-tipped pastette. 140 µl of cold diluted matrigel was aliquoted into each transwell and even distribution of the extracellular matrix was ensured by tilting. The matrigel was allowed to gel by incubating at 37 °C for 30 minutes.

The cells to be used were trypsinised and washed 3 times in with cold culture medium and resuspended at a concentration of 10^5 cells per 0.5 ml. 1 ml culture medium was aliquoted into the lower compartment and 0.5 ml (10^5) cells were dispensed into the upper compartment. The transwell plate was incubated in a humidified incubator for a varying time course in order to optimise the time for the invasion assay.

The number of cells on the upper and under-surface of the porous membrane were assessed using the MTT assay. 50 and 100 µl of MTT stain were added to the upper

and lower compartments of the transwell respectively and incubated for 3 hours. The crystals were removed from the under-surface of the porous membrane using a 1 cm diameter circular Whatman filter paper, and the crystals were solubilised by immersing it in 1 ml DMSO. The medium was decanted from the upper chamber and the transwell was placed in a culture well containing 1 ml DMSO, and rotated on a clinical specimen rotator. 200 μ l aliquots of solubilised crystals were transferred to a 96 well plate and read at 570 nm on a spectrophotometer. Invasion was determined as the ratio of the under-surface A570 reading relative to the upper surface A570.

On the basis of the previous experiment in our lab, 48 hours and 72 hours were used for OVCAR3 and OAW42 invasion in matrigel respectively.

2.5.7 Transwell migration assay

The purpose of this assay was to quantify the haptotactic response of the ovarian cancer cells to extra-cellular matrix, i.e. observation and quantification of extra-cellular matrix component directed stimulation of cellular migration. This assay allows assessment of the relative contribution of the components to stimulation of migration and also allows an assessment of the effect of the Barx2 gene introduction on this process.

Cells were prepared so they were in the log phase of growth.

2 mg/ml stock solution of MTT in PBS was prepared and then filtered (0.22 μ) it and stored it at 4 ⁰C wrapped in foil to protect from light.

The solutions of collagen IV and BSA were prepared at a concentration of 10 μ g/ml in PBS and then the BSA solution was filtered.

250 μ l of the 10 μ g/ml of collagen IV solution per well was added in a 24 well tissue culture tray.

The transwell cell culture insert was placed in the tissue culture well so the underside of the well was in contact with the solution. Then incubated for 1 hour at 37 °C.

The transwell was transferred to 24 well plate contained 250 µl of 0.1% BSA in PBS per well and then incubated at room temperature for 1 hour.

The cells were trypsinised then washed the cells three times in 10 ml of serum free RBMI 1640 for OAW42 and DMEM for OVCAR3. The cells were syringed four times then counted and the cell density was adjusted to 5×10^4 cells per 100 µl in serum free RPMI 1640 for OAW42 and DMEM for OVCAR3.

The under surface of the well insert was washed twice and the BSA with 250 µl of PBS was replaced.

400 µl of serum free RPMI 1640 for OAW42 and DMEM for OVCAR3 was placed in the lower compartment.

100 µl of serum free medium containing 5×10^4 cells was added to the upper compartment, and incubated for 48 hours.

10 and 40 µl of 2mg/ml MTT was added to the top and bottom compartments respectively. Then incubated 3 hours at 37 °C with the plate wrapped in foil.

The untreated MTT was aspirated off from the lower well and pipetted off from the upper well.

The crystals were removed from the under surface of the well by using a 1 cm diameter circular whatman filterpaper and soaked in a 1 ml of spectroscopic grade DMSO. The crystals were solubilised by placing on a shaking platform for 15 minutes.

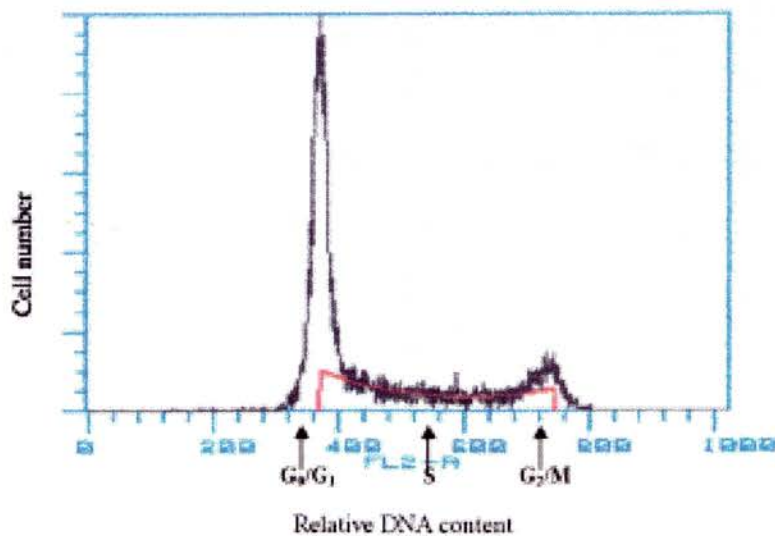
The transwell was placed in a culture well containing 1 ml DMSO and the crystals were solubilised by placing on a shaking platform for 15 minutes.

200 μ l aliquots of solution per sample were transferred into a 96 well plate and read at A570 nm . DMSO blanks were placed in the first column of tray to blank the plate reader. Colorimetric analysis (see above) was performed and the percentage migration was quantified compared with control plates (transwells coated with 0.1% BSA only on their under-surface).

2.5.8 *The analysis of cell cycle*

A single cell suspension was prepared by trypsinisation. Cells were fixed and stained with propidium iodide to bind DNA, then passed through the flow cytometer. Relative DNA content was assessed and distribution of the population of the cells with respect to the cell cycle was observed. A typical DNA histogram is shown in Figure 2-12. Cells are predominantly $1n$ with respect to DNA content, i.e., most are in G_0/G_1 . The second peak is for those in G_2/M phase post replication, when cell DNA content is $2n$. Relatively few cells exist in S phase.

Figure 2-12 Typical FACS DNA Histogram



2.5.9 *The FACS annexin-V assay for apoptotic cells*

This assay is devised to incorporate the total number of apoptotic cells released into the media rather than looking at the rate at which cells are being committed into apoptosis; cells which have undergone apoptosis and which are subsequently floating around in the media are therefore included along with the early-stage apoptotic cells which have not yet become detached.

The media was removed from each petri dish and transferred to labelled FACS tubes then spun at 1700rpm for 4 min and discarded to leave cell pellet at the bottom of tube.

The petri dishes were washed with 2 ml of PBS, and then 1.5 ml of trypsin was added then incubated in incubator until cells have become detached. 1.5 ml of RPMI with 10 %FCS was added to stop the trypsin. 3 ml of cell suspension was added to each of the relevant cell pellets, and then spun at 1700rpm for 4 min.

The pellet was resuspend in 1 ml of RPMI with 10 % FCS and left in an incubator for 5 min, and then spun at 1700rpm for 4 min.

The pellet was resuspend in 1 ml of ice cold PBS and then spun at 1700rpm for 4 min. The pellet was resuspend in 100 μ l of ice cold Annexin-v buffer, and then 10 μ l of and 1 μ l of FITC were added respectively in each tube as appropriate, and incubated in the dark for 15 min at 4 $^{\circ}$ C.

400 μ l of Annexin-v buffer was added and then the samples were run on the FACS within 1 hour.

Apoptotic cells were then read on a display of red fluorescence at flow cytometry. Apoptotic cells have high fluorescence and side scatter. Necrotic cells have intermediate fluorescence and low side scatter.

2.5.10 *Growth inhibition assay of PEO1/PEO1cDDP with Cisplatin.*

The objective of this part of work was to explore whether the Barx2 gene plays a role in multidrug resistance in ovarian cancer cells. Two cell lines were used in this assay. The sensitivity of the two cell lines to varying concentrations of cisplatin was directly compared in growth assay for each other. A one cell line was PEO1 platinum sensitive parent line; the controls vector only transfected clonal derivative line PEO1-Hyg1.6 and the Barx2 transfected clonal derivative line PEO1-BX11.1. The other cell lines were PEO1 cDDP parent line that is derived from PEO1 but is resistant to cisplatin about 2-3 fold, a vector control only transfected clonal derivative line PEO1c DDP-Hyg2.2 and a Barx2 transfected clonal derivative line PEO1cDDP-BX1.3.

A single cell suspension was prepared by trypsinisation and syringing for each cell lines.

For each cell line, 2.5×10^4 cells/well /1 ml of IPMR/10% FCS/P+S was seeded in 24 well tray in quadruplicate per each concentration of cisplatin.

The cells were incubated at 37 °C and 5% CO₂ for 2 days to adhere, and then 4 wells cells were harvested for each cell line to count the cells by the coulter counter method.

The media was then replaced with fresh media containing varying concentration of cisplatin between 300nm to 3 µm /ml The cells were exposed to cisplatin at 37 °C and 5% CO₂ and incubated for further 72 hours. The media containing Cisplatin was removed from the wells. The cells were refed with IPMR/10% FCS/P+Sand and then the media without cisplatin was changed for every 4 days thereafter. The number of colonies per plate was counted every 4 days up to 12 days by using the coulter counter method (see above).

**3 LOH ANALYSIS FOR THE CHROMOSOME 11 q BETWEEN
D11S1894 AND D11S1309 IN CANCER**

The previous LOH data suggested disruption of chromosome 11 q23.3 – 24.3 (D11S934 to D11S1320) region (4.5 Mb) frequently occurs in ovarian cancer and colorectal cancer, and is associated with poor prognosis in epithelial ovarian cancer patient (Gabra H, et al. 1996). It has also been confirmed that the Barx2 gene is located within this minimal region at between the markers D11S912 and D11S1320 (Krasner A, et al. 2000). Therefore, 14 polymorphic which all were across the 4.5 Mb region within chromosome 11q24-25, were chosen to repeat analysis of the chromosome 11 q24 region D11S1894 and D11S1309 in colorectal cancer in order to more refine the LOH analysis and clinicopathological associations.

3.1 Re-arrangement of Polymorphic Microsatellite Repeat Markers Order

The study used 14 polymorphic microsatellite repeat to screen a population of patients with colorectal cancer. These polymorphic markers are outlined in Table 3-1

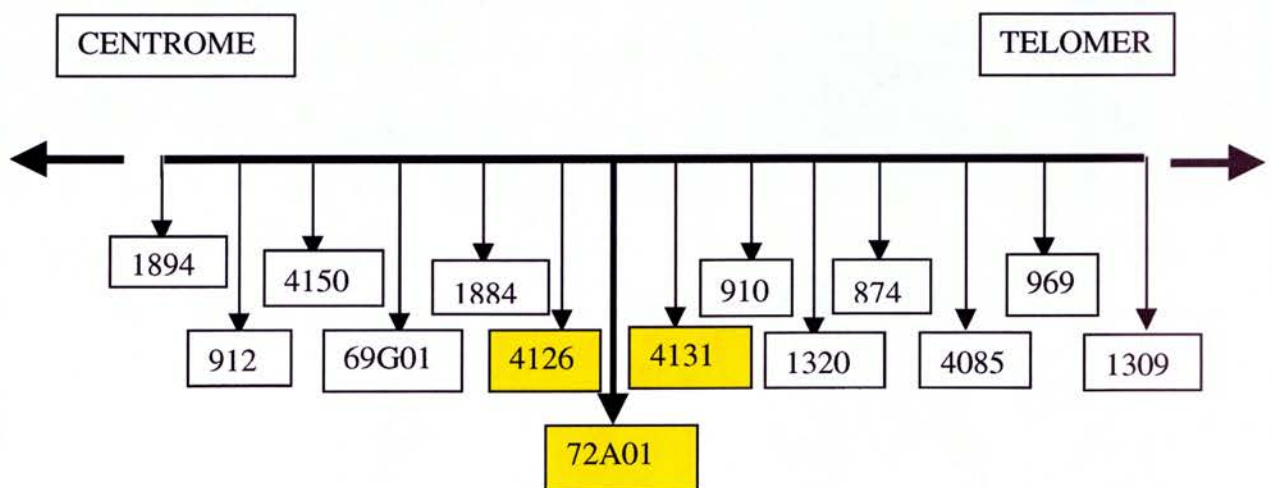
Table 3-1 Polymorphic Microsatellites: Identity and Location

Locus	Location	Name
D11S1894	11q24	
D11S912	11q24.1	AFM157xh6
D11S4150	11q24	
GATA69G01	11q24	
D11S1884	11q24	
D11S4126	11q24	
GATA72A01	11q24	
D11S4131	11q24	AFMb002vd1
D11S910	11q24	AFM154yh2
D11S1320	11q24	AFM234yf10
D11S874	11q24	
D11S4085	11q24	SHGC-20717
D11S969	11q24 –25	
D11S1309	11q24 –25	AFM200vg5

The order of markers in the region was re-analysed by Dr Grant C. Sellar and Dr Karen P. Watt. Bacterial artificial chromosomes (BACs) containing the markers were identified. Genbank data base was searched using BLAST, entering the primary sequence of repeat markers. These were then subjected to Nucleotide identify X (NIX)

analysis. BAC clones are in the process of being sequenced as part of the Human Genome Project. This allowed a tiling path of markers to be developed. D11S969 and D11S1309 are not identified on any BACs and therefore NIX analysis has not been possible for these. D11S1894 has been identified to be located in chromosome 11q23 centromeric to D11S912. The centromeric markers (D11S1894 to D11S1884 and D11S910 to D11S1309) have been ordered with respect to each other. GATA72A01, D11S4126 and D11S4131 cannot yet be ordered with respect to each other but they are represented on the same BAC and they therefore spread over a maximum of 200 kb (see Figure 3-1).

Figure 3-1 the Order of Microsatellite Markers Used in LOH Study



yellow box = these polymorphic microsatellites marker can not be ordered with respect to each other but they are represented on the same BAC and they therefore spread over a maximum of 200 kb.

3.2 Loss of heterozygosity rates of within Region Between D11S1894 and D11S1309 for all subgroups













































































































































































Molecular analyses of blood-tumour pairs from 39 patients with colorectal cancer were used for this part of analysis.

Loss of heterozygosity was observed with variable frequency using different markers. The colorectal cancer samples were classified into many subgroups depending upon the location of the disease, Dukes' stages, differentiation, vascular invasion, and perineural invasion and mucin production. The comparisons were made between LOH and the subgroup classification of colorectal cancer of the samples analysed. LOH results for all markers in this study are displayed in Figure 3-2 and LOH results in subgroups are summarised at Table 3-2 and Table 3-3. Clear examples of LOH at D11SGATA69G01 and D11S1884 are shown in Figure 3-3.

In Figure 3-2, Top and Bottom, case numbers for patients' tumour/blood pairs. Left, microsatellite loci used to detect LOH. Hatched boxes represent homozygous; filled boxes represent LOH; empty boxes represent retention of heterozygosity. ND means not determinable.

Figure 3-2 The Summary Of LOH Analysis for Colorectal Cancer

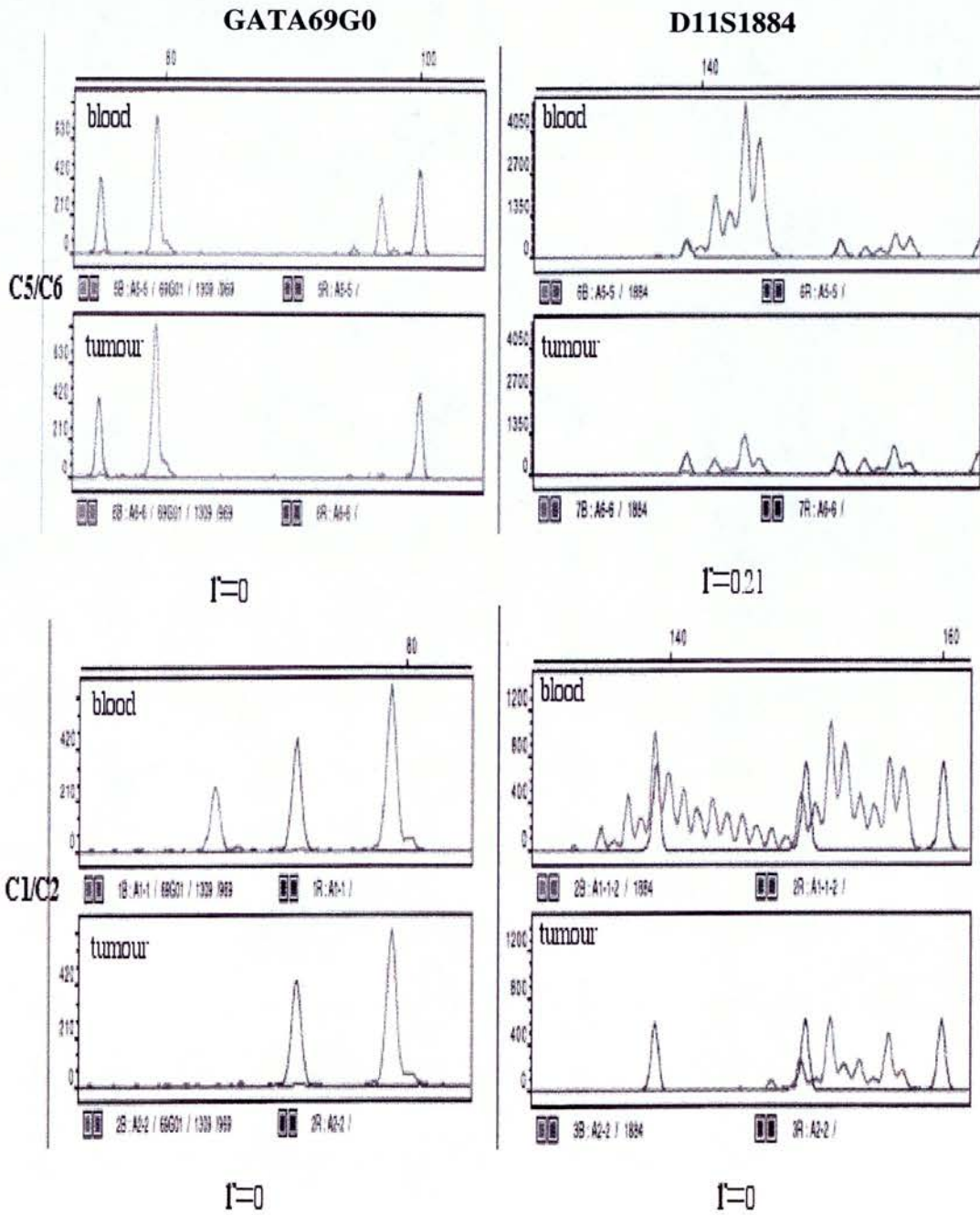
	C1/C2	C3/C4	C5/C6	C7/C8	C9/C10	C11/C12	C13/C14	C15/C16	C17/C18	C19/C20	C21/C22	C23/C24
D11S1894	■	▣	▣	■		▣	▣	▣		▣	□	□
D11S912	■	■	■	▣		□	■	▣		■	■	□
D11S4150	□	▣	■	▣		□	▣	□		ND	□	▣
GATA69G01	■	▣	■	▣		▣	▣	□		▣	▣	▣
D11S1884	■	▣	■	▣		▣	▣	ND		▣	▣	ND
D11S4126	■	▣	■	□		□	▣	□		□	□	□
GAT72A01	■	▣	■	▣		▣	■	▣		▣	▣	□
D11S4131	■	▣	■	■		□	■	■		□	□	□
D11S910	■	■	▣	▣		▣	▣	□		▣	□	▣
D11S1320	■	■	▣	■		▣	▣	□		▣	□	▣
D11S874	■	■	▣	ND		▣	■	▣		□	ND	□
D11S4085	■	■	▣	□		□	▣	□		▣	□	□
D11S969	■	▣	■	▣		□	▣	▣		▣	□	▣
D11S1309	■	▣	▣	▣		▣	▣	▣		▣	▣	▣

C25/C26	C27/C28	C29/C30	C31/C32	C33/C34	C35/C36	C37/38	C39/C40	C41/42	C43/C44	C45/C46	C47/C48	C49/C50
ND 	 ■ 	■  ■ 	  ■ 	 ■  	 ■  	   	   	ND  ND 	  ■ 	   	 ■  	■ ■ ■ 
ND 	 ■  	■  ■ 	  ■ 	 ■  ■	 ■  	   	   	ND  ND 	  ■ 	   	 ■  	■ ■ ■ 
ND 	 ■  	■  ■ 	  ■ 	 ■  ■	 ■  	   	   	ND  ND 	  ■ 	   	 ■  	■ ■ ■ 
ND 	 ■  	■  ■ 	  ■ 	 ■  ■	 ■  	   	   	ND  ND 	  ■ 	   	 ■  	■ ■ ■ 
ND 	 ■  	■  ■ 	  ■ 	 ■ ■	 ■ 	   	   	ND  ND 	  ■ 	   	 ■  	■ ■ ■ 

C51/C52	C53/C54	C55/C56	C57/C58	C59/C60	C61/C62	C63/C64	C65/C66	C67/C68	C69/C70	C71/C72	C73/C74	C75/C76
		ND	ND							ND	ND	
		ND									ND	
		ND									ND	
		ND									ND	
		ND						ND		ND	ND	
		ND							ND			
		ND								ND	ND	ND
		ND									ND	
											ND	
		ND									ND	
		ND									ND	
C51/C52	C53/C54	C55/C56	C57/C58	C59/C60	C61/C62	C63/C64	C65/C66	C67/C68	C69/C70	C71/C72	C73/C74	C75/C76

C77/C78	C79/C80	C81/C82	C83/C84	C85/C86	C87/C88	C89/C90	C91/C92	C93/C94	C95/C96	C97/C98	C99/C100	
	ND			ND	ND			ND				D11S1894
	ND	ND	ND									D11S912
												D11S4150
ND												GATA69G01
ND		ND	ND	ND		ND	ND					D11S1884
												D11S4126
												GAT72A01
						ND	ND					D11S4131
												D11S910
												D11S1320
						ND	ND					D11S874
												D11S4085
												D11S969
C77/C78	C79/C80	C81/C82	C83/C84	C85/C86	C87/C88	C89/C90	C91/C92	C93/C94	C95/C96	C97/C98	C99/C100	

Figure 3-3 Example of LOH in Colorectal Cancer



Primary LOH data from two cases critical to the definition of the 11q24 locus. Blood, normal DNA; Tumor; tumor DNA shown at left top of each picture from patient's blood/tumor pairs C1/C2 and C5/C6 (left). Microsatellite loci are shown at top. Densitometric ratios of allele peak area were calculated (shown at bottom), and values between 0.0 and 0.7 are taken to indicate LOH

Table 3 -2 Loss of Heterozygosity Rates within Region between D11S1894 and D11S1309 in Colorectal Cancer

Locus	No. Analysed	No. informative	No. LOH	%. LOH
D11S1894	39	12	7	58.33
D11S912	39	26	11	42.31
D11S4150	39	25	9	36.00
GATA69G01	39	9	3	33.33
D11S1884	39	9	5	55.56
D11S4126	39	28	7	25.00
GAT72A01	39	16	7	43.75
D11S4131	39	28	13	46.43
D11S910	39	19	5	26.31
D11S1320	39	20	10	50.00
D11S874	39	19	10	50.80
D11S4085	39	25	8	32.00
D11S969	39	12	5	41.67
D11S1309	39	12	4	33.30

Informative number = LOH number plus retention of heterozygosity number

Table3-3 Loss of Heterozygosity Rates Within Region Between D11S1894 and D11S1309 for All Subgroups (1)

LOCUS	LOCATION					
	Ascending	Sigmoid	Transverse	Descending	Rectum	Unknown
D11S1894	1/1	4/6	0/2	1/1	0/1	1/1
D11S912	3/5	4/9	0/3	0/0	2/6	2/3
D11S4150	1/4	3/7	0/2	0/0	2/8	3/3
GATA69G0	2/2	0/3	0/1	0/0	1/3	0/0
D11S1884	2/3	0/2	1/1	0/0	1/2	1/1
D11S4126	2/5	2/9	0/2	0/1	2/8	1/3
GATA72A01	1/1	5/8	1/1	0/0	1/5	0/1
D11S4131	3/5	4/9	0/3	1/1	3/8	2/2
D11S910	1/3	2/8	0/1	0/0	1/5	1/2
D11S1320	2/2	3/6	0/0	1/1	1/8	3/3
D11S874	3/4	6/9	0/1	0/0	1/4	0/1
D11S4085	1/3	4/10	0/4	0/1	2/5	1/2
D11S969	2/3	0/2	0/1	0/0	2/5	1/1
D11S1309	1/2	1/5	0/0	0/0	1/4	1/1

LOCUS	DUKES' STAGE				VASCULAR INVASION		
	A	B	C1	Unknown	No	Yes	Unknown
D11S1894	0/0	3/7	3/4	1/1	7/12	0/0	0/0
D11S912	0/0	4/12	5/11	2/3	7/20	2/3	2/3
D11S4150	0/1	3/11	3/10	3/3	9/25	0/0	0/0
GATA69G01	0/0	2/4	1/5	0/0	3/8	0/1	0/0
D11S1884	1/1	3/6	1/1	1/1	5/9	0/0	0/0
D11S4126	1/1	3/13	2/10	1/4	7/28	0/0	0/0
GATA72A01	0/0	3/8	4/7	0/1	7/16	0/0	0/0
D11S4131	0/1	4/14	7/11	2/2	12/27	1/1	0/0
D11S910	0/0	3/9	1/8	1/2	4/18	1/1	0/0
D11S1320	0/1	2/6	5/10	3/3	9/19	1/1	0/0
D11S874	0/0	6/1	4/6	0/1	9/18	1/1	0/0
D11S4085	0/0	2/11	5/12	1/2	7/23	1/2	0/0
D11S969	0/0	3/6	1/5	1/1	5/11	0/1	0/0
D11S1309	0/1	3/7	1/4	0/0	4/11	0/1	0/0

Values given are the number of cases with LOH in that subgroup/total number of informative cases in that subgroup.

Table3-3 Loss of Heterozygosity Rates Within Region Between D11S1894 and D11S1309 for All Subgroups (2)

LOCUS	DIFFERENTIATION-OVERALL			
	Moderate	Poor	Well	Unknown
D11S1894	5/8	1/1	0/2	1/1
D11S912	7/14	2/2	0/3	2/2
D11S4150	5/17	1/4	0/1	3/3
GATA69G01	2/6	1/3	0/0	0/0
D11S1884	3/7	1/1	0/0	1/1
D11S4126	5/20	1/4	0/1	1/3
GATA72A01	5/9	2/3	0/3	0/1
D11S4131	6/17	5/6	0/2	2/3
D11S910	4/12	0/3	0/2	1/2
D11S1320	5/12	1/3	1/2	3/3
D11S874	9/14	1/3	0/1	0/1
D11S4085	7/16	0/4	0/3	1/2
D11S969	3/8	1/2	1/1	1/1
D11S1309	3/9	0/2	0/0	1/1

LOCUS	PERINEURAL INFILTRATION			MUCIN PRODUCTION		
	No	Yeas	Unknown	No	Yes	Unknown
D 11S1894	7/12	0/0	0/0	6/11	1/1	0/0
D11S912	10/25	1/1	0/0	11/21	0/4	0/0
D11S4150	9/25	0/0	0/0	8/20	1/5	0/0
GATA69G01	3/8	0/1	0/0	3/8	0/1	0/0
D11S1884	5/9	0/0	0/0	5/9	0/0	0/0
D11S4126	5/26	2/2	0/0	6/25	1/3	0/0
GATA72A01	6/15	1/1	0/0	7/16	0/0	0/0
D11S4131	13/27	0/1	0/0	12/23	1/5	0/0
D11S910	5/18	0/1	0/0	4/14	1/5	0/0
D11S1320	10/20	0/0	0/0	9/17	1/3	0/0
D11S874	9/18	1/1	0/0	9/18	1/1	0/0
D11S4085	7/24	1/1	0/0	8/21	0/4	0/0
D11S969	5/12	0/0	0/0	5/11	0/1	0/0
D11S1309	3/11	1/1	0/0	4/12	0/0	0/0

Values given are the number of cases with LOH in that subgroup/total number of informative cases in that subgroup.

In total, of 39 cases of colorectal cancer which could be analysed, 28 cases (71.18 %) had evidence of LOH involving at least one locus on chromosome 11 q24-25 between D11S1894 to D11S1309.

For all colorectal cancer, high levels of LOH (> 40 %) were found for seven loci (see Table 3-2): D11S1894 demonstrated LOH in 7 of 12 informative (58.33%) of cases; D11S874 in 10/19 (50.80 %) informative tumors; D11S1320 in 10/20 (50.00 %) informative tumors; D11S4131 in 13/28 (46.43 %) informative tumors; GATA69G01 in 7/16 (43.75 %) informative tumors; D11S912 in 11/26 (42.30.2 %) informative tumors and D11S969 in 5/12 (41.67%) informative tumors. The lowest frequencies of allele loss in this region were detected at D11S4126 with only 25% LOH in colorectal cancer.

If one focuses on the most centromeric markers and identifies tumors with secure loss in this region, a consensus region of deletion can be identified and the shortest region of overlap (SRO) determined. These are shown in Figure 3.3 to 3.5 (Hatched boxes = homozygous, filled boxes = LOH, empty boxes = retention of heterozygosity). In cases where a locus with LOH is separated by an uninformative locus from another that remain heterozygous, that uninformative locus was included within the shaded bars as part of the deletion since this region could have been lost. This type of analysis suggests 3 shortest region of overlap corresponding to 3 consensus regions of deletion/allele imbalance a) between GAT72A01 and D11S4131; b) between D11S912 and D11S4150 and c) between D11S1320 and D11S4085 (see Figure 3.4 to 3-6). The size of these region is <200kb, 200kb and 2Mb respectively. The frequency of LOH was 58.6% at D11S912 to D11S4150, 50.0 % at D11S1320 to D114085 and 42.1 % at GAT72A01 to D11S4131. Of the total 39 cases, 10 cases (25.6 %) had LOH at all loci. 11 cases (28.2%) showed no LOH, and 18 cases (46.2%) showed partial LOH in this region.

Figure 3-4 Subchromosome Consensus Region of LOH

		D11S912 To D11S4150							
		C47/C48	C67/C68	C19/C20	C93/C94	C27/C28	C33/C34	C43/C44	C21/C22
D11S1894					ND				
D11S912									
D11S4150				ND					
GATA69G01									
D11S1884			ND						
D11S4126									
		C47/C48	C67/C68	C19/C20	C93/C94	C27/C28	C33/C34	C43/C44	C21/C22
<p>The centromeric extent of this region is defined by blood/tumour pair C33/34 and C43/C44. The telomeric extent is defined by pair (c21/c22 contained with in this region are the markers D11S912 and D11S4150.</p>									

Figure3-6		Region of LOH Between D11S1320 to D11S4085									
	Subchromosome Consensus	C47/C48	C57/C58	C55/C56	C7/C8	C67/C68	C83/C84	C43/C44	C39/C40	C69/C70	
D11S4126		□	□	▣	□	□	□	□	▣	▣	D11S4126
GAT72A01		▣	□	ND	▣	▣	□	□	□	ND	GAT72A01
D11S4131		▣	▣	□	▣	□	□	▣	□	□	D11S4131
D11S910		▣	▣	ND	▣	▣	□	□	□	□	D11S910
D11S1320		▣	□	ND	▣	▣	▣	▣	▣	▣	D11S1320
D11S874		ND	▣	▣	ND	▣	▣	□	ND	▣	D11S874
D11S4085		▣	▣	ND	□	▣	▣	□	□	□	D11S4085
D11S969		▣	▣	ND	▣	▣	▣	▣	▣	□	D11S969
D11S1309		▣	▣	ND	▣	▣	□	▣	▣	□	D11S1309
		C47/C48	C57/C58	C55/C56	C7/C8	C67/C68	C83/C84	C43/C44	C39/C40	C69/C70	

The centromeric extent of this region is defined by blood/tumour pair C43/C44 and C39/C40. The telomeric extent is defined by pair C69/C70. Contained within this region are markers between D11S1320 and D11S4085

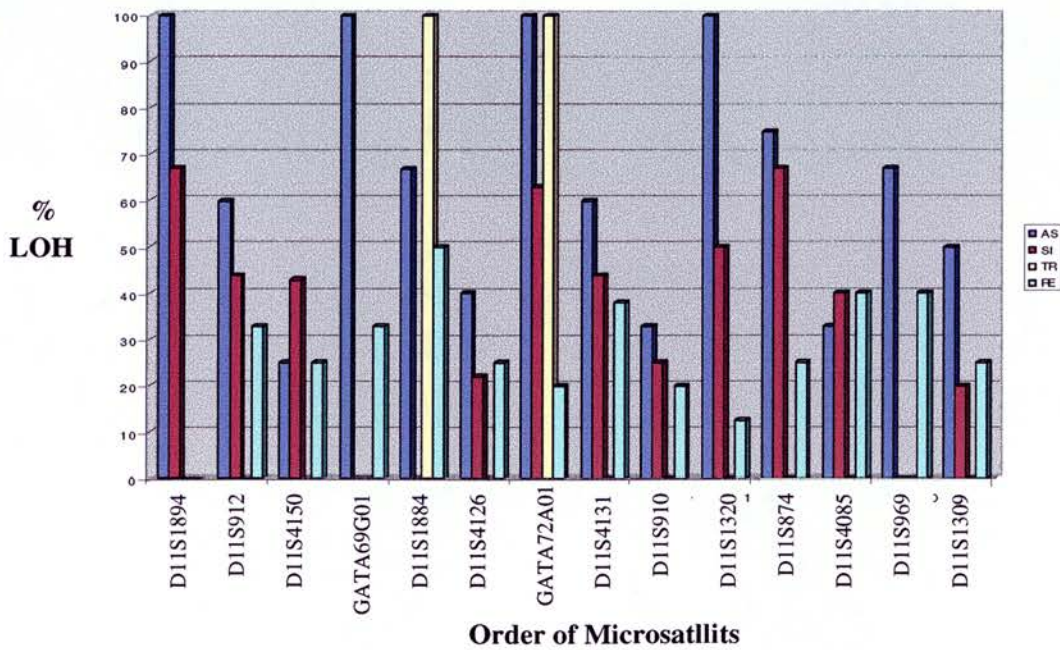
3.3 The relationship between LOH and clinicopathological features of colorectal cancer.

Fisher's exact test was used to analyse whether loss of individual markers or of the SROs was significantly associated with the clinicopathological features of colorectal cancer.

3.3.1 Allele imbalance and location of disease

No association was evident between LOH at any of the loci tested and the site of colorectal cancer with respect to the colon (see Figure 3-7).

Figure 3-7 The Relationship Between % LOH and Site of disease

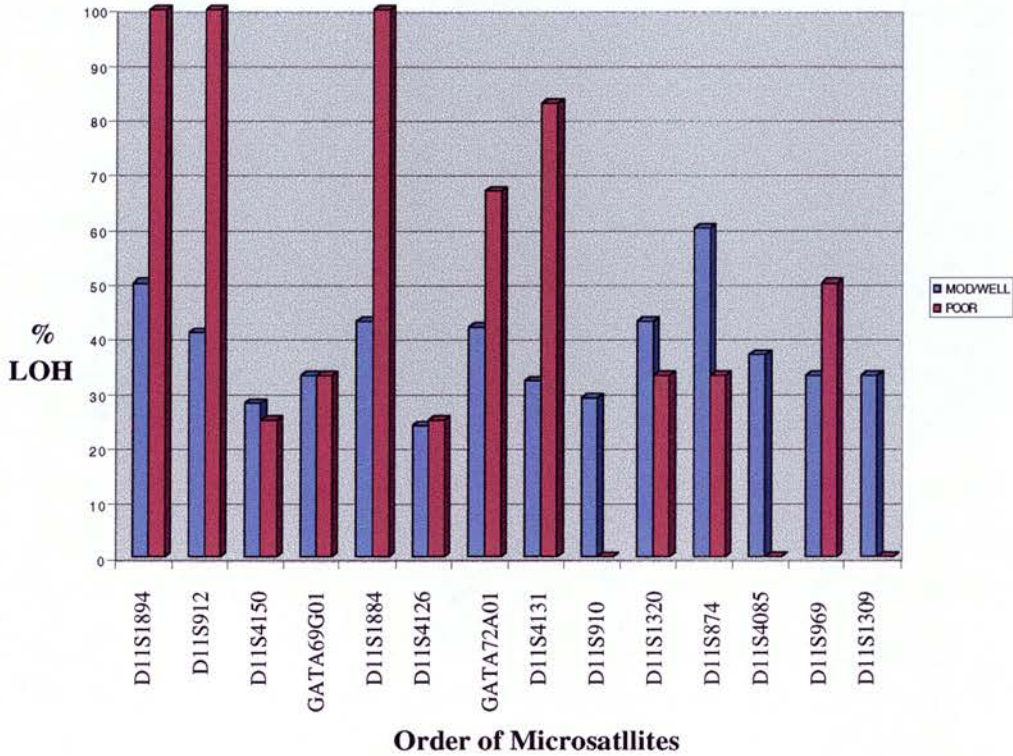


Location of disease in colorectal cancer. AS; ascending. SI; sigmoid. TR; transverse. RE; rectum

3.3.2 Allele imbalance and differentiation grade

The only trend towards significance was between D11S 4131 LOH and pathologic differentiation grade (p= 0.0561). The poorly differentiated tumor had particularly high levels of LOH at D11S 4131 (See Figure 3-8).

Figure 3-8 The Relationship Between % LOH and Differentiation Grade

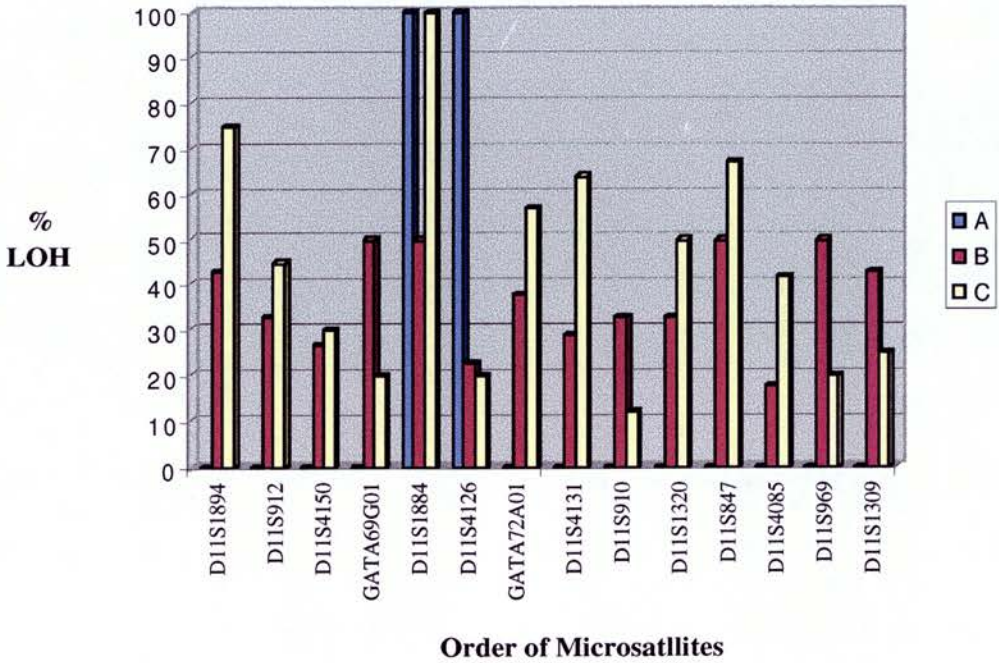


Differentiation grade of colorectal cancer. Mod; moderate differentiation. Poor; poor differentiation

3.3.3 Allele imbalance and clinical stage

No significant difference was seen at any locus, comparing early stage with lately stage in colorectal cancer patients. The only apparent trend towards significance was between D11S4085 LOH and Dukes' stage. More advanced stage had higher levels of LOH at D11S 4085 ($p=0.07$)(Figure 3-9).

Figure 3-9 The Relationship Between % LOH and Clinical Stage



Clinical stage of colorectal cancer. A; Dukes' stage A. B; Dukes' stage B; C; Dukes' stage C1

3.3.4 The relationship between minimal deleted regions(SROs) and clinicopathological features of colorectal cancer.

There were no significant difference between LOH at SROs and clinicopathological features of colorectal cancer. These results were outlined at Table 3-4

Table 3-4 The Relationship between LOH at SROs and Clinicopathological Features of Colorectal Cancer.

SRO	Dukes' Stage			Differentiation Grade				
	A	B	C	NK	Well	Moderate	Poor	NK
D11S912								
-D11S4150	0	8/15 (53.3)	6/11 (54.6)	3/3	0/3	11/22 (50.0)	3/4	3/3
GAT72G01								
-D11S4131	0/1	6/19 (31,6)	8/16 (50.0)	0/1	0/4	9/28 (32.1)	5/7	0/1
D11S4131								
-D11S4085	0/0	8/20 (40.0)	8/15 (53.3)	3/3	1/3	13/28 (46.4)	3/7	3/3

Values given are the number of cases with LOH in that subgroup/total number of informative case in that subgroup (% LOH in brackets).

NK = not known

3.4 Relationship between survival of patient with colorectal cancer and LOH

In order to assess effects on survival of LOH at individual markers, Kaplan-Meier/Log Rank analysis was performed. There was no significant correlation between losses at individual markers and survival of patients with colorectal cancer. The analysis results were summarized in Table 3-5

Table 3-5 The Summary of Kaplan-Meier/Log Rank Analysis for Individual Marker

Locus	p value
D11S1894	0.9311
D11S912	0.7808
D11S4150	0.6632
GATA69G01	0.5458
D11S1884	0.5088
D11S4126	0.9118
GATA72A01	0.3298
D11S4131	0.1071
D11S910	0.7148
D11S1320	0.8068
D11S874	0.6793
D11S4085	0.1916
D11S969	0.2919
D11S1309	0.4982

Kaplan-Meier/Log rank analysis was also performed to assess effects on survival of LOH at SROs. Deletions of the SROs were not significantly associated with survival of patients with colorectal cancer. The results are outlined in Table 3-6.

Table 3-6 The Results of Kaplan-Meier/Log Rank Analysis for the Consensus Region

Consensus Region	p value
D11S912—D11S4150	0.5829
GATA72A01—D11S4131	0.2450
D11S1320—D11S4085	0.7403

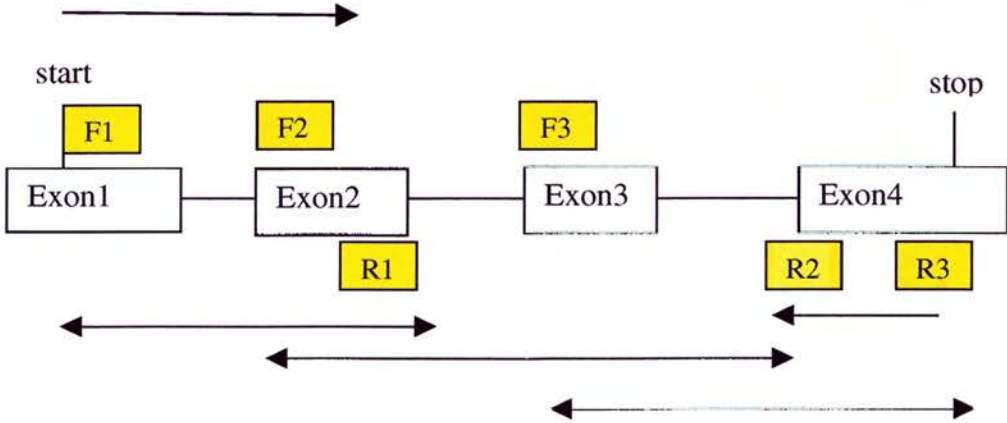
4. EXPRESSION OF BARX2 IN OVARIAN CANCER

The preceding investigation showed that frequent LOH was present at chromosome 11 q24-25 in cancer (Gabra H, et al. 1996), suggesting the presence of tumour suppressor genes in this regions. Barx2 is also located within the critical 8.5 Mb LOH region of chromosome 11 q24-25 (Krasner A, et al. 2000). This raises the question; is Barx2 a TSG? To begin to address this question, it is important to understand the expression of Barx2 in normal ovarian epithelium tissues, ovarian cancer cell lines and ovarian cancer tissues. Fresh samples were obtained from 10 cases of normal ovarian tissue, 28 cases of primary epithelial ovarian cancer tissue and 10 ovarian cancer cell lines. RNA of these samples was extracted, and then RT-PCR and Northern blotting were performed according to standard methods as described in section of the materials and methods.

4.1 Results of reverse transcription polymerase chain reaction.

A total of 4 pair of RT-PCR primer (F1/R1, F2/R2, F3/R3 and F1/R3) were used in this project. According to the cDNA sequence of Barx2 gene, we designed four pair of primer for Barx2 (see Figure 4-1) in order to analyse expression for each exon as well as full length cDNA of Barx2 in ovarian cancer cell lines and tissue.

Figure 4-1 Human Barx2 RT-PCR Primers



4.1.1 The expression of Barx2 in normal ovarian epithelium

Figure 4-2 shows the expression of Barx2 (F1/R3) in 10 normal ovarian epithelium tissues. These samples came from the patients with uterine leiomyoma. After total hysterectomy and unilateral salpingo-oophorectomy, the pathologist confirms the ovaries as normal. RNA was isolated from preoperatively stripped ovarian epithelium. The results show that there is a 780bp PCR product in all normal ovarian epithelium.

Figure 4-2 the Expression of Barx2 (F1/R3) In Normal Ovarian Epithelium



4.1.2 The expression of Barx2 in ovarian cancer cell lines.

A total 4 separate experiment of semiquantitative RT-PCR have been performed to test the expression of the Barx2 in 10 ovarian cancer cell lines. Figure 4-3 to Figure 4-7 show the results of a representative experiment of semiquantitative RT-PCR, in which the Barx2 expression is assayed in ovarian cancer lines using different primer

combinations (F1/R1, F2/R2, F3/R3 and F1/R3). All of results of expression of Barx2 in ovarian cancer lines are outlined in Table 4 –1

Figure 4 – 3 The Expression of Barx2 F1/R1 in Ovarian Cancer Cell Lines

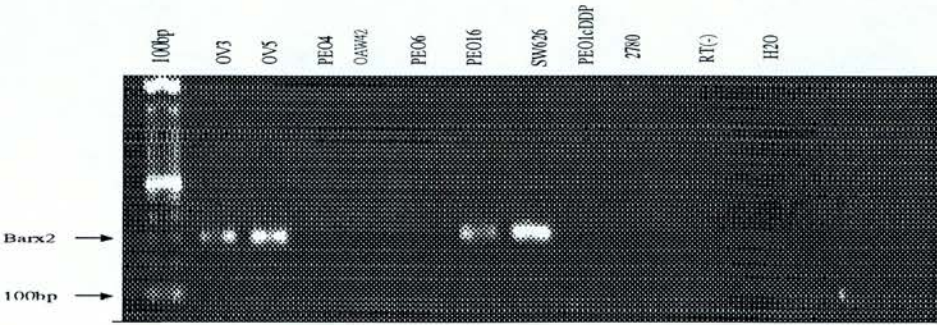


Figure 4 – 4 The Expression of Barx2 F2/R2 in Ovarian Cancer Cell Lines

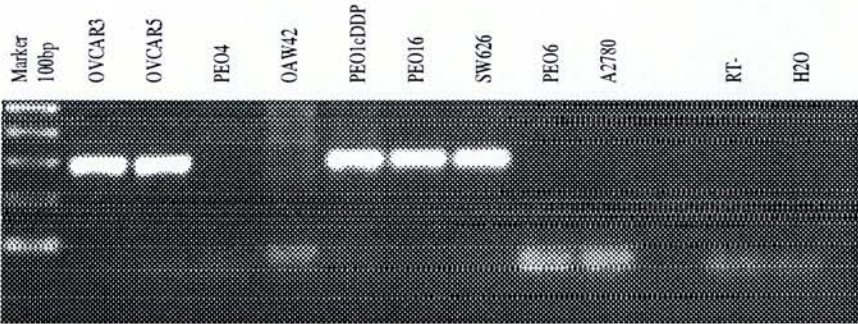


Figure 4 – 5 The Expression of Barx2 F3/R3 in Ovarian Cancer Cell Lines

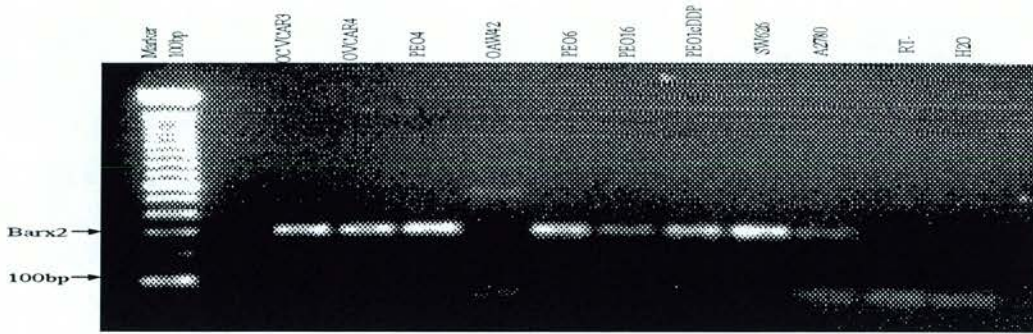


Figure 4 – 6 The Expression of Barx1 F1/R3 in Ovarian Cancer Cell Lines

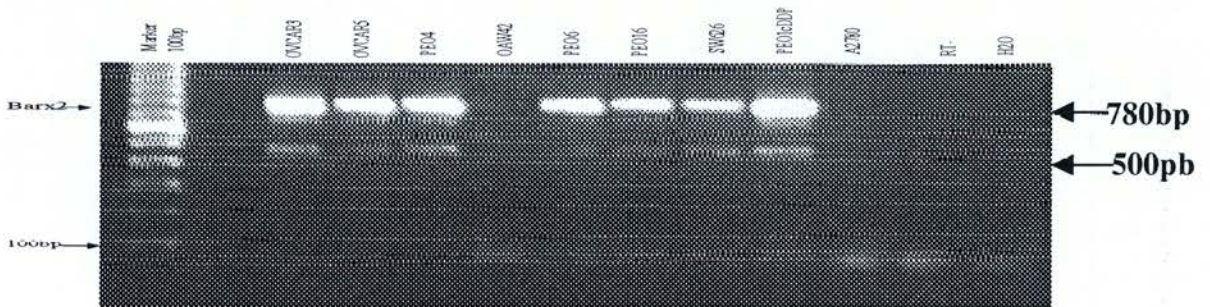


Figure 4- 7 The Expression of γ -act In Ovarian Cancer Cell Lines

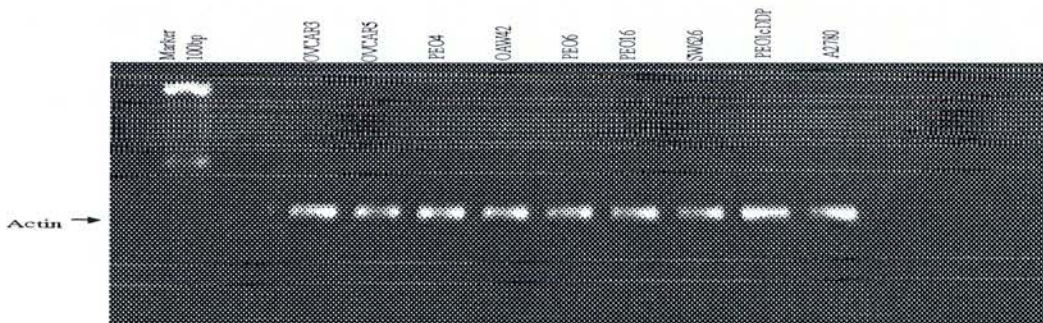


Table 4 - 1 The Expression of Barx2 in Ovarian Cancer Cell Lines

Cell Line	Barx2			
	F1/R1	F2/R2	F3/R3	F1/R3
OAW42	-*	-	-	-
A2780	-	-	+*	-
OVCAR3	+	+	+	+
OVCAR5	+	+	+	+
PEO1	+	+	+	+
PEO1cDDP	-	+	+	+
PEO4	-	-	+	+
PEO6	-	-	+	+
PEO16	+	+	+	+
SW262	+	+	+	+

*- = *Negative for expression of Barx2*

*+= *Positive for expression of Barx2*

In summary, (1) In a total of 10 ovarian cancer cell lines, 5 of 10 are downregulated/absent for expression of Barx2 F1/R1, and 4 cell lines are downregulated/absent in expression of Barx2 F2/R2, and only 1 cell line is downregulated/absent in expression of Barx2 F3/R3. It is worth noting that in most of the ovarian cancer cell lines, downregulated/absent expression of Barx2 is principally at F1/R1. One explanation for this difference observed between the primer pairs is an alteration in the nucleotide sequence bound by the F1/R1 primer in exon2. Alternately, we may just be observing a variation in the sensitivity of different primer pairs to discriminate levels of Barx2 expression. The expression of Barx2 as detected by F1/R3 RT-PCR is downregulated/absent in OAW42 and A2780. (2) When expression of Barx2 full-length cDNA (F1/R3) was tested, two products were generated in all of ovarian cancer cell lines running at 780bp and 500bp. (Figure 4-6).

4.1.3 The expression of Barx2 in ovarian cancer tissue

4.1.3.1 The frequency of the Barx2 in ovarian cancer tissue

Figure 4-8 to Figure 4-10 show the expression of Barx2 (F1/R1, F2/R2 and F3/R3) in 28 ovarian cancer tissue respectively. Figure 4-11 show the expression of g-actin in the same ovarian cancer tissue The result was summarised in Table 4-2.

Figure 4-8 The Expression of Barx2 (F1/R1) In Ovarian Cancer Tissue

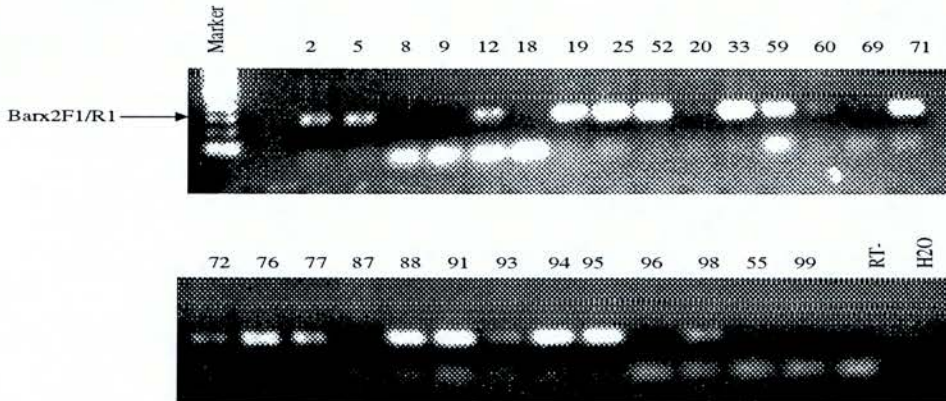


Figure 4-9 The Expression of Barx2 (F2/R2) In Ovarian Cancer tissue

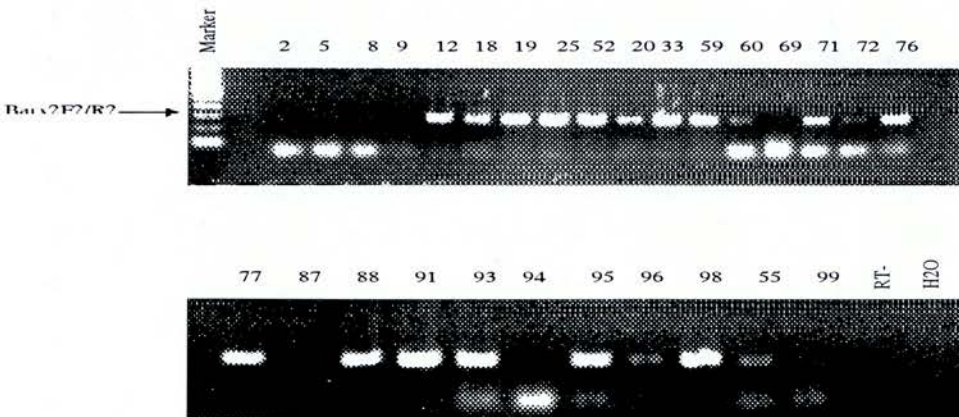


Figure 4-10 The Expression of Barx2 (F3/R3) In Ovarian Cancer Tissue

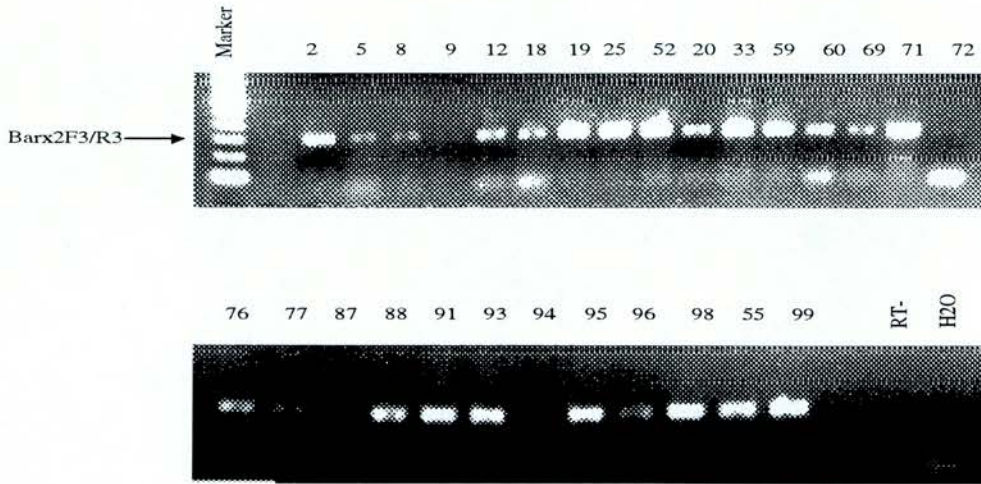


Figure 4-11 The Expression of γ -actin In Ovarian Cancer Tissue

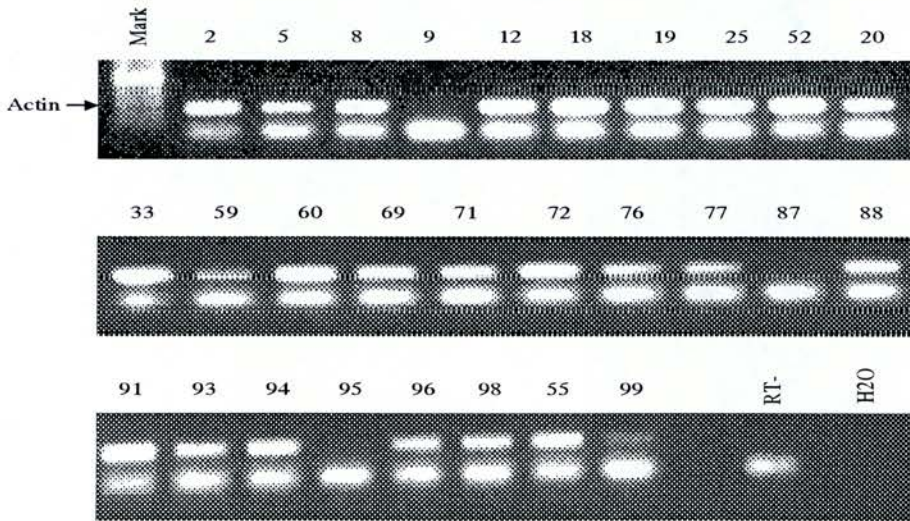


Table 4-2 The Expression of Barx2 in Ovarian Cancer

Primer	No. analysed	No. informative	Positive		Negative	
			N	%	N	%
F1/R1	28	25	13	52.0	12	48.0
F2/R2	28	25	15	60.0	10	40.0
F3/R3	28	25	17	68.0	8	32.0

In summary, 28 examples of ovarian cancer tissue were tested for expression of Barx2 by RT-PCR using three different primer pairs and the expression of γ -actin in same tissues was assessed by RT-PCR as a control. The relative intensity of RT-PCR products was determined using Lab-works (UVP) software and producing a ratio of Barx2 to Actin in each sample. In a total of 28 samples, 25 were analyzed except 3 cases where expression of γ -act was negative. The positive rate of F1/R1, F2/F2, F3/R3 and F1/R3 was 52.0%, 60.6 %, and 68.7 % respectively. Ovarian tumor samples overall show a decreased level of Barx2 expression compared with the normal ovarian epithelium.

4.1.3.2 Relationship between expression of Barx2 and pathological parameters in epithelial ovarian cancer

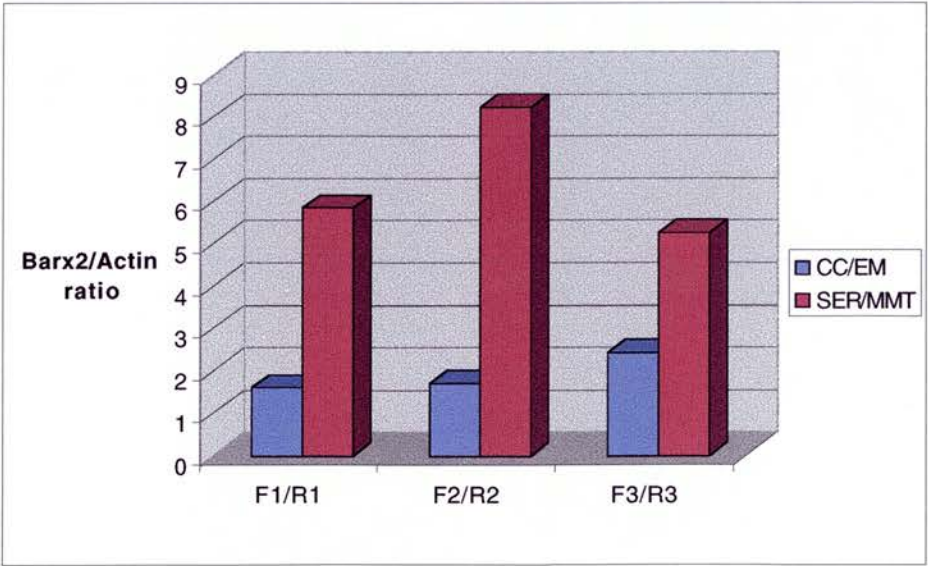
The 25 cases of ovarian cancer analysed in this studies comprised of 5 endometrioid, 15 serous, 2 clear cell carcinoma, 3 mixed mesodermal tumour (MMT). The expression level of Barx2 in clinicopathological subgroups (pathology, stage and differentiation grade) by RT-PCR as a continuous variable is summarised in Table 4- 3. Figure 4-12 to 4-14 shows the relationship between the expression level of Barx2 in ovarian cancer and the clinicopathological parameters.

Table 4- 3 The Expression of Barx2 in Clinic-pathological Subgroups of Ovarian Cancer

Clinicopathological subgroups	No	Barx2/Actin ratio(Mean ±SD)		
		F1/R1	F2/R2	F3/R3
<u>Pathology</u>				
CC/EM	7	1.61 ±1.4	1.70 ±1.6	2.43 ± 0.9
SER/MMT	18	5.86 ±13.2	8.25 ±19.4	5.27 ±15.9
<u>Stage</u>				
I + II	8	7.94 ± 17.4	9.9 ± 24.5	10.7 ± 23.6
III+IV	13	3.16 ± 7.2	4.7 ± 11.9	1.5 ± 1.0
Unknown	4			
<u>Grade</u>				
Poor	13	2.94 ±7.2	5.1 ±11.9	1.4 ±1.1
Mod/Well	8	8.5 ±17.2	11.5 ±25.6	10.6 ±23.6
Unknown	4			

CC= clear cell carcinoma, EM = endometrioid carcinoma, SER= serous carcinoma, MMT = mixed mesodermal tumour Mod/well = moderation or well differentiation grade of ovarian cancer. Poor = poor differentiation grade of ovarian cancer

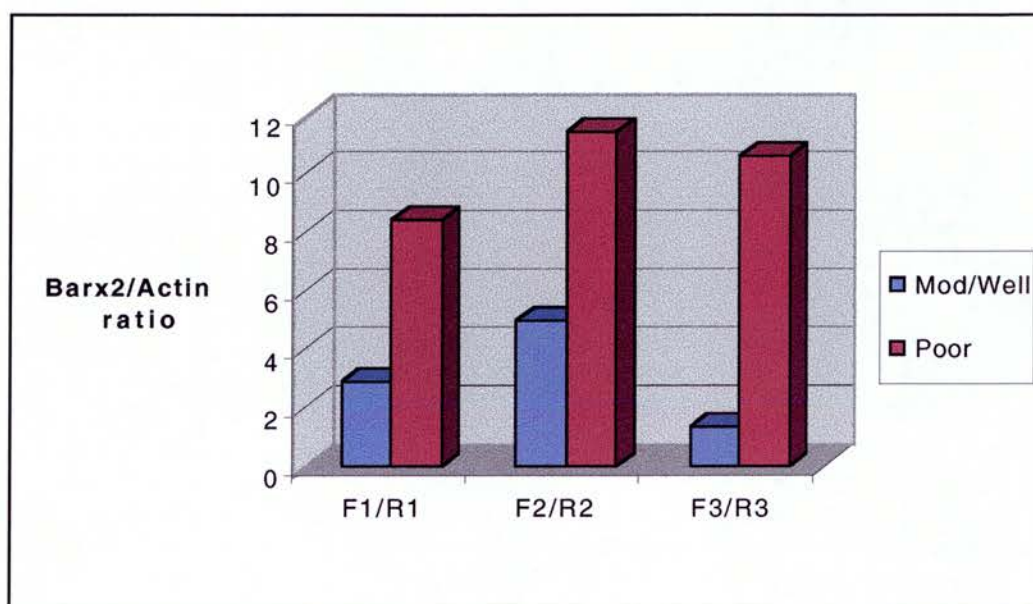
Figure 4- 12 The Relationship between The Expression Level of Barx2 and Pathologic Type of Ovarian Cancer



CC= clear cell carcinoma, EM = endometrioid carcinoma, SER= serous carcinoma, MMT = mixed mesodermal tumour

Compared the expression level of Barx2 by RT-PCR using the F1/R1, F2/R2 and F3/R3 primer pairs with pathological subtypes of ovarian cancer, although mean Barx2/actin ratio of F1/R1, F2/R2 and F3/R3 in clear cell carcinomas and endometrioid carcinoma were lower than these in serous carcinoma and mixed mesodermal tumour (1.61 vs 5.9 in F1/R1, 1.7 vs 8.3 in F2/R2 and 2.4 vs 5.3 in F3/R3 respectively), there is no statistically significant difference (p value is 0.95, 0.84 and 0.16 respectively).

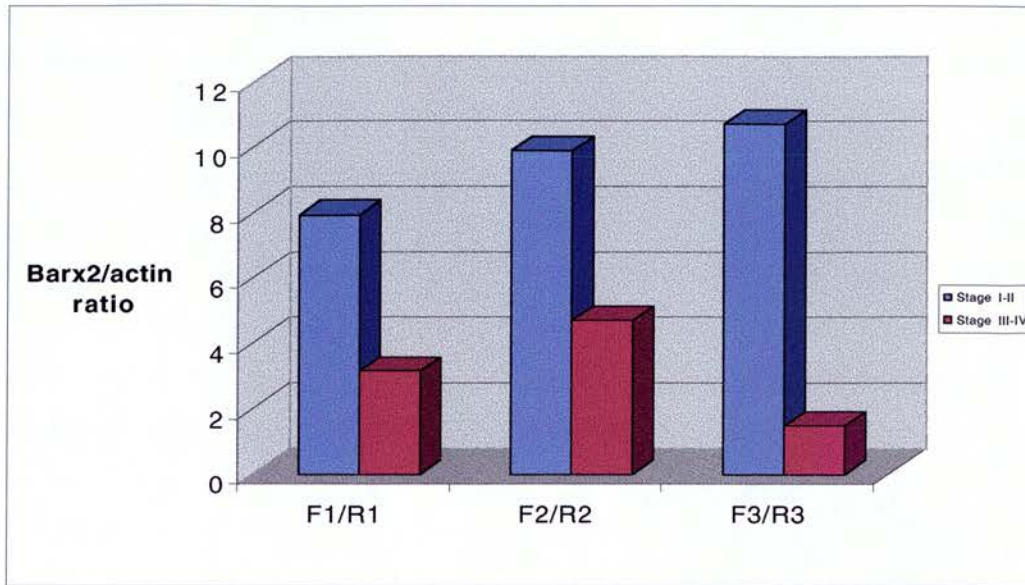
Figure 4- 13 The Relationship between the Expression Level of Barx2 and Pathologic Grade of Ovarian Cancer.



*Mod/well = moderation or well differentiation grade of ovarian cancer.
 Poor = poor differentiation grade of ovarian cancer*

Although no significant association was evident between the expression of Barx2 and pathologic grade of ovarian cancer, there is a trend towards higher Barx2 expression levels in moderation or well differentiation grade of ovarian cancer compared with poor differentiation grade of ovarian cancer. p value is 0.064 (F1R1), 0.37 (F2/R2) and 0.064 (F3/R3) respectively.

Figure 4- 14 The Relationship between the Expression Level of Barx2 and Clinic Stage of Ovarian Cancer



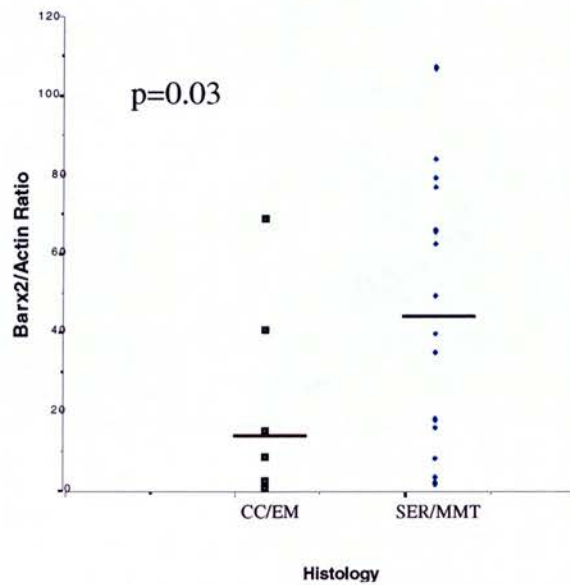
The mean Barx2/actin ratio of F1/R1, F2/R2 and F3/R3 in stage I-II is 7.94, 9.89 and 10.7 respectively. Compared with the mean Barx2/actin ratio of F1/R1, F2/R2 and F3/R3 in stage III-IV (3.16, 4.70 and 1.49 respectively), there is not statistically significant difference. ($p = 0.27, 0.6, \text{ and } 0.064$ for F1/R1, F2/R2 and F3/R3 respectively)

In summary, the expression level of Barx2 F1/R1, F2/R2 and F3/R3 in the serous carcinomas carcinomas and mixed mesodermal tumors were compared to endometrioid carcinomas and clear cell carcinoma, P values are 0.95, 0.84 and 0.16 respectively. There is also no significant difference in the expression level of Barx2 F1/R1, F2/R2 and F3/R3 between moderately or well differentiated and poor differentiated tumors. Although the expression level of Barx2 in early clinical stage (I – II) was seemed higher than that in late clinical stage (III-IV), the difference were not significant ($p = 0.27, 0.6, \text{ and } 0.064$ for F1/R1, F2/R2 and F3/R3 respectively). From these data, the expression of the Barx2 does not appear to be associated with pathological type and grade or clinical stage in epithelial ovarian cancer.

Subsequently, the expression of Barx2 in ovarian cancer was reanalyzed by Barx2 F1/R3

RT-PCR as a continuous variable in a series of ovarian cancer that comprised many of the tumor samples analyzed in this study. Barx2 expression (mean Barx2/actin ratio) by semiquantitative PT-PCR was found to be significantly lower for endometrioid/clear cell variants of ovarian cancer ($p=0.03$) (see Figure 4- 15) as compared to serous/mixed mesodermal tumor variants of ovarian cancer.

Figure 4 – 15



Semiquantitative RT-PCR, showing Barx2/Actin ratio for each of 22 samples of RNA extracted from primary ovarian cancer classified according to histology. Mean ratios for each group are indicated by a horizontal bar

CC=clear cell carcinoma, EM= endometrioid carcinoma, SER= serous carcinoma, MMT= mixed mesodermal

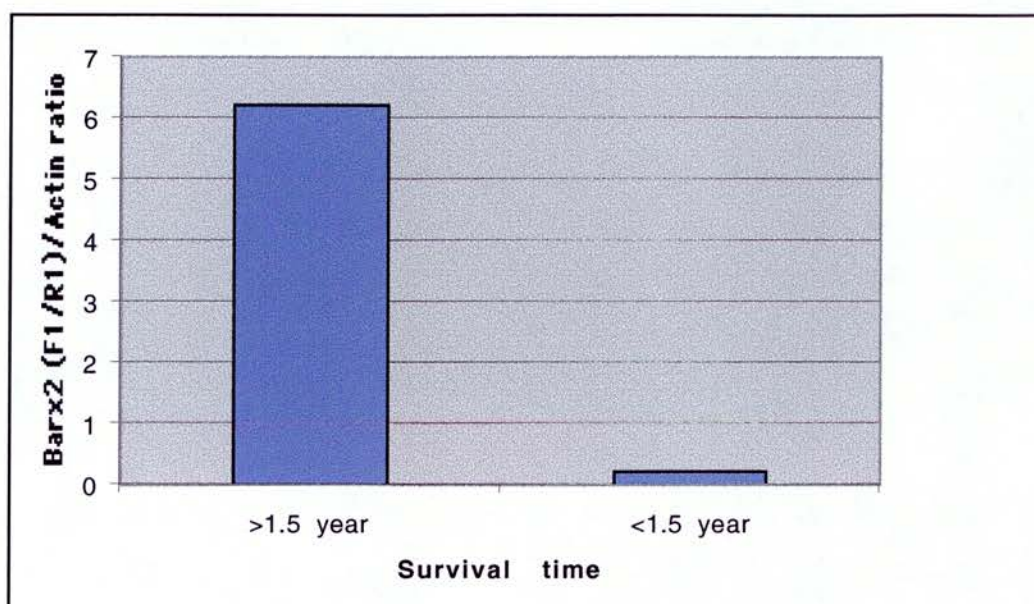
4.1.3.3 Relationship between expression of Barx2 and survival of patient with ovarian cancer.

All patients were on continuing follow-up, which ranged between 30 and >2780 days. The relationship between the expression of Barx2 and survival of patients with epithelial

ovarian cancer was analyzed.

The mean Barx2 (F1/R1)/actin ratio of patients with survival more than 1.5 year is higher (6.22) than that of patients with survival less than 1.5 year (0.29). At these survival cutoffs, the difference in expression of Barx2 was very significant ($p=0.0048$) (see Figure 4-16). In contrast, however, no significant relationship to be found between the expression level of Barx2 F2/R2 and F3/R3 and survival of patients ($p = 0.097$ and 0.977 respectively).

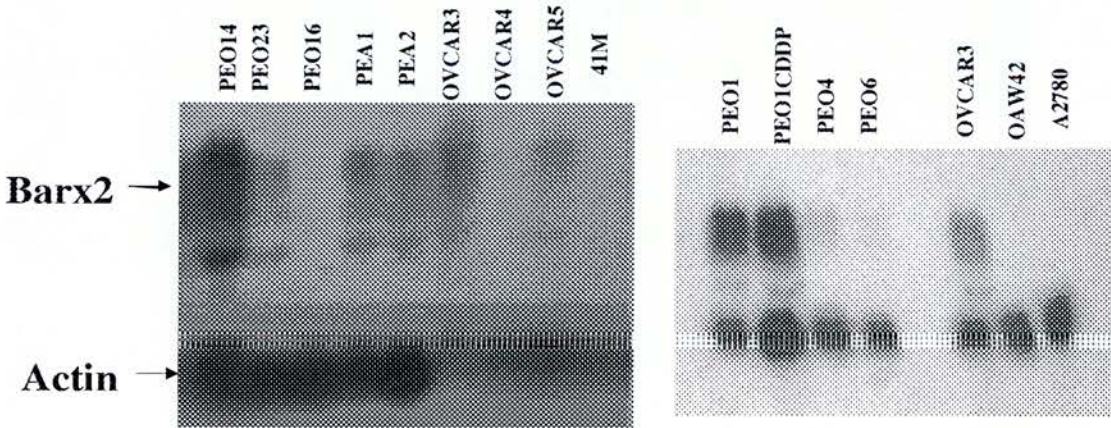
Figure 4-16 Relationship between Expression Level of Barx2 F1/R1 and Survival of Patients with Ovarian Cancer.



4.2 Barx2 Expression by Northern blotting

The results of RT-PCR have shown that expression of Barx2 is absent in some ovarian cancer tissues and cell lines. In order to confirm and quantitate further the results, Northern blotting was performed to investigate the expression of Barx2 in ovarian cancer cell lines. The Northern analysis was performed twice and the expression levels found to be reproducible. Figure 4-17 show the results of Northern blot for expression of Barx2 in ovarian cancer cell lines.

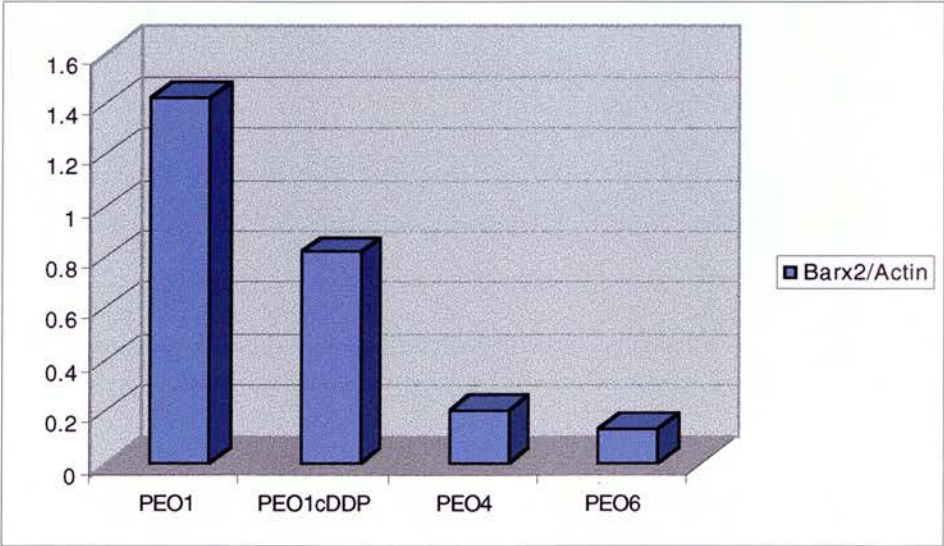
Figure 4- 17. The Results of Northern Blot for Testing the Expression of Barx2 in Ovarian Cancer Cell Lines.



Northern blot of ovarian cancer cell lines probed with Barx2. This result showed strong expression of a 1.74 - 2.2 Kb band in ovarian cancer cell lines PEO1, PEO14 and OVCAR3. PEO1 and PEO14 represent ovarian cancer cell lines from two patients early

in the course of their disease. PEO4/ PEO6; and PEO23 were derived from the same two patients following relapse after cisplatin chemotherapy. PEO1cDDP was derived from PEO1 by *in-vitro* cisplatin exposure. The *in vivo* and *in vitro*-derived cisplatin resistant cell lines showed obviously reduced Barx2 expression (normalised to γ -actin), compared with the corresponding platinum sensitive cell line. Figure 4-18 showed that expression of Barx2 is downregulated in the PEO1 series. Several other ovarian cancer lines showed low or absent Barx2 expression, particularly OAW42 and A2780. As OAW42 does not endogenously express Barx2 (as determined reproducibly by RT-PCR and Northern blotting studies), it was a suitable cell line to explore function analysis of Barx2 following transfection of a Barx2 expression construct (see section 6)

Figure 4- 18 Downregulation Expression of Barx2 in Ovarian Cancer Cell Lines PEO1 series



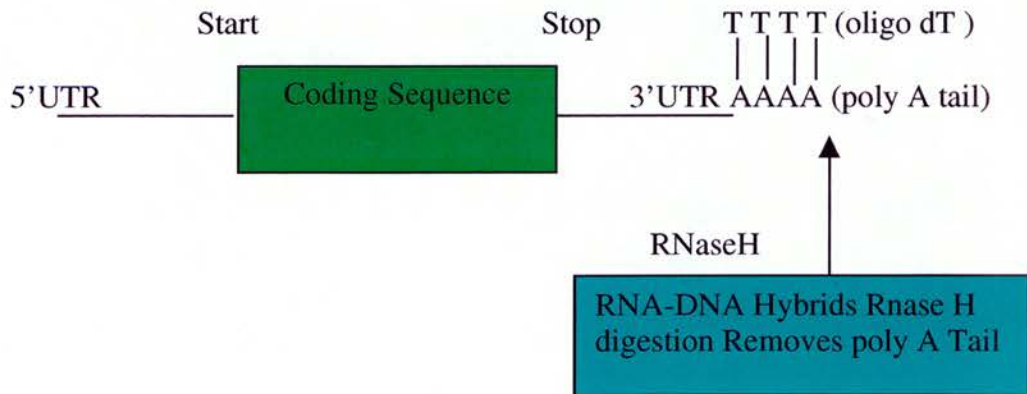
Comparing the expression of Barx2 in PEO1, PEO1cDDP, PEO4 and PEO6 have only 57.7 %, 14.6 % and 9.5 % of the Barx2 expression level (relative to actin) of PEO1 respectively.

4.3 The results of RNaseH experiment

A broad band corresponding to Barx2 was detected in the ovarian cancer cell lines Northern blotting suggested the existence of multiple transcriptional isoforms (Figure 4-17). In addition, RT-PCR using the full length coding region primer pairs F1/R3 detected at least two Barx2 transcript (780bp and 500bp RT-PCR products; Figure 4-5). These apparent isoforms may result from a) variation in length of Barx2 transcript poly A tail in different cell lines; b) different Barx2 isoform due to alternative exon or splicing of an alternative 3'UTR.

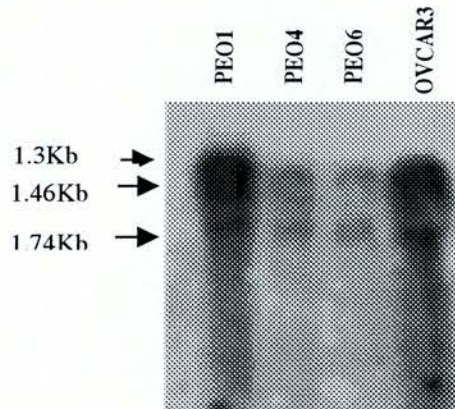
The purpose of performing a RNaseH experiment was to remove the Poly A tail from the total population of transcripts, thereby normalising for any variation poly A tail length. RNA/DNA hybrids formed between transcript poly A tails and oligo- dT primers following incubation are recognised and digested by Rnase H (see Figure 4-19).

Figure 4- 19 The Principle of RNaseH Experiment



A Northern blot of RnaseH- oligo dT RNA hybridized with a full length Barx2 probe reveals three apparent isoforms, (see Figure 4-20), which arise due to a mechanism other than variation in poly A tail length.

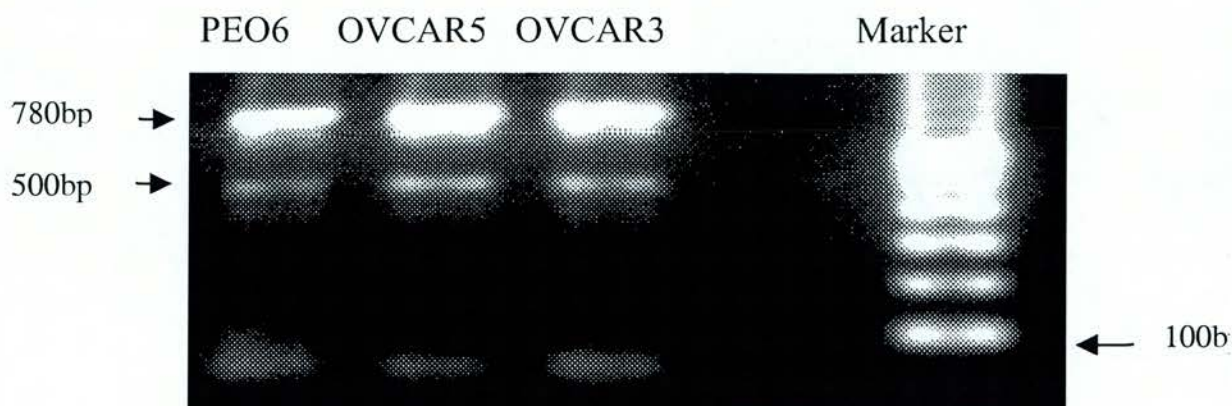
Figure 4- 20 The Expression of Different Barx2 Isoforms in Ovarian Cancer Cell Lines



4.4 Sequencing of Barx2 cDNA

Semiquantitative RT-PCR and Northern blot data together for Barx2 expression in ovarian cancer cell lines have provided evidence for the existence of at least two transcript isoforms, one was about 700 to 780 bp and other one was about 440bp to 500. It suggested that there would be two transcription isoform in the Barx2 gene. In order to investigate further the difference between the two transcription isoforms of Barx2 in ovarian cancer, Barx2 F1/R3 RT-PCR products from OVCAR3, OVCAR5 and PEO6 (see Figure 4-21) were directly sequenced or subcloned and then sequenced.

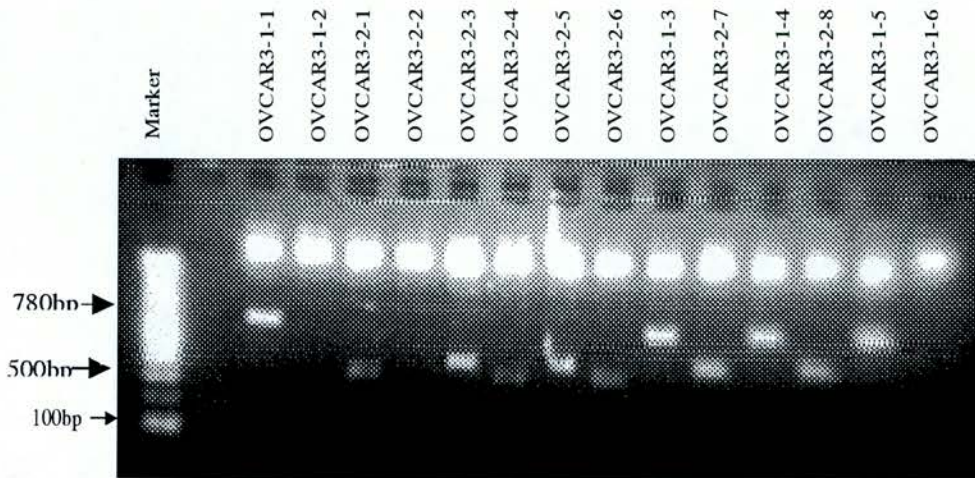
Figure 4-21 the Expression of Barx2 (F1/R3) In Ovarian Cancer Cell Lines



The pGEM-T Easy vector systems Promega were used to subclone these RT-PCR products. The vectors of this system are prepared by cutting Promega's pGEM-5Zf(+) and pGEM-T easy vector with EcoR V and adding a 3' terminal thymidine to both ends. These single 3'-T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR product generated by certain thermostable polymerases. They also contain T7 and SP6 RNA polymerase promoter flanking a multiple cloning site (MCS) within the α -peptide coding region of the enzyme p-galactosidase. Insertional inactivation of the α -peptide allows recombinant clones to be directly identified by color screening on indicator plates.

These vectors contain multiple restriction sites within the MCS, which allow for the release of the insert by digestion with a single restriction enzyme. Figure 4-22 shows the results of EcoRI digestion subcloned Barx2 F1/R3 RT-PCR products from OVCAR3, showing presence of different insert size

Figure 4-22 the Result of Digestion with EcoRI for Subclones of Barx2 F1/R3 RT-PCR Products of OVCAR3



OVCAR3-1 and -2 represent that this subclone was derived from 780 bp or 500 bp product of RT-PCR respectively. The final digit (1 to 8) represents the clonal number.

Plasmid DNA was isolated from a total 35 subclone , Insert were then sequenced using Barx2 F1 and R3 primer flanking the MCS.

All sequence was analyzed with ABI-seq analysis package and compared with Barx2 mRNA sequence in GenBank (NCBI) nucleotide database by BLAST 2.0 searching. The results are outlined in Table 4-3.

Table 4-3 Results of RT-PCR Products Sequencing in Ovarian Cancer Cell Line

cell line	RT-PCR product size(bp)	sequencing primer	Genbank of Barx2 sequence match(bp)	Exon			
				1	2	3	4
OVCAR3	700-780	F1	224-920	+	+	+	+
			210-282	+	-	-	-
			582-908	-	-	+	+
	440-500	R3	185-865	+	+	+	+
			231-282	+	-	-	-
			582-878	-	-	+	+
OVCAR5	700-780	F1	223-792	+	+	+	+
			218-282	+	-	-	-
			582-928	-	-	+	+
	440-500	R3	185-878	+	+	+	+
			218-282	+	-	-	-
			582-878	-	-	+	+
PEO6	700-780	F1	223-792	+	+	+	+
			219-282	+	-	-	-
			582-928	-	-	+	+
	400-500	R3	219-757	+	+	+	+
			179-281	+	-	-	-
			582-856	-	-	+	+

+= sequence can match with exon

-= sequence mismatch with exon

The results showed that the sequence of 780 bp Barx2 F1/R3 RT-PCR product cover Barx2 exons 1 to 4. However, the 500bp RT-PCR product was found to be deleted for Barx2 exon2 (285bp) (see Figure 4-22).

Computer analysis shows that deletion of exon 2 would result in a frame shift mutation in exon3, introducing a termination codon. As exon 2 and exon 3 encode the homeodomain , such a protein product (if ever, made) would be lacking the homeodomain and may consequently act as a dominant negative isoform. The presence of such an isoform may severely disrupt the normal function of the Barx2 protein in its regulation of specific target genes.

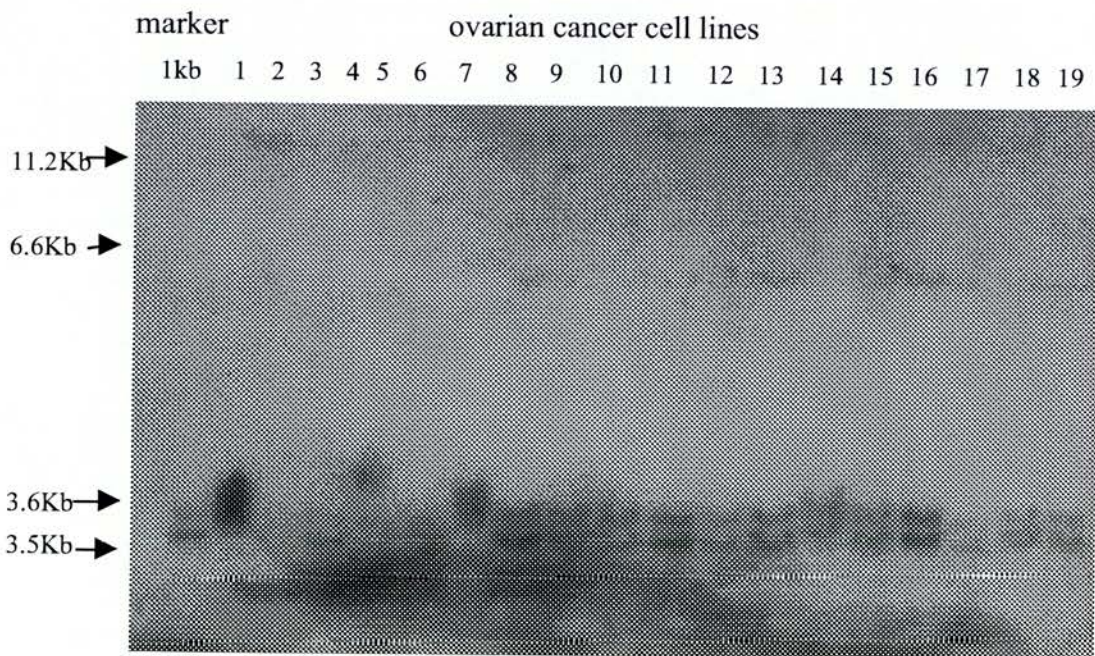
5. STRUCTURAL ANALYSIS OF BARX2

Northern blot and RT-PCR analysis data have shown that expression of Barx2 is downregulated/absent in proportion ovarian cancer tissue and cell lines. We decided to look for mutation in these sources expressing Barx2 and to identify whether sequence alterations occurred. Southern blot, pulsed-field gel electrophoresis (PFGE), mutation and methylation analyses were performed to determine whether any structural alterations of the Barx2 gene are present in ovarian cancer tissues and cell lines.

5.1 Southern blotting analysis

High molecular weight (HMW) genomic DNA was extracted from 19 ovarian cancer cell lines. DNA was digested with EcoR I or BamH I. Size separated after running agarose gel and Southern blotting was hybridized with a full length Barx2 prob. The results are showed in Figure 5-1 (EcoR I) and Figure 5-2 (BamH I). The result of Southern blotting (EcoR I) was summarized in Table 5-1

Figure 5-1 The Result of Southern Blot (EcoR I)



Southern blot of EcoR I digested HMW DNA probed with Barx2 full length probe shows that restriction fragment (3.5 Kb and 3.6 Kb) are present in most of the cancer cell lines tested. Some of the cell lines also show evidence of two larger hybridizing fragments of 11.2 Kb and 6.6 Kb

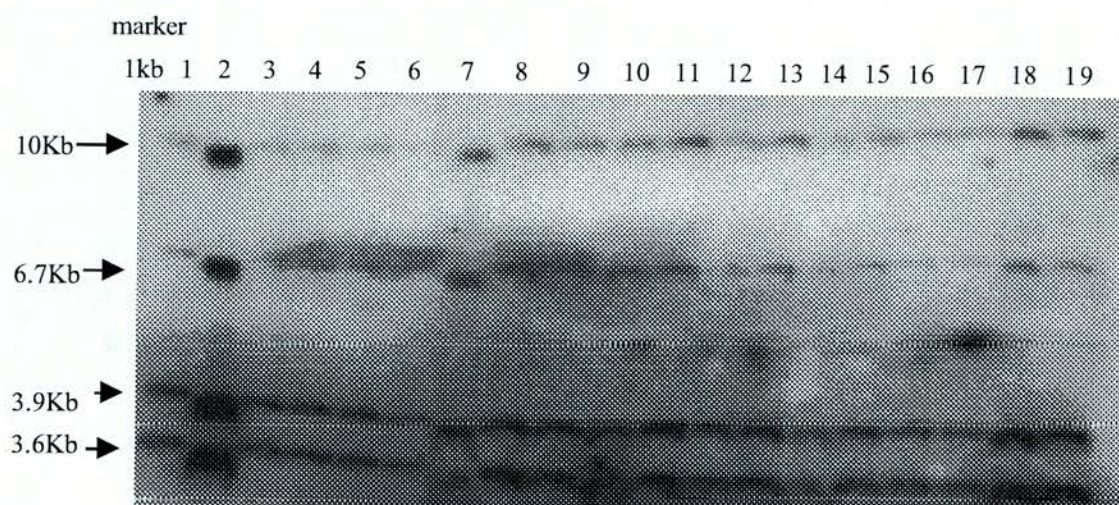
Table 5-1 the Result of Southern Blot (EcoR I)

Southern No	CELL INE	11.2Kb	6.6Kb	3.6Kb	3.5Kb
1	SW48	ND	ND	+	+
2	LOVO	ND	ND	+	+
3	HT115	ND	ND	+	+
4	HT117	+	+	+	+
5	HT215	+	+	+	+
6	HTC18	+	+	+	+
7	A2780	+	+	+	+
8	PEO23	+	+	+	+
9	OVCAR5	+	+	+	+
10	OVCAR4	ND	ND	+	+
11	OVCAR4	+	+	+	+
12	OVCAR3	+	+	+	+
13	SW626	+	+	+	-
14	OAW28	ND	ND	+	+
15	OAW42	ND	ND	+	+
16	PEO1cDDP	ND	ND	+	+
17	PEO1	ND	ND	+	+
18	PEO6	ND	ND	+	-
19	PEO4	ND	ND	+	+

ND= not determinable

17/19 cancer cell lines have two strong restriction fragments of 3.6 Kb and 3.5 Kb. However, the 3.5 Kb fragment is not detectable in OAW28 and PEO6, although the 3.6 Kb fragment is in all cases.

Figure 5-2 the Result of Southern Blot (BamH I)



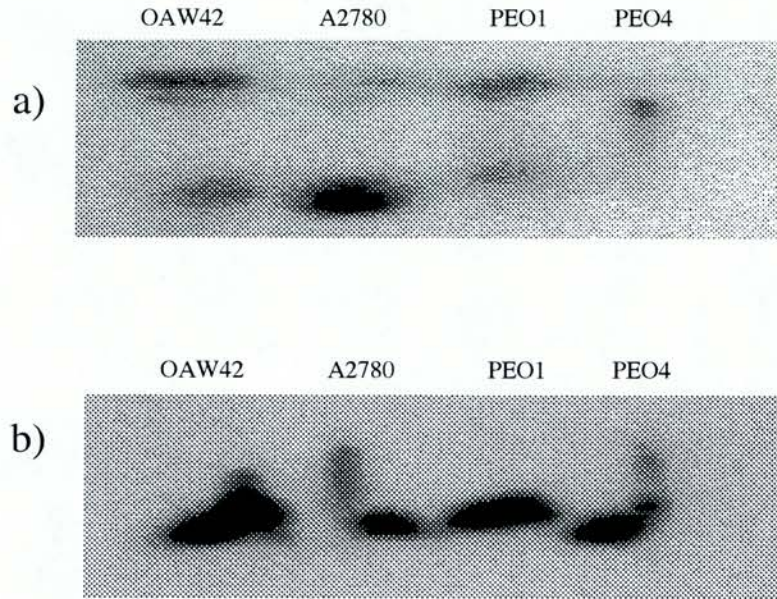
Southern blot of BamH I digested HMW DNA probed with Barx2 full length probe shows that two strongly restriction fragments (3,9Kb and 3.6Kb), and two weakly hybridizing restriction fragments (10Kb and 6.67Kb)

The conclusion is therefore that the Barx2 gene, at this level of detection, is not rearranged in any of the 19 cell lines tested. Therefore, this is unlikely to be the mechanism underlying affected Barx2 expression in these cell lines.

5.2 The Result of Pulsed-Field Gel Electrophoresis (PFGE).

In preceding investigations, the data has shown that expression of Barx2 is downregulated/absent in the ovarian cancer cell lines OAW42, A2780, PEO1-CDDP, PEO4 and PEO6. But there were no difference between these ovarian cancer cell lines in Southern Blot. The experiment of Pulsed-Field Gel Electrophoresis was performed in order to analyze further whether there would be altered long-range structure in OAW42, A2780, PEO1, and PEO4 ovarian cancer cell lines. Figure 5-3 showed the results of PFGE.

Figure 5- 3 The Result of Pulsed-Field Gel Electrophoresis



The cell lines digested with Hind III for southern blot probed with Barx2 full length probe showed two restriction fragments (Figure 5-3 a). The cell lines digested with SacI for southern blot probed with Barx2 full length probe showed only one restriction fragment (Figure 5-3 b). There are no any differences between the cell lines.

5.3 Mutation analysis

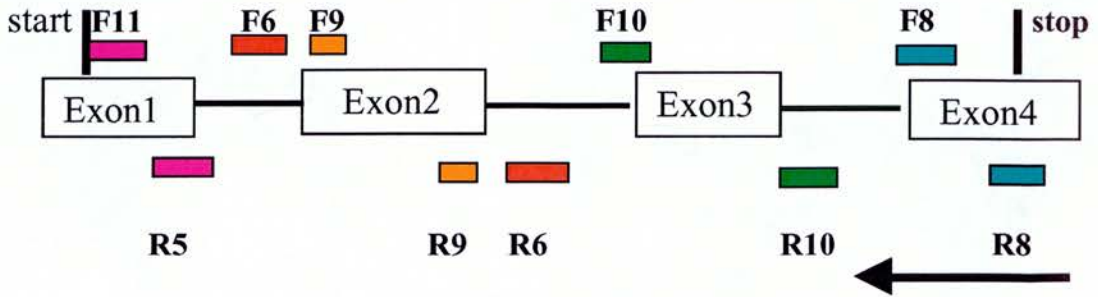
5.3.1 *The single stranded conformation polymorphism analysis (SSCP) /heteroduplex analysis (HDA) for Barx2 in ovarian cancer cell lines.*

Physical mapping demonstrated that Barx2 was located within the chromosome 11q25 region that was associated with frequent loss of heterozygosity in ovarian cancer. In addition, Barx2 expression was absent or reduced in some ovarian cancer cell lines and tumor tissue. To fulfill the Knudson's two-hit hypotheses for inactivation of a tumour suppressor gene, investigations of possible mutation of Barx2 as a likely method of inactivation or altered function was explored.

SSCP and HDA are quick, simple techniques which provide a good starting point for mutation analysis. The DNA segment of interest is amplified by polymerase chain reaction, the strands of the PCR product are denatured, then separated by gel electrophoresis under nondenaturing conditions. Each individual strand may self-anneal and fold to achieve the most thermodynamically stable conformation. Electrophoretic differences occur with as little as a single base change (Orita M, et al. 1989). I used a protocol (provided by Richard Axton, MRC Human Genetics Unit Edinburgh) whereby SSCP and HAD can be carried out together. This relies on the observation that under SSCP conditions, a significant proportion of a PCR product can reanneal prior to electrophoresis. The capture of both single and double stranded DNA on the same polyacrylamide gel allows data from both techniques to be gathered at the same time.

We designed four pairs of PCR primer to allow amplification of each of the 4 exon of Barx2 individually. Although not publicly available at the time, our collaborator Dr Barry Nelkin (John Hopkins University, USA) provided us with the genomic organization and partial nucleotide sequence of the Human Barx2 gene. The sequence information encompassed the exon-intron boundaries, permitting design of PCR primers from within introns. Therefore, PCR amplification was possible allowing for analysis of coding of complete exon sequence and also splice junction sites where nucleotide changes may alter normal transcriptional splicing event (see Figure 5-4).

Figure 5 – 4 The Primer of Barx2 for PCR of Genomic DNA



F= forward R= reverse

Exon-specific genomic PCR products from 9 ovarian cancer cell lines were analyzed four by the combined SSCP/HAD method. A total of 4 experiment were performed. Representative results are shown in Figure 5-5 to Figure 5- 8, and are all summarized in Table 5-2

Figure 5-5 The SSCP of Barx2 (exon1) In Ovarian Cancer Cell Lines

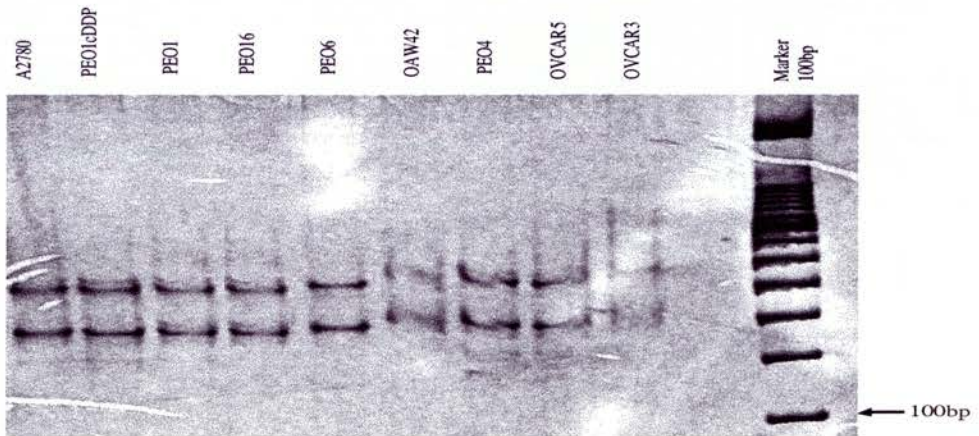


Figure 5-6 The SSCP of Barx2 (exon2) In Ovarian Cancer Cell Lines

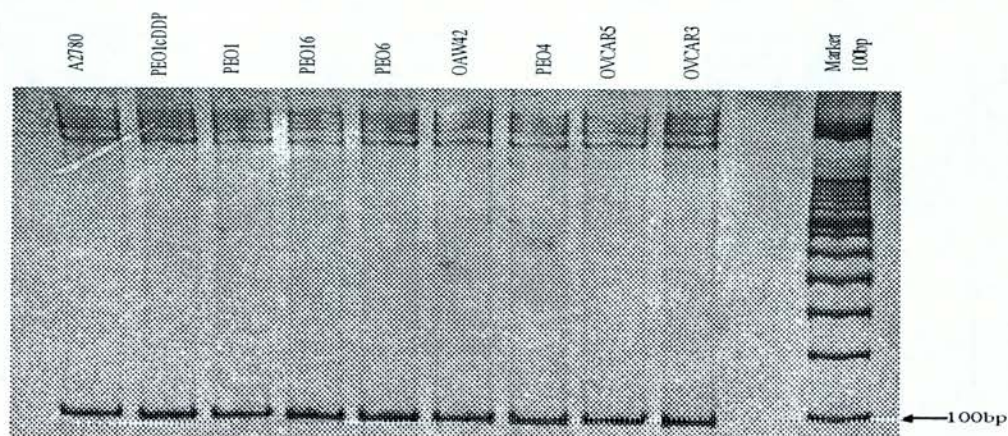


Figure 5-7 The SSCP of Barx2 (exon3) In Ovarian Cancer Cell Lines

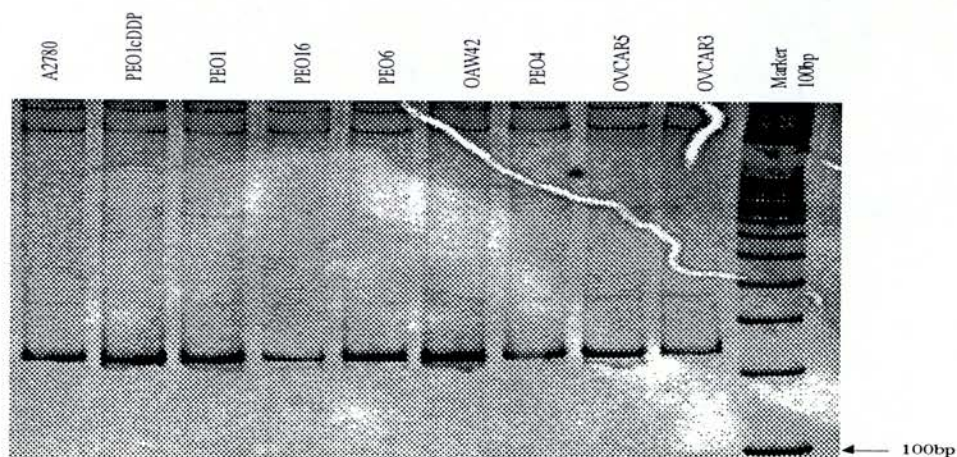
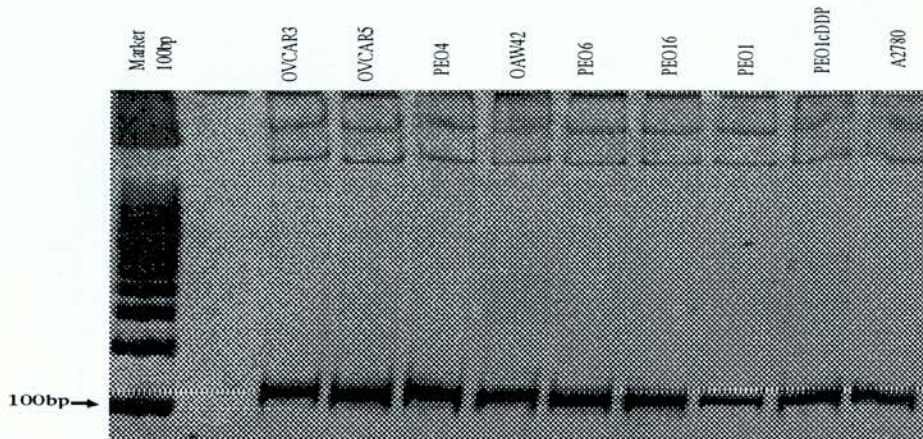


Figure 5-8 The SSCP of Barx2 (exon4) In Ovarian Cancer Cell Lines



The top bands represent single stranded DNA bands and the bottom Bands are double stranded in the gels. There are no band shifts in the gels

Table 5- 2 The Results of SSCP/HAD for Barx2 in Ovarian Cancer Cell Lines

ovarian cancer lines	F 11/R5 exon 1			F6/R6 exon 2			F8/R8 exon 3			F10/R10 exon 4		
	S	D	SH	S	D	SH	S	D	SH	S	D	SH
A2780	+	+	-	+	+	-	+	+	-	+	+	-
OAW42	+	+	-	+	+	-	+	+	-	+	+	-
PEO1	+	+	-	+	+	-	+	+	-	+	+	-
PEO4	+	+	-	+	+	-	+	+	-	+	+	-
PEO1cDDP	+	+	-	+	+	-	+	+	-	+	+	-
PEO6	+	+	-	+	+	-	+	+	-	+	+	-
OVAR3	+	+	-	+	+	-	+	+	-	+	+	-
OVAR5	+	+	-	+	+	-	+	+	-	+	+	-
PEO16	+	+	-	+	+	-	+	+	-	+	+	-

S= Single stranded band, D= Double stranded band, SH= Shifted band.

In all of the cell lines tested, no shifted bands were detected, indicating that no mutations were present

It is also possible that under the experimental conditions, particular mutations would not be detectable and only become evident by repeating the SSCP/HDA assay using altered sets of condition.

The SSCP/HDA experiment was further repeated. The concentration of PA gel was decreased to 8 % and run time increased to 20 hours but at 4 0C in order to separate top single stranded band better. These results are shown in Figure 5-9 to Figure 5-10 and summarized in Table 5-3.

Figure 5-9 The Results of SSCP for Barx2 (exon 4 and 2) In Ovarian Cancer Cell Lines

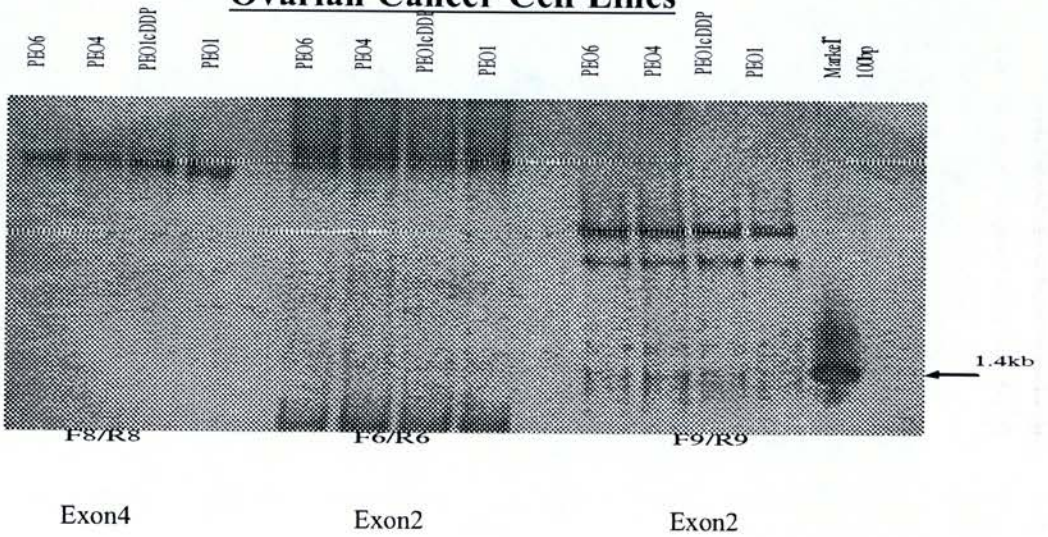
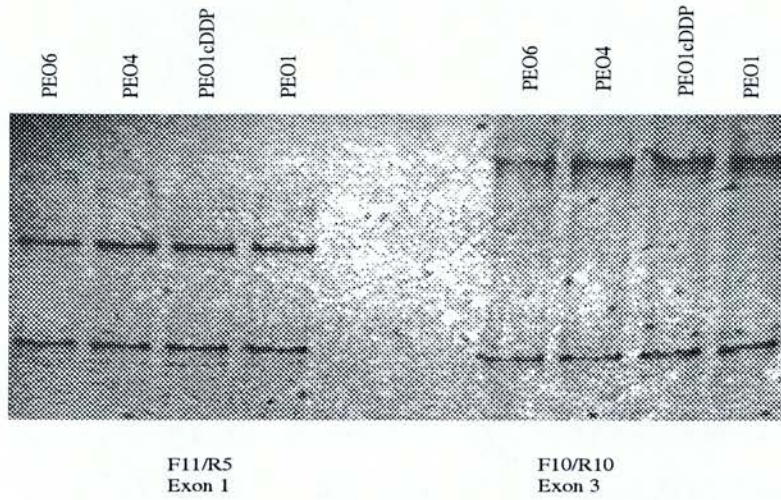


Figure 5-10 The Results of SSCP for Barx2 (exon 1 and 3) In Ovarian Cancer Cell Lines



All of band in the gel is single stranded band

Table5 –3 The result of SSCP/HAD for Barx2 in PEO1,PEO4,PEO1cDDP and PEO6

ovarian cancer lines	exon 1			exon 2			exon 3			exon 4		
	S	D	SH	S	D	SH	S	D	SH	S	D	SH
PEO1	+	+	-	+	+	-	+	+	-	+	+	-
PEO4	+	+	-	+	+	-	+	+	-	+	+	-
PEO1cDDP	+	+	-	+	+	-	+	+	-	+	+	-
PEO6	+	+	-	+	+	-	+	+	-	+	+	-

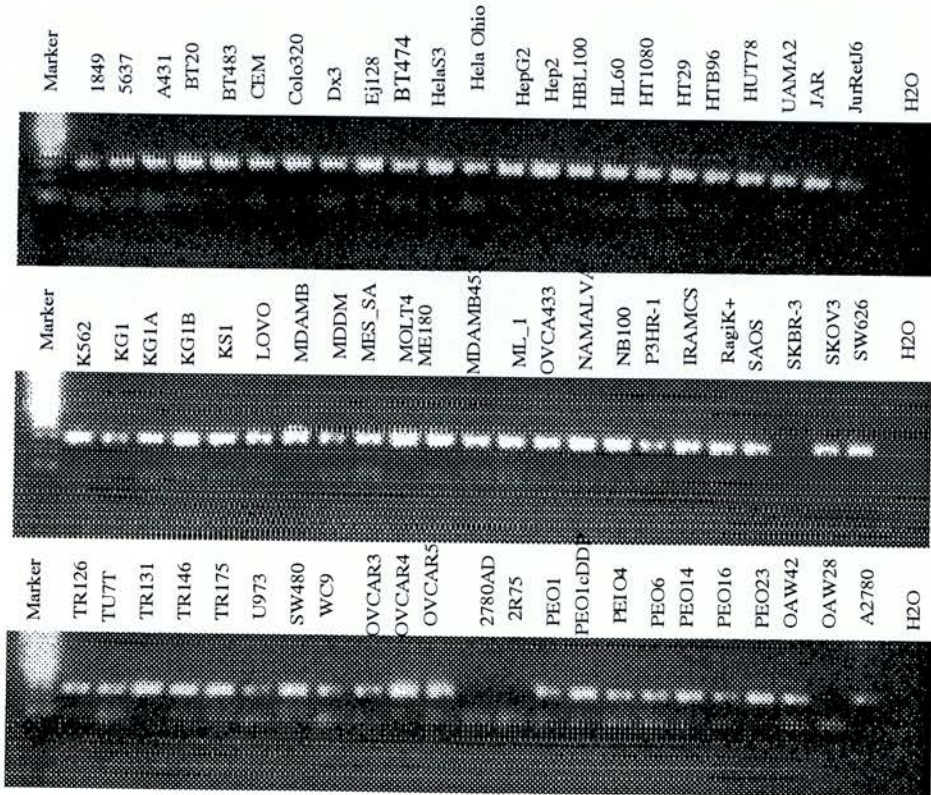
S= Single stranded band, D= Double stranded band, SH= Shifted band.

In all cell lines, no shifted bands were found for any exon, indicating that there was no Barx2 mutation in these ovarian cancer cell lines.

5.3.2 Mutation analysis of Barx2 and identification of single nucleotide polymorphism of cancer cell lines and tumour tissue using the Transgenomic Wave™

PCR primers were designed to allow amplification of individual Barx2 exons. Larger PCR fragments were used for Denaturing High Pressure Liquid Chromatography (DHPLC) analysis of cancer cell lines and colorectal tumour/blood pair DNA (high molecular weight). Figure5-11 showed the results of genomic PCR of Barx2 (exon2) in cancer cell lines. The genomic PCR of Barx2 exon1 and exon2 was performed individually in a total of 69 cancer cell and 48 colorectal tumour/blood pairs.

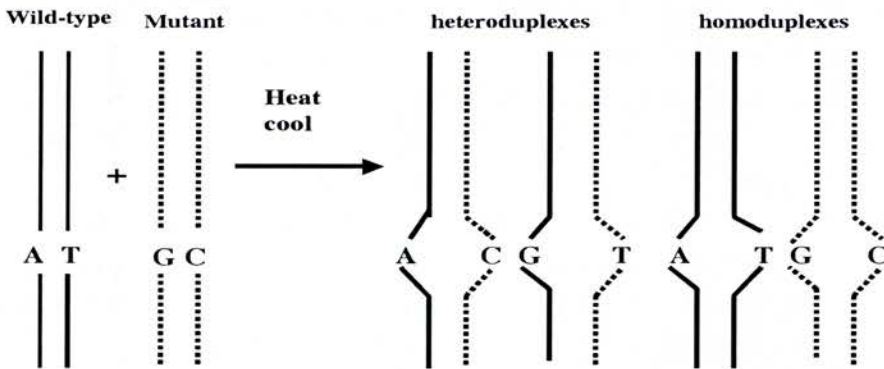
Figure5-11 The Results of Genomic PCR of Barx2 (exon2) In Cancer Cell Lines.



DHPLC was performed using a Transgenomic Wave machine. For cell line DNAs, alteration of the known sequence from wild type was assessed by mixing Exon PCR products from each of the cell lines with Exon PCR products derived from monoallelic Barx2 PAC DNA in a 1:1 mix for heat denaturation and re-annealing to identify heteroduplex.

Sequence alteration detection is based on the principle that individuals who are heterozygous in a mutation or polymorphism have a 1:1 ratio of wild-type and mutant DNA. A mixture of hetero- and homoduplexes is formed when the PCR product is hybridised by heating to 95 °C and cooling slowly. The DNA from individuals who have two mutant alleles (homozygous mutation) will only form homoduplexes and must therefore be mixed with wild-type DNA and hybridised in order to detect heteroduplexes. After this treatment, a sample will contain a mixture of hetero- and homoduplexes (see Figure 5-12). However, DNA from homozygous wild-type individuals will form only one species, the homoduplex wild-type (Underhill PA, et al.1996)

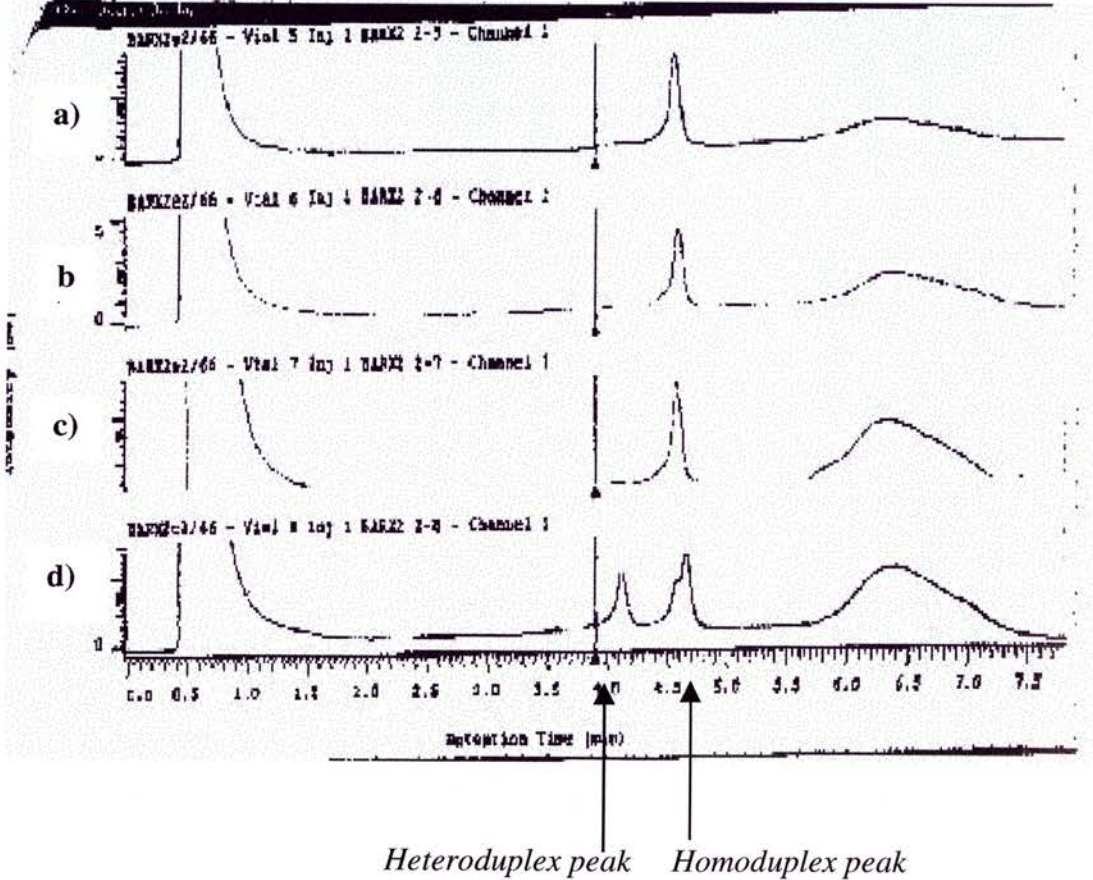
Figure 5-12 Creation of A Mixture of Hetero- and Homoduplexes Though Hybridization



Mutation are visualised on the Wave™ as a characteristic pattern of peaks corresponding to the mixture of heteroduplexes and homoduplexes formed when wild-type and mutant DNA are hybridized.

Total 69 cancer cell lines expect for 4 cell lines that PCP did not work, PCR products of individual Barx2 exons in 65 cancer cell lines and 48 colorectal tumour/blood pairs were screened using Wave™ for detecting mutation of Barx2 respectively. Genomic PCR products from the cell lines were analysed both with and without addition of the PAC clone derived wild-type product. Heterozygous alterations in Barx2 sequence should be detectable without addition of any wild type product Figure 5-13 shows an example for mutations by DHPLC using Transgenomic Wave in ovarian cancer cell lines. The results are summarised in Table 5-4

Figure 5-13 an Example of Result of Mutation Scanning in Cancer Cell Lines.



a) Cancer cell line DNA without spiking with wild-type DNA. Homoduplex peak only b) Spiking cell line DNA from (a) with wild-type DNA, A homoduplex peak, indicates that cell line (a) is homozygous wild-type. c) Another cancer cell line DNA without spiking with wild-type DNA. Homoduplex peak only. d) Spiking cell line DNA from (c) with wild-type DNA. Heteroduplex peak beside homoduplex peak. indicates either heterozygous mutation or polymorphism.

Heteroduplex formation was observed by DHPLC in ovarian cancer cell line SKOV3 following spiking with wild-type PAC-derived PCR product, indicating that presence of a sequence difference between the cancer cell line exon 2 of Barx2 and wild-type exon 2 (wild-type Barx2 PAC-derived PCR product). Sequencing of cell lines SKOV3 exon 2 PCR product revealed the presented missense: Ser--- Pro creating a Hae III site (The sequencing work was done by Dr Karen Watt in our lab). This missense mutation was not contained within the homeobox sequence encoded by exon 2, and is not a clear example of an inactivating mutation.

Table 5-4 the Summary of Identification of Single Nucleotide Polymorphisms of Cancer Cell Lines and Tumour Tissue on the WaveTMes

Sample	N	exon1 HE	exon2 HE	exon3 HE	exon4 HE
cancer cell line	65	2	5	0	0
colorectal tumour/ blood pair	48	4	6	0	0

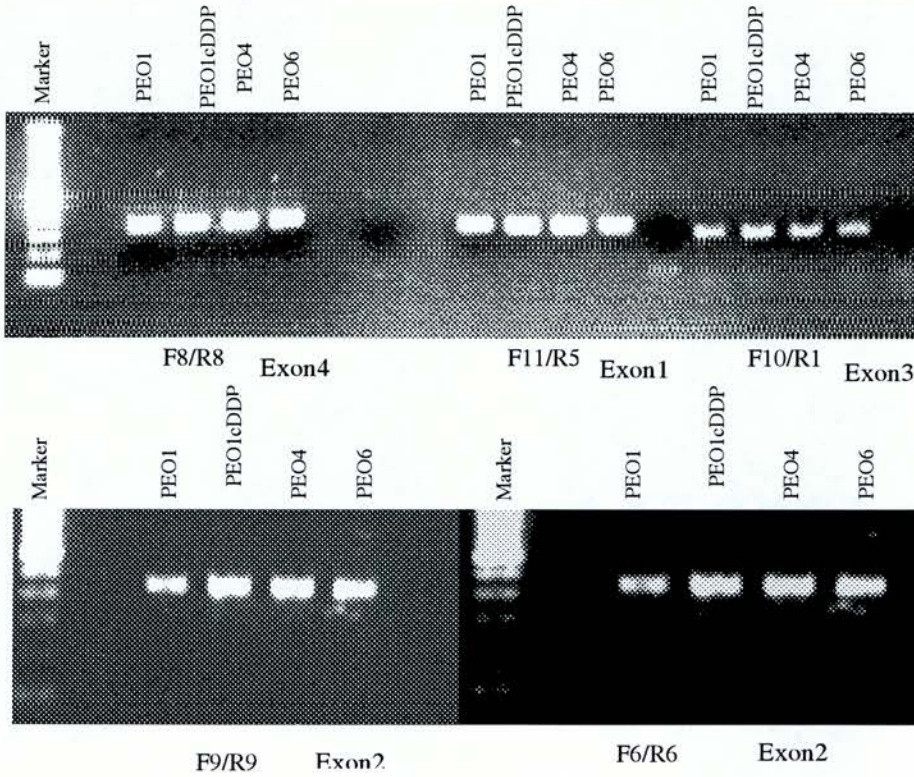
5.3.3; Direct DNA sequencing analysis for Barx2 in ovarian cancer cell lines

Although RT-PCR and Northern blot data have shown that expression of Barx2 is downregulated or absent in several ovarian cancer cell lines, there was no evidence to demonstrate inactivating mutation of Barx2 in these ovarian cancer cell lines.

Sequencing of PCR products is a powerful technique that enables a direct search for mutations in a defined fragment of DNA.

Genomic PCR products for individual exons from the PEO series, PEO1, PEO1cDDP, PEO4 and PEO6 were generated (Figure 5-14) and sequenced directly.

Figure 5-14 the Results of Genomic PCR of Barx2 in PEO Series



CLUSTAL X multiple sequence alignment was used to compare PEO1 sequence with PEO1cDDP, PEO4 and PEO6. In addition, DNA from fibroblasts isolated during the initial derivation of PEO1 was also used to generate Barx2 exon-specific PCR products. PEO1 fibroblast DNA represented normal DNA in this comparison.

The alignment analysis of genomic PCR products for Barx2 gene in PEO-Fibroblast PEO1, PEO1-CDDP, PEO4 and PEO6 was summarised in Figure 5-15.

Figure 5-15 The CLUSTAL X window in multiple sequence alignment. An alignment of Barx2 exon 2(F6/R6, F9/9) sequences in PEO-Fibroblast PEO1, PEO1-CDDP, PEO4 PEO6 and OVCAR3 (from top to bottom in order) were displayed. Exceptional sequences were shown as a white background. The overall direct sequencing of genomic PCR products did not reveal any difference in coding sequence between PEO1, PEO1-CDDP, PEO4 PEO6, OVCAR3 and PEO-Fibroblast. In conclusion no somatic inactivating mutations are present in these ovarian cancer cell line series.

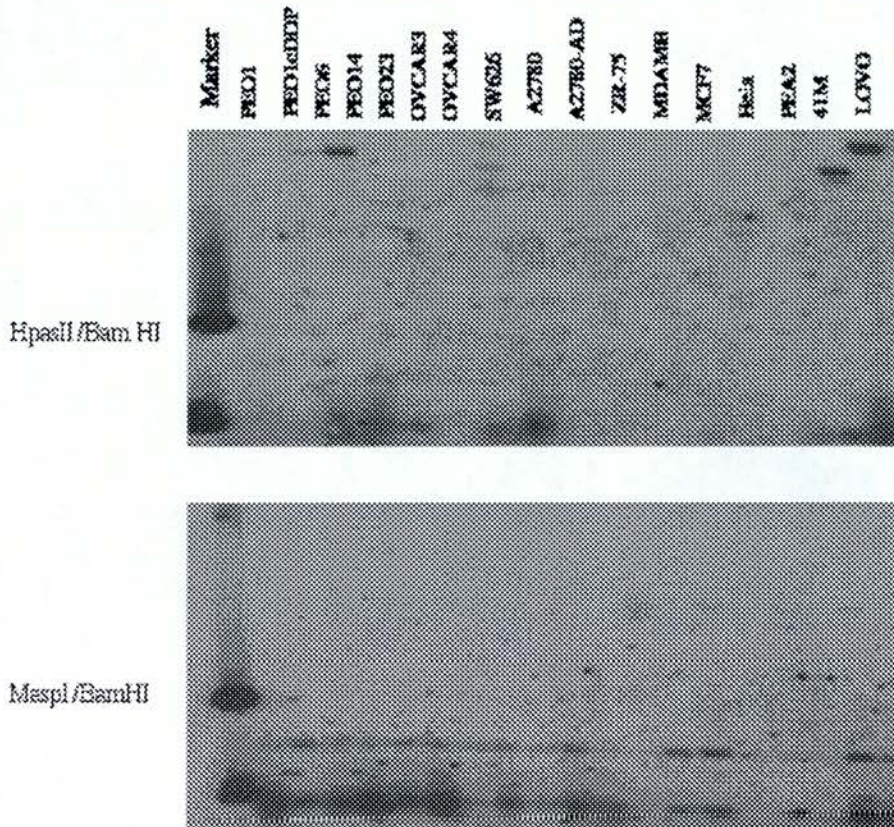
5.4 Methylation analysis for Barx2 in cancer cell lines

In the absence of somatic inactivation mutations, a study of the methylation status of the CpG islands at 5' end of the Barx2 gene was undertaken to investigate its possible role in downregulated/absent expression of Barx2 in ovarian cancer cell lines.

Methylation of cytosine located 5' to guanosine is known to have a profound effect on the expression of genes. In normal cells, methylation occurs predominantly in CG-poor regions, while CG-rich areas, CpG islands, remain unmethylated. Aberrant methylation of normally unmethylated CpG islands has been associated with transcriptional inactivation of defined tumour suppressor genes in human cancers (Mompalao RL and Bovenzi V. 2000). Many studies have confirmed that methylation of DNA may contribute to tumorigenesis through silencing of genes which are usually hypomethylated and critical in control of the cell cycle (e.g. tumour suppressor genes) and cellular differentiation (Kay PH, et al. 1997).

DNA digested with methylation sensitive restriction enzymes is one way that determines the methylation status of cytosine (Laird PW and Jaenisch R. 1996). In order to demonstrate whether methylation is a possible mechanism for gene silencing, Southern blots were prepared. DNA of cancer cell lines digested firstly with BamHI and Hind III and then digested with methylation sensitive and non-sensitive restriction enzymes MspI and HpaII respectively. Blots were then probed with the Barx2 F11/R5 (5' UTR to exon2). The results were summarised in Figure 5-16.

Figure5-16 The Result of Barx2 5' CpG island methylation in Cancer Cell Lines



The alteration in the hybridisation pattern observed between HpaII and MspI-digested cell line DNA in the Southern analysis suggested that Barx2 5' CpG island is methylated in PEO1cDDp, PEO6, PEO14 PEO16, OVCAR3, 2780 AD, HeLa PEA2 41M and MDA. Cell lines previously demonstrated to have downregulated Barx2 expression apparently show evidence of CpG island methylation, thereby providing a mechanism for gene silencing.

Following this study, however, methylation-specific PCR, a new and highly sensitive technology for the detection of gene methylation (Herman JG, et al. 1996) was performed by Dr Grant C. Sellar and Genevieve J. Rabiasz in our lab to determine the methylation status of cytosine in ovarian cancer cell lines and tumour tissue. PCR primers spanning the CpG-rich MseI fragments were designed to assess CpG island somatic methylation. The six primers covered a region covering the two MseI fragments and extending from -1097 to +282 with respect to the translational start site. Eighty epithelial ovarian normal/tumour pairs and 38 matched ovarian cancer biopsies prior to and following relapse after cisplatin chemotherapy were analysed. The results showed that the homeobox gene Barx2 is not methylated in ovarian cancer, in contrast to the initial findings from the southern blot analysis presented here.

6. FUNCTIONAL ANALYSIS OF BARX2

Frequent LOH at chromosome 11q24-25 was associated with poor survival in epithelial ovarian cancer (Gabra H, et al 1996). Microcell mediated transfer of chromosome 11 to the ovarian cancer line NIH:OVCAR3 had revealed a phenotype of invasiveness suppression and suppression of cell migration and adhesion associated with 4.5 Mb region on the chromosome 11q24 between D11S912-D11S1320 (Gabra H, et al.unpublished). Barx2 is located at chromosome 11q25 between the markers D11S912 and D11S1320 (Krasner A, et al. 2000), suggesting that Barx2 as a functional candidate epithelial ovarian tumor suppressor gene. Transfections of Barx2 into OVCAR3, OAW42, PEO1and PEO1cDDP were performed in order to further analyze the function of the Barx2 gene in ovarian cancer.

6.1 Transfecting Barx2 into Ovarian Cancer Cell lines.

6.1.1 Setting up the optimal lethal concentration of hygromycin B for all ovarian cancer cell lines used.

An expression construct (pBABE Barx2) containing the full length human Barx2 cDNA subcloned in pBABE hygro was obtained from Dr Barry Nelkin. The parental vector contains the hygromycin selectable marker and therefore, the optimal lethal concentration of hygromycin B for all ovarian cancer cell lines transfection expression construct was determined.

Several ovarian cancer cell lines were characterized with respect to their toxicity profiles to hygromycin B in order to determine the most utilitarian lines. Thus, survival was empirically calculated for several ovarian cancer cell lines for hygromycin B. The optimal lethal concentrations of hygromycin B for all lines used is outlined in Table 6-1

Table 6-1 the Optimal Lethal Concentrations of Hygromycin B for All Lines Used

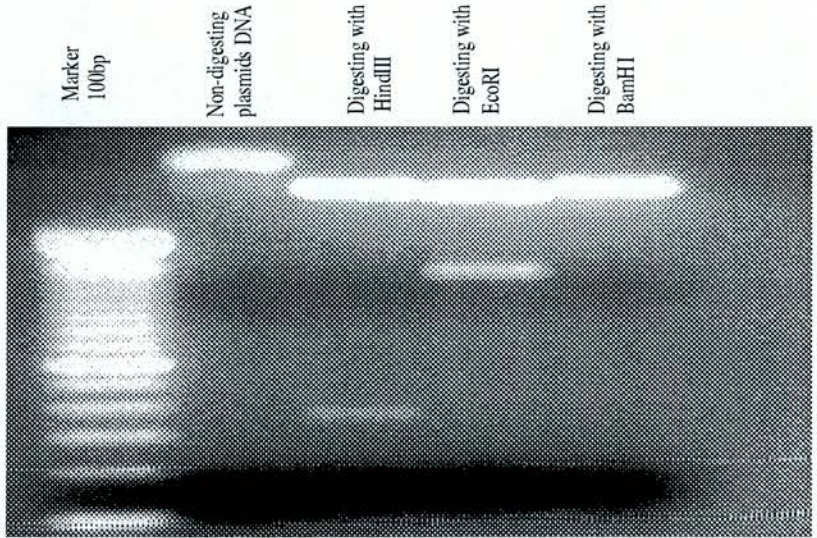
Cell Lines	Toxicity
PEO1	75 µg/ml for 3 week
PEOcDDP	75 µg/ml for 3 week
PEO4	80µg/ml for 3 week
PEO6	80µg/ml for 3 week
OVCAR3	128µg/ml for 2 week
A2780	60µg/ml for 2 week
OAW42	150µg/ml for 2 week
ON3	125µg/ml for 2 week

6.1.2 Preparation of pBABE Barx2 and pBABE Hygro plasmid DNA

pBABE Barx2 and pBABE Hygro plasmid DNA were initially transformed into competent JM109 cells. Isolation of plasmid DNA from transformed single colonies was performed using QIAGEN QIAquick mini spin kits. To check the integrity of the Barx2 insert purified plasmid DNAs were digested with restriction enzyme (EcoR I, Hind III and BamH I) and also amplified by Barx2 F1/R3 PCR. Figure 6 –1 to 6-2 shows these results.

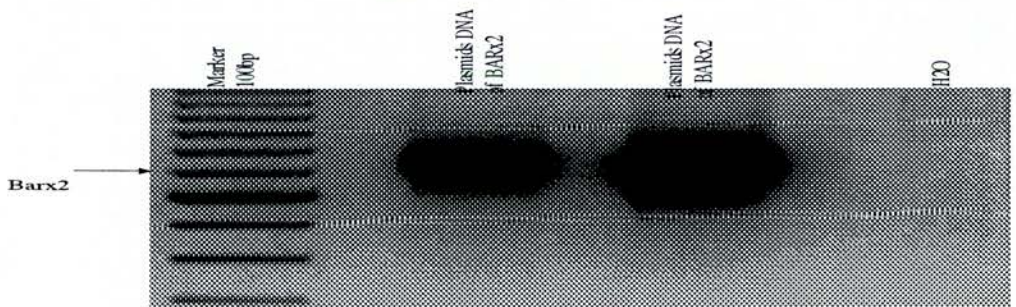
Construct DNAs were also linearized with NotI to increase the effect of transfection. Figure 6-3 shows the result of linearization of pBABE Barx2 plasmid DNA with NotI.

Figure 6-1 The Results of Digesting pBABE Barx2 Plasmid DNA with Restriction Enzymes



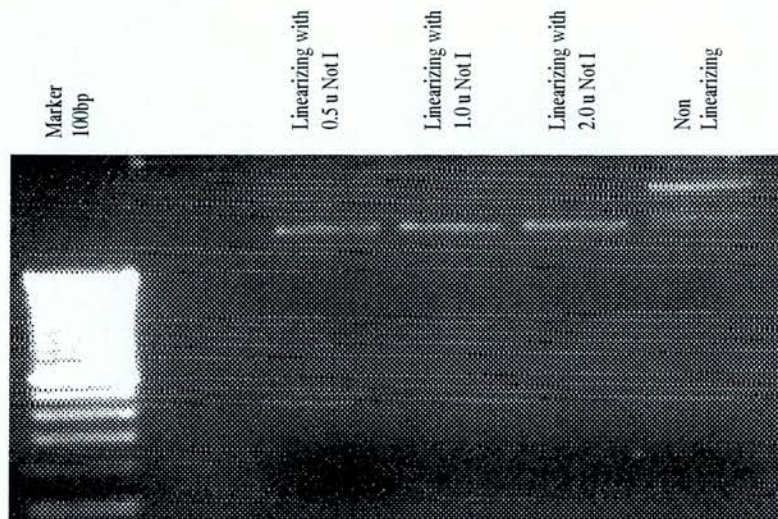
>1.4kp band and 380bp band (with Hind III); >1.4kb and 1.2kb band (with EcorI); and >1.4 Band (with BamH I). These sizes of fragments tallied with those expected from the plasmid construct. Non-digested plasmid DNA appears as a band that is higher than that of digested plasmid DNAs.

Figure 6-2 The Result of PCR (F1/R3) for pBABE Barx2 Plasmid DNA



PCR (F1/R3) for plasmid DNA showed a specific 780 bp Barx2 fragment.

Figure 6-3 NotI Linearization of pBABE Barx2 Plasmid DNA for Transfection



6.1.3 Determining optimal effective of transfection condition for ovarian cancer cell lines used

Initially, optimal effectene condition for transfection were established empirically for these ovarian cancer cell lines (outlined in section 2) These are outlined in Table 6-2

Table 6-2 Optimal Effectene Conditions for Ovarian Cancer Cell Lines Used

Cell line	effectene volume(μ l)	enchance volume(μ l)	DNA(μ g)
OAW42	10, 25, 50	8.0	0.2
PEO1	25	8.0	0.2
PEO1cDDP	25	8.0	0.2
PEO4	10, 25, 50	8.0	0.2
PEO6	10, 25, 50	8.0	0.2
A2780	10, 25, 50	8.0	0.2

However, no clones were produced for OAW42, A2780, PEO4 and PEO6 in 2 separate Barx2 and vector transfection experiment. In order to improve transfection

efficiency, the transfection selector kit was used. The results of OAW42 transfection selector kit experiment are outlined at Table 6-3

Table 6-3 The Results of OAW42 Transfection Selector

No	effectene volume(μ l)	enhancer volume(μ l)	DNA(μ g)	cell density
1	2	1.6	0.2	++
2	5	1.6	0.2	+++
3	10	1.6	0.2	+++
4	4	3.2	0.4	+++
5	10	3.2	0.4	+++++
6	20	3.2	0.4	+++
7	8	6.4	0.8	++++
8	20	6.4	0.8	++
9	40	6.4	0.8	+
10	Control (serum + effectene)			+
11	Control (serum)			+

This data showed that the suitable conditions of transfection in OAW42 are; effectene 10 μ l, enhancer 3.2 μ l and transfecting DNA 0.4 μ g.

Lipofectin was used to transfect pBABE Barx2 into OVCAR3, ON3 and A2780. The lipofection conditions use the lipofection protocol (outlined in section 2). The optimal lipofectin conditions were established empirically for these ovarian cancer cell lines and are outlined in Table 6-4.

Table 6-4 Optimal Lipofectin Conditions for Ovarian Cancer Cell Lines Used

Cell line	Lipofectin (μ l)	DNA(μ g)	Incubation time (hour)
OVCAR3	10	2	6
ON3	10	2	6
A2780	10	2	12

6.1.4 The transfection of the human Barx2 gene into ovarian cancer cell lines

Between 2 and 4 separate transfection experiments were performed for each cell line. For each experiment, the ovarian cancer cells were seeded in three plates per lipofectin or effectene condition. The results are summarized in Table 6-5

Table 6-5 Summary of Transfection Experiment

Cell line	Experiment No	Method	Result
OAW 42	1	effectene	not transfectable
	2	effectene	not transfectable
	3	effectene	2 pBABE-Hygro-Barx2 clones, 1 distinct population, 1 distinct. no pBABE-Hygro clone produced
	4	effectene	Distinct clones in pBABE -Hygro no pBABE -Hygro- Barx2 clone produced.
A2780	1	Lipofectin	not transfectable
	2	effectene	not transfectable
	3.	effectene	distinct clones in pBABE-Hygro no pBABE-Hygro- Barx2 clone produced distinct population clones in pBABE-Hygro, distinct clones
PEO1	1	effectene	in pBABE-Hygro- Barx2
	2	effectene	Distinct population clones in pBABE -Hygro, distinct clones in pBABE-Hygro- Barx2.
PEO1 cDDP	1	effectene	distinct population clones in pBABE -Hygro, distinct clones in pBABE-Hygro- Barx2
	2	effectene	distinct population clones in pBABE-Hygro, distinct clones in pBABE-Hygro-Barx2
PEO4	1	effectene	not transfectable
	2	effectene	not transfectable
	3	effectene	not transfectable
PEO6	1	effectene	not transfectable
	2	effectene	not transfectable
	3	effectene	not transfectable
ON3	1	Lipofectin	distinct population clones in pBABE-Hygro-Barx2
	2	Lipofectin	distinct population clones in pBABE -Hygro, distinct clones in pBABE -Hygro- Barx2
OV3	1	Lipofectin	distinct population clones in pBABE -Hygro, distinct clones in pBABE-Hygro-Barx

The transfection experiment for OVCAR3 was performed by Dr Hani Gabra.

Several cell lines were derived following transfection using either the lipofection or effectene protocol, and these are summarised in Table 6-6.

Table 6-6 Designation of Clonally Derived Cell Lines Obtained by Plasmids Transfection

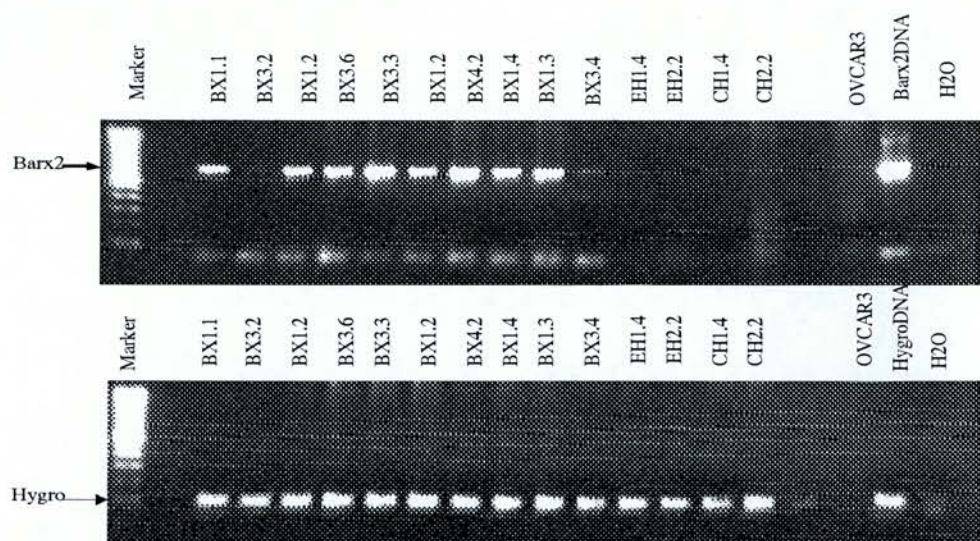
Cell Lines	pBABE-Hygro Cell Line	pBABE-Barx2 Cell Line
OVCAR3	EH	BX
PEO1cDDP	CH	CB
PEO1	PH	PB
ON3	ONH	OB
OAW42	42H	42B

6.2 Molecular analysis of ovarian cancer cell lines transfected with Barx2

6.2.1 Detection of Barx2 full length cDNA by genomic PCR (using primer F1/R3) in ovarian cancer cell lines transfected with Barx2.

The objective of this part of the work was to determine whether the Barx2 gene has been transfected into the cancer cell lines. As the plasmid expression construct became genomically integrated, genomic PCR was used to amplify the inserted fragment of Barx2 in a series of clones derived from different separate transfection experiments. PCR amplification of the Hygromycin (HmB) selectable marker from transfection derived clones was also performed to amplify HmB fragment in these clones as a control. Figure 6- 4 and 6-5 shows examples of genomic PCR for Barx2 (F1/R3) in clones derived from transfection with pBABE-Barx2 or pBABE-Hygro only. All results are summarized in Table 6- 7.

Figure 6-4 Genomic PCR for Amplifying Insert Fragment of Barx2 or Hygro in OVCAR3 transfected BX and EH



Top panel shows the results of genomic PCR (F1/R3) for amplifying Barx2 insert in BX and EH OVCAR3-derived clonal transfected cell lines. The majority of clones derived from Barx2 OVCAR3 transfection are positive for insert of Barx2, but BX3.2 is negative, suggesting that it is a transfection revertant that has lost the Barx2 insert. Also all clones derived from control vector transfected cell lines are negative for Barx2 insert, but positive for Hygromycin (see bottom panel). As expected, OVCAR3 is negative for both Barx2 insert and Hygromycin.

**Figure 6-5 Genomic PCR for Insert Fragment of Barx2 or Hygro in
CB and CH**

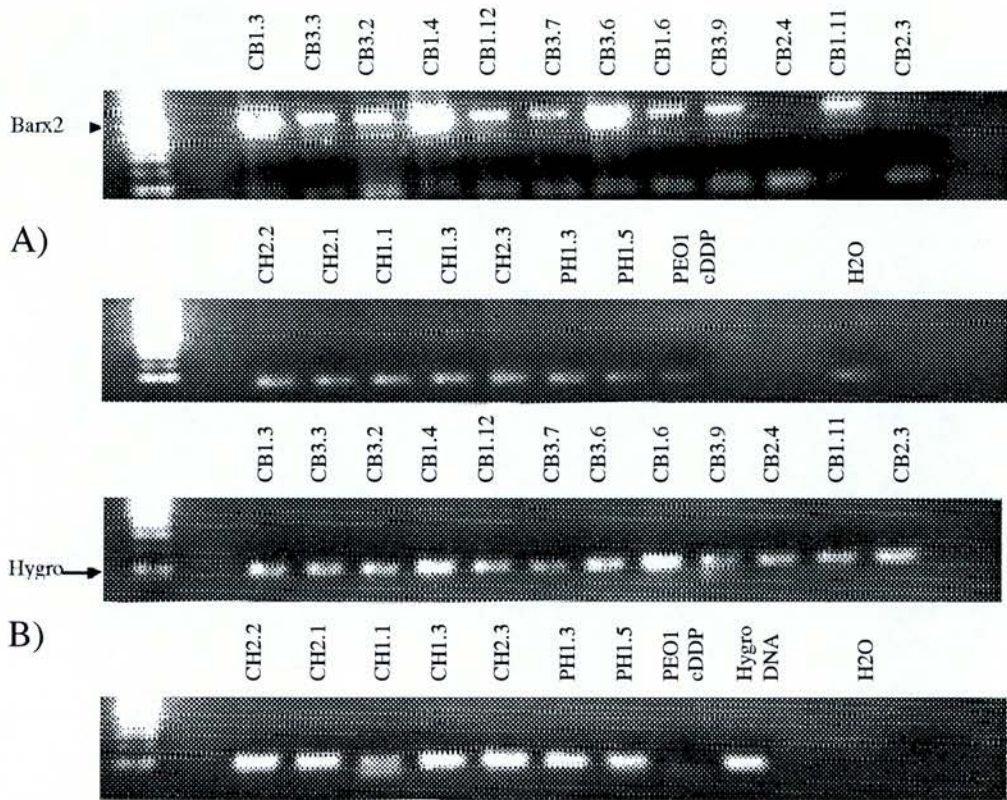


Figure 6.5 A) Examples of genomic PCR (F1/R3 for the Barx2 insert in CB (PEO1cDDP/pBABE-Barx2) and CH (PEO1cDDP/pBABE-Hygro) clone series. Most of clones derived from PEO1cDDP transfected with Barx2, except for CB 2.4 and CB2.3, are positive for the Barx2 insert. All clones derived from control vector transfected cell lines are negative for the Barx2 insert, but positive for Hygromycin marker (see Figure B). PEO1cDDP is negative for Barx2 insert and also for the Hygromycin marker.

Table 6-7 Summary of Genomic PCR Results for Amplification of Barx2 Insert or Hygromycin Marker in Clonal Cell Lines Following Transfection

Cell Lines	Insert Fragment of Hygro	Insert Fragment of Barx2
<u>OVCAR3</u>		
BX 1.1	+++	++
BX 3.2	+++	+/-
BX 1.2	+++	++
BX 3.6	+++	++
BX 3.3	+++	+++
BX 4.2	+++	+++
BX 1.4	+++	+++
BX 1.3	+++	+++
BX 3.4	+++	+
EH 1.4	+++	-
EH 2.2	+++	-
<u>PEO1 cDDP</u>		
CB 1.3	++	+++
CB 3.3	++	++
CB 1.7	++	++
CB 1.4	++	+++
CB 1.12	++	+
CB 3.7	++	++
CB 3.6	++	+++
CB 3.9	++	++
CB 1.6	++	++
CB 2.4	++	-
CB 1.11	++	++
CB 2.3	++	+/-
CH 1.2	++	-
CH 2.2	+++	-
CH 1.4	+++	-
CH 1.5	++	-
CH 2.1	++	-
CH 1.1	++	-
CH 1.3	++	-
CH 2.3	++	-
<u>PEO1</u>		
PB 1.7	+++	++
PB 2.1	+++	++
PB 6.1	+++	+++
PB 7.1	+++	++

PB 8.1	+++	++
PB 11.1	+++	+++
PB 13.1	+++	++
PB 15.1	+++	++
PB 15.2	+++	++
PB 16.2	+++	+
HY 1.6	+++	-
HY 2.7	+++	-
HY 1.3	+++	-
HY 1.5	+++	-
<u>OAW42</u>		
42B 1.1	+++	++
42B 1.2	+++	+++
42B 1.10	+++	+/-
42B 1.4	+++	+++
42B 1.6	+++	-
42B 1.7	+++	+
42B 1.3	+++	+++
42H 7.2	++	-
42H 7.3	++	-
42H 7.4	++	-
42H 7.5	++	-
<u>ON3</u>		
OB 1.2	++	++
OB 1.4	++	++
OB 3.4	++	++
OB 3.1	++	++
OB 5.2	++	++
OB 6.1	++	+
OB 6.2	++	+++
OB 7.1	++	+
OB 7.2	++	+
OB 8.2	++	+/-
OB 9.1	++	+
OB 10.1	++	+/-
OB 12.1	++	+

+++ - ++ = *strong PCR product*

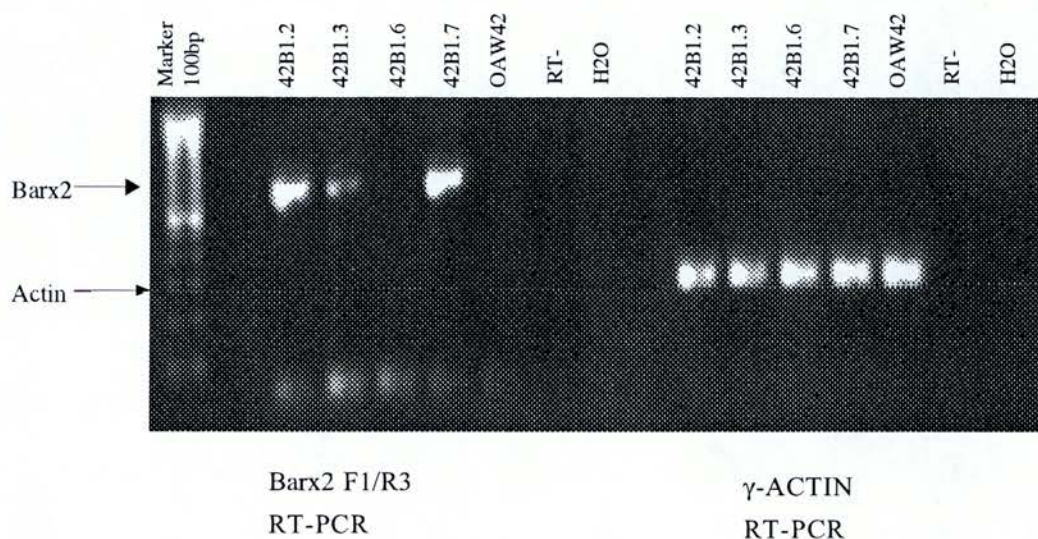
+ = *weak PCR product*

- = *No PCR product*

6.2.2; Detecting *Barx2* expression in clones derived from OAW42 transfected with *Barx2*.

As mentioned above (see section 5), OAW42 does not endogenously express *Barx2*. After transfecting *Barx2* full length cDNA into OAW42, *Barx2* F1/R3 RT-PCR was used to detect expression of *Barx2* in series of clones derived. (Figure 6-6)

Figure 6-6 *Barx2* expression in clones derived from OAW42 transfected with *Barx2*.



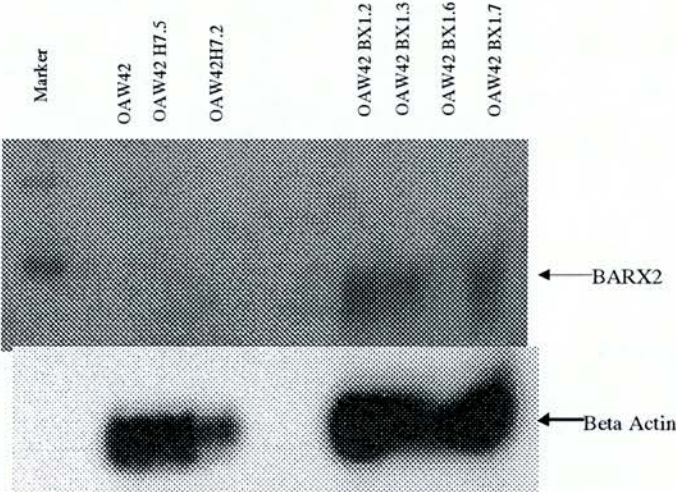
*These data show that there were different levels of expression of *Barx2* in clones derived from OAW42 transfected with *Barx2*. The expression of *Barx2* was strongly positive in 42B 1.2 and 42B 1.7. Weak in 42B1.3 and absent in 42B1.6. Actin control RT-PCR are positive for all cell lines.*

6.2.3; Detecting the expression of *Barx2* in clones derived from OAW42 transfected with *Barx2* by Northern Blot

To more fully quantify the different levels of *Barx2* expression, a Northern blot of

total RANs isolated from the series of OAW42 Barx2 transfected cell lines was hybridized with a full length Barx2 cDNA probe, following by γ -actin. (Figure 6-7).

Figure 6- 7 Northern Blot Analysis of Barx2 Expression in clones derived from OAW42 transfected with Barx2.



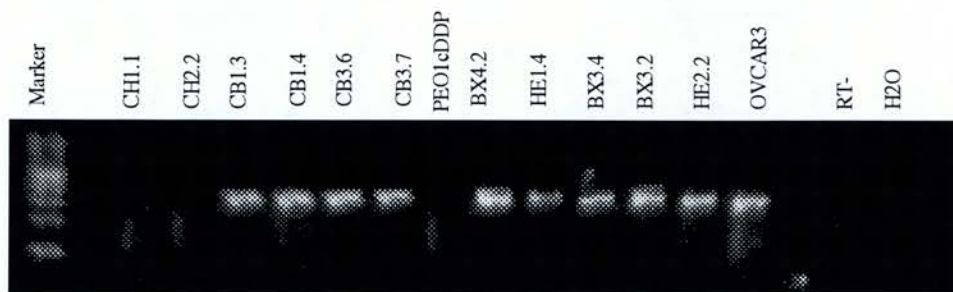
Northern blot comparing RNA from OAW42 parent cell line, Hygromycin control vector transfected clonal derivatives 42H 7.5 and 42H 7.2, and four Barx2 transfected clonal derivatives 42B 1.2, 1.6, 1.3 and 1.7 sequentially probed with Barx2 and Actin. 42B 1.2, 1.3 and 1.7 clearly express Barx2. 42B 1.6 did not express Barx2 consistent with both RT-PCR data (Figure 6-6) and also the lack of integrated Barx2 insert from genomic PCR (Table 6-7).

6.2.4 Detecting the expression of Barx2F1/R1 in clones derived from PEO1cDDP transfected with Barx2 by RT-PCR

The expression of Barx2 by F1/R1 RT-PCR is negative in PEO1 cDDP cell line

(Figure 4-2). Therefore, in order to confirm that the plasmid DNAs had been transfected into this cell line, Barx2 F1/R1 RT-PCR could be used to detect whether Barx2 is expressed in these Barx2-transfected PEO1cDDP clones. (Figure 6-8)

Figure 6-8 Expression of Barx2 F1/R1 in Barx2 Transfected Clonal derivatives, Hygro Transfected Clonal derivatives and Their Parent Cell Lines



The expression of Barx2F1/R1 detectable by F1/R1 RT-PCR is present in “CB” Barx2-transfected PEO1cDDP cell lines. The expression of Barx2 is still absent in PEO1cDDP parent cell line and Hygromycin control vector transfected derivatives—CH cell lines.

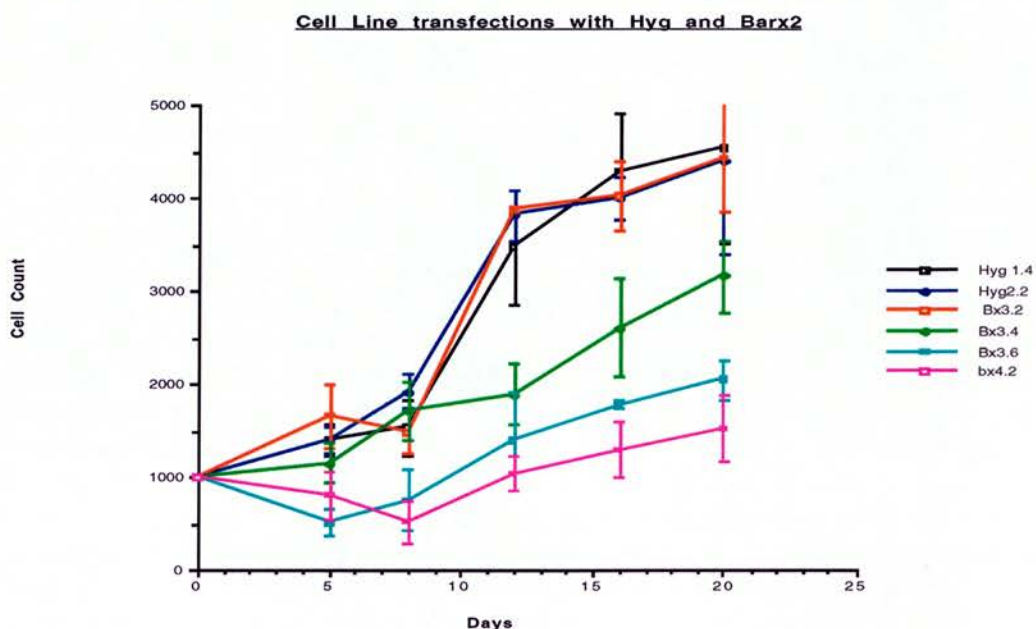
6.3 The functional analysis of Barx2 in ovarian cancer cell lines

6.3.1 In-vitro growth of ovarian cancer cell lines transfected with Barx2

For each of the cell line used for transfection, three or four Barx2 transfections, two pBABE Hygro transfections and parent cell lines were analyzed in an *in vitro* growth

assay. Experiments were performed three times, and results obtained were similar in each case. Representative experiments (1×10^4 cells initially seeded) with quadruplicate samples shows Figure 6-9 to 6- 12

Figure 6- 9 *In-vitro* Growth of pBABE Hygro Control Transfected pBABE Barx2 Transfected OVCAR3 Cell Lines

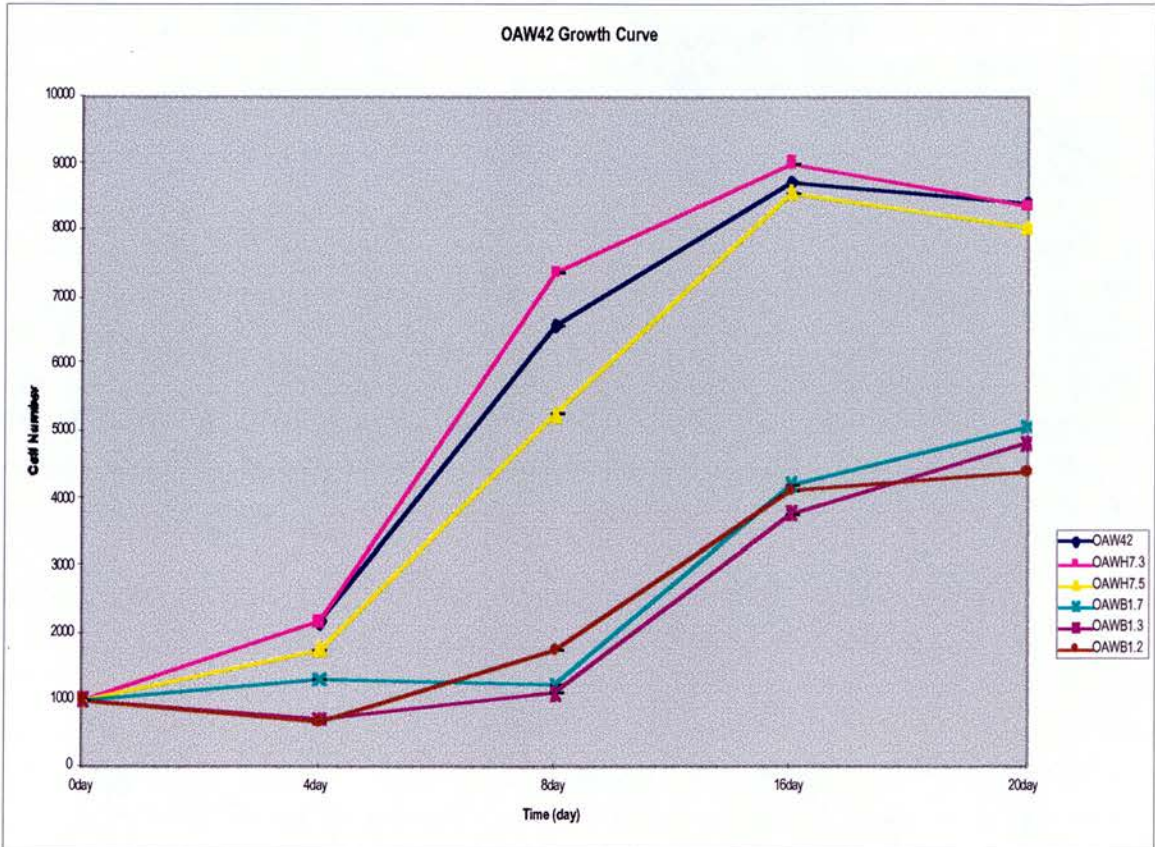


Transfection of OVCAR3 cell line yielded several lines with variable expression of Barx2. BX4.2 and BX3.6, the highest pBABE-Barx2 copy number clones, were strongly suppressed for cell growth compared with controls. BX3.4, a clone with lower pBABE-Barx2 copy number clones, was also suppressed for cell growth comparing with controls, but much less than BX4.2 and BX3.6 (see Figure 6-4). This suggests that the extent of cell growth-inhibition was dependent on exogenous Barx2 expression level in OVCAR3.

BX3.2, EH1.4 and EH2.2 growth characteristics are superimposable, with no significant difference detectable. It was shown previously that BX3.2 is a transfection revertant , having lost the Barx2 insert (Table6-7), thereby explaining why their

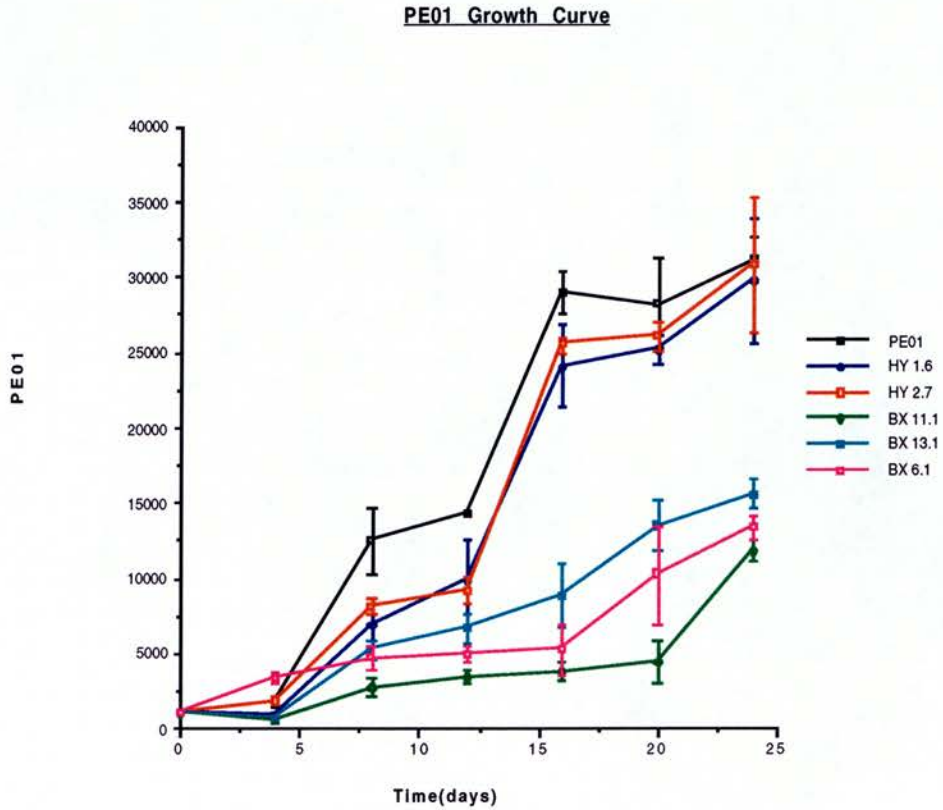
growth characteristics were similar.

Figure 6- 10 *In-vitro* Growth of pBABE Hygro Control Transfected pBABE Barx2 Transfected OAW42 Cell Lines



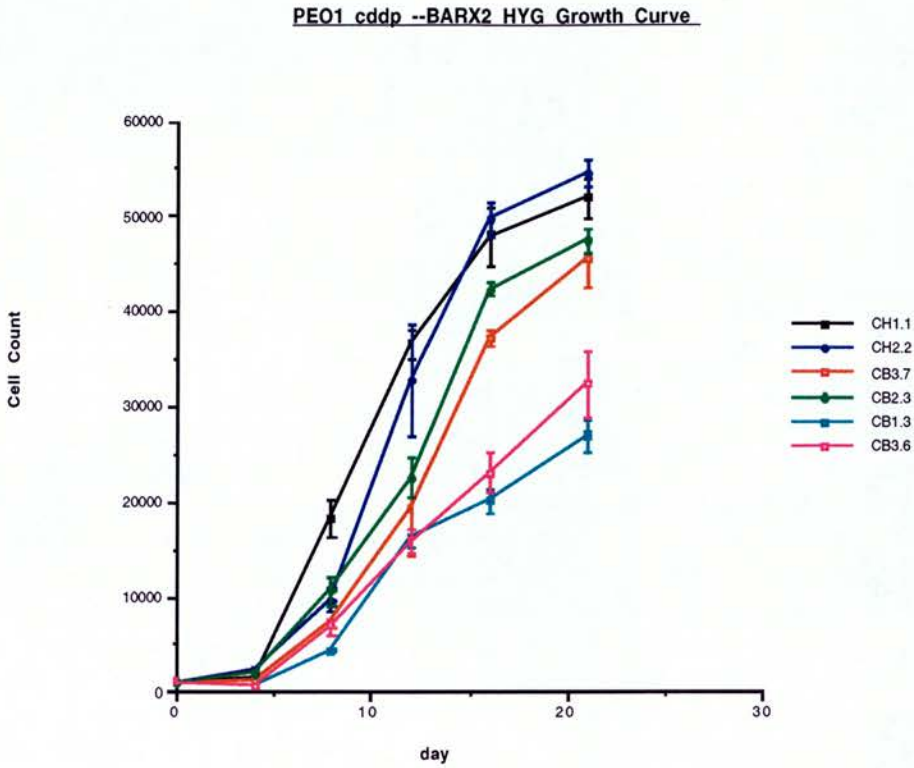
These data showed that there was no significant difference in cell growth comparing 42H 7.5 and 42H 7.3 derived from control vector transfected OAW42 with parent cell line. 42B1.3, 42B1.2 and 42B1.7, the Barx2 expressing transfected clones (Figure 6-6) were suppressed for cell growth compared with parent and clones derived from vector control transfection.

Figure 6- 11 *In-vitro* Growth of pBABE Hygro Control Transfected pBABE Barx2 Transfected PEO1 Cell Lines



Vector only control transfections PH 1.6 and PH 2.7 showed no significant difference in cell growth compared with PEO1 parent cell line. PB11.1, PB13.1 and PB 6.1, the highest pBABE-Barx2 copy number clones clones (Table 6-7), were strongly suppressed for cell growth compared with PEO1 parent and control vector clones PH1.6 and 2.7.

Figure 6- 12 *In-vitro* Growth of pBABE Hygro Control Transfected pBABE Barx2 Transfected PEO1cDDP Cell Lines

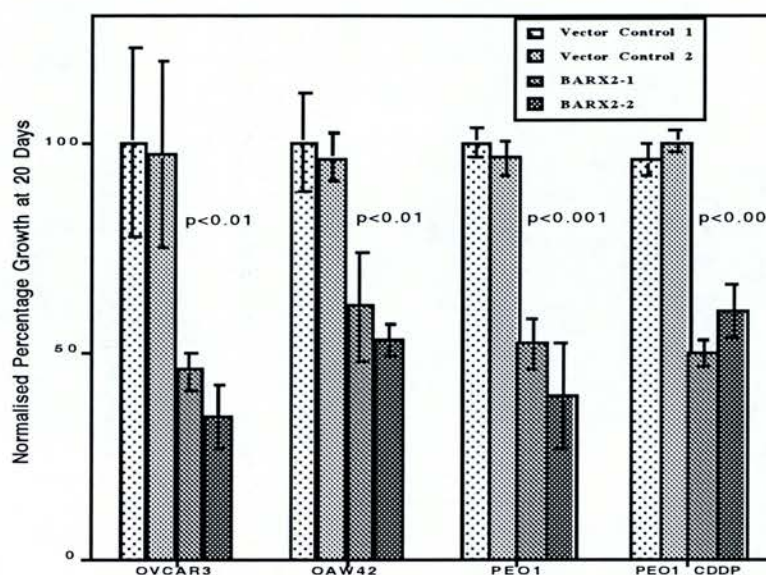


Transfection of Barx2 into PEO1cDDP cell line yielded several lines with variable expression of Barx2. CB1.3 and CB3.6, the highest pBABE-Barx2 copy number clones, were strongly suppressed for cell growth compared with control. CB3.7 and CB2.3, clones with lower pBABE-Barx2 copy number clones, was also suppressed for cell growth compared with controls, but much less than CB and CB.

In summary, compared with cell lines transfected with control pBABE Hygro vector and parent cell lines, all of the cell lines transfected with Barx2 and demonstrating strong expression of Barx2 were equally growth suppressed (see Figure 6-13). The cell lines transfected with Barx2 showing low pBABE-Barx2 copy number clones were also growth suppressed but a much lesser than those with high pBABE-Barx2

copy number clones did. The Barx2 transfection revertant clones were not growth suppressed and behaved like their respective vector-only control transfections and parental cell lines. These data show that Barx2 gene may play important role in suppressing ovarian cancer cell line growth.

Figure 6-13 Growth Assay for 4 Ovarian Cell Lines



Vector control 1 and 2 represent independently derived pBABEHygro transfected control clonal lines for each cell line. Barx2-1 Barx2-2 represents two independently derived pBABEBARX2 transfected clonal cell lines for each parent cell line. The growth assay is normalized for the control values on day 20, and Barx2 transfections are proportional to this.

6.3.2 Cell-cycle distribution of ovarian cancer cell lines OAW42 transfected with Barx2 by flow cytometry

The reasons whereby Barx2 transfection resulted in cell growth inhibition was further investigated. It was postulated that the suppressive effect may be the consequence of

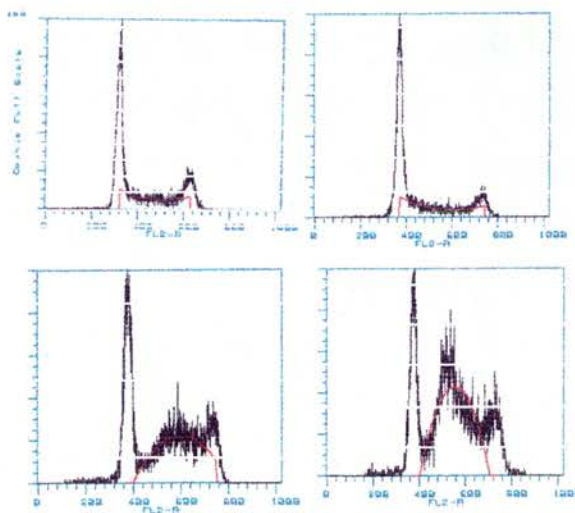
either a delay in progression through the cell cycle or as a result of increased apoptosis (cell death). The results of cell cycle distribution analyses and apoptosis assays are presented.

Analysis of the cell cycle was performed on two separate occasions for OAW42 parent cell line, Hygromycin control vector transfected clonal derivatives 42H 7.5 and 42H 7.2, and Barx2 transfected clonal derivatives 42B 1.2 and 1.7.

Flow-cytometric cell-cycle analysis was performed using propidium iodide staining of cells. (Figure 6-14)

FACS analysis demonstrates that transfection of Barx2 into OAW42 caused clustering in S-phase compared with the parent cell line (see Figure 6-14). This suggested that the Barx2 transfection was associated with a delay in this part of the cell cycle.

Figure 6-14 Transfection of Barx2 into OAW42 Caused Clustering in S-phase



FACS cell-cycle analysis of OAW42 (top left) and control vector transfected OAW42 line 42H 7.5 (top right). Compared with these, two Barx2 transfected clonal lines are shown, 42B 1.2 (bottom left) and 42B 1.7 (bottom right). There are clearly more cells in the S-phase population after Barx2 transfection than in controls suggesting delay of progression through this part of the cycle.

This information is summarized in Table 6-8; the results of statistical analysis for this data are outlined in Table 6-9.

Table 6- 8 DNA-FACS Analysis of Clones Derived from OAW42 transfected with Barx2 or Hygro control vector

Cell Lines	G0 +G1		S-Phase		G2+M	
	Mean	SD	Mean	SD	Mean	SD
OAW42	89.20	2.49	9.77	2.57	1.03	0.15
42H 7.3	85.27	2.03	12.97	2.35	1.73	0.64
42H 7.5	86.60	0.46	11.93	0.70	1.47	0.57
42B 1.2	22.90	9.01	69.00	19.58	8.13	10.77
42B 1.7	22.00	4.54	68.43	12.66	11.57	8.16

Table 6-9 the Results of Statistical Analysis for DNA-FACS

Group	F value	p value
OAW42 Vs 42H7.3	1.196	0.4554
OAW42 Vs 42H7.5	13.479	0.0691
OAW42 Vs 42B1.2	58.044	0.0169*
OAW42 Vs 42B1.7	24.216	0.0396*
42H 7.3 Vs 42H7.5	11.270	0.0850
42B 1.2 Vs 42 B1.7	2.392	0.2948
42B 1.2 Vs 42H 7.3	69.421	0.0142*
42B 1.2 Vs 42H 7.5	782.40	0.0013*
42B 1.7 Vs 42H 7.3	29.022	0.0333*
42B 1.7 Vs 42H 7.5	327.09	0.0030*

* represent significant difference between two groups.

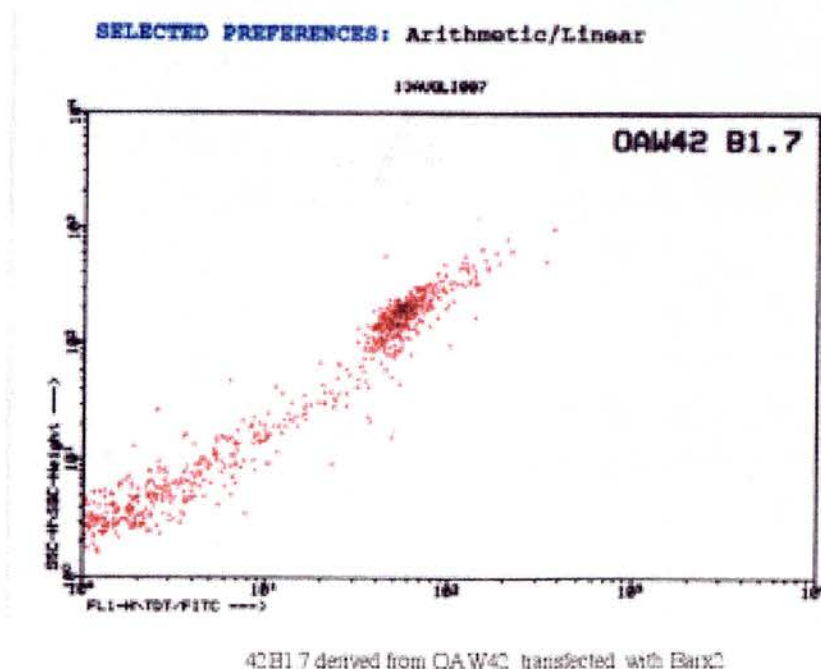
6.3.3 The apoptosis analysis for Clones Derived from OAW42 transfected with Barx2

Barx2 transfection of OAW42 was also analyzed for apoptosis by the annexin V FACS assay. The results are summarized in Table 6-10. Figure 6-15 shows an example of the annexin V FACS assay for apoptosis in OAW42 pBABE Barx2 transfected clone 42B1.7. There was no evidence of either increased cell death or apoptosis in this assay to explain the observed growth suppression phenotype, suggesting that these effects were not obviously due to non-specific cytotoxicity.

Table 6-10 Annexin V-FACS assay for Clones Derived from OAW42 transfected with Barx2

Cell Line	% Apoptosis
OAW42	0.16
42H 7.3	0.17
42H 7.5	0.44
42B 1.3	0.00
42B 1.7	0.26

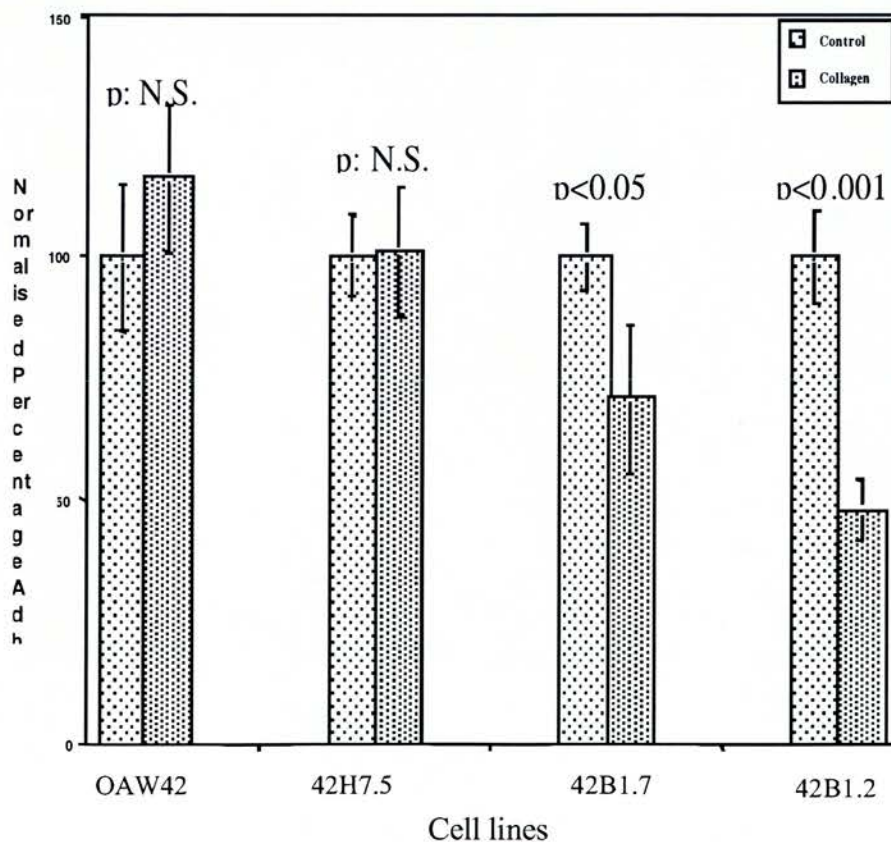
Figure 6-15 The Annexin V-FACS assay for 42B 1.7 Cell Line



6.3.4 Cellular attachment assay for Clones Derived from OAW42 transfected with Barx2 or Hygro control vector

The ability of the cell lines to attach to components of the extra-cellular matrix was tested. The OAW42 parent cell line, Hygromycin control vector transfected clonal derivatives 42H 7.5 and Barx2 transfected clonal derivatives 42B 1.2, 1.7 were allowed to attach to tissue culture plastic coated plates with and without collagen IV. (Figure 6-16) Introduction and expression of Barx2 in OAW42 was significantly associated with inhibition of attachment to collagen IV coated plastic. No such effect was observed on plastic alone. Furthermore, the higher expressing Barx2 clone had greater inhibition of adhesion to collagen IV (40% of control, $P < 0.001$)

Figure 6-16 the Attachment Assay Comparing Control with Clones Derived from OAW42 Transfected with Barx2

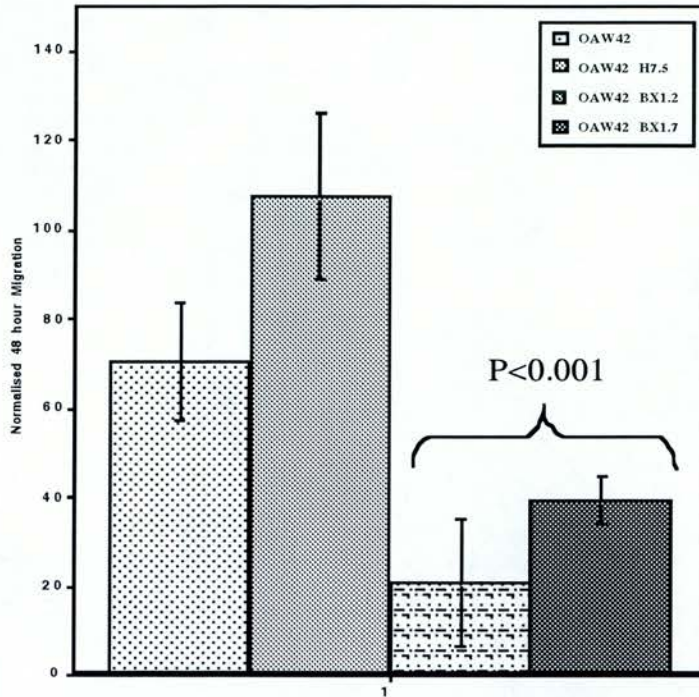


OAW42 cell adhesion to uncoated and collagenIV coated plastic. Barx2 transfected clonal lines 42B 1.2 and 42B 1.7 are significantly suppressed for cell adhesion collagen IV coated plastic, as compared with OAW42 and vector control lines 42H 7.5. There is no significant difference in adhesion to non-coated plastic between the cell lines tested.

6.3.5 Cell migration assay for clones derived from OAW42 transfected with Barx2 or Hygro control vectors.

In order to determine the affect of Barx2 on cellular migration in response to components of the extracellular matrix, the under surface of 8 µm pore size Nuclepore filters was coated with collagen IV. An assessment was made of migration of the Barx2 transfected derivative cell lines over 72 hours in response to those extracellular matrix components using the method outlined in section 2. The results showed that introduction and expression of Barx2 in OAW42 was significantly ($p < 0.001$) associated with inhibition of transwell cellular migration to a collagen IV haptotactic signal. The higher expressing barx2 clone 42B had greater inhibition of cellular migration. The data is summarized in Figure 6- 17

Figure 6- 17 Transwell Migration Assay for Comparing Control with Clone Derived from OAW42 Transfected with Barx2



OAW42 haptotactic cell migration to a collagen IV signal. Barx2 transfected clonal lines 42B 1.2 and 42B 1.7 are significantly suppressed for migration as compared with OAW42 and vector control line 42H 7.5.

6.3.6 Matrigel Invasion analysis of OAW42 and OVCAR3 following transfection with Barx2 or Hygro control vector

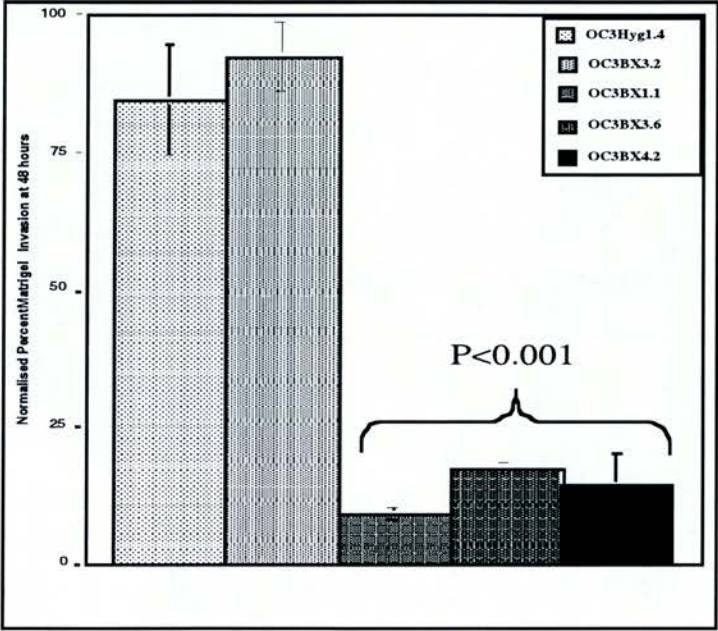
Two separate experiments were performed and similar results obtained from both. The initial experiment was carried out by Dr Hani Gabra.

The object of this part of the work was to assess and compare the ability of tumor cell invasion into matrigel between parent cell lines and Barx2 or Hygro control vector transfected clonal lines.

Introduction of barx2 into OVCAR3 cell lines produced a highly significant ($p < 0.001$)

in all cases) and profound inhibition of matrigel invasion to approximately 20 % of controls. BX3.2 was found to be a Barx2 transfection revertant that had lost the Barx2 insert, and it was not suppressed for matrigel invasion. (Figure 6-18)

Figure 6- 18 Matrigel Invasion Assay for Comparing Control with Clone Derived from OVCAR3 Transfected with Barx2

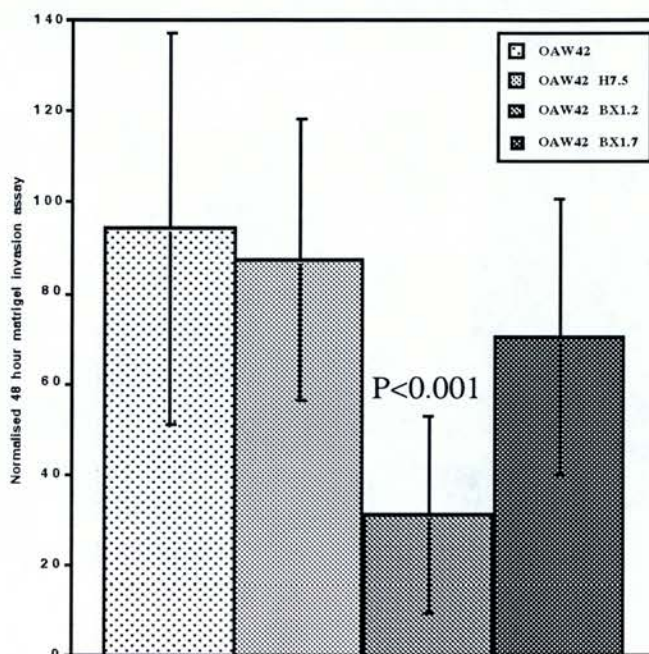


Matrigel invasion of OVCAR3 vector transfected control (EH 1.4) and three Barx2 transfected clonal lines. Barx2 transfected lines BX 1.1, 3.6 and 4.2 are profoundly suppressed for matrigel invasion. BX 3.2 has lost the Barx2 insert but has retained hygromycin resistance, and has the same characteristics in this assay as the vector only control OC3Hyg1.4

OAW 42 does not endogenously express Barx2. Transfection of this cell line yielded several cell lines with variable expression of Barx2. 42B 1.2, the highest Barx2 expressing was significantly suppressed for matrigel invasion compared with controls ($p < 0.001$ Figure 6-19). 42B 1.7, a clone with lower Barx2 expression than 42B1.2,

was also suppressed for matrigel invasion, but to a much less than 42B 1.2 and this result was not statistically significant. This suggests that the extent of matrigel invasion-inhibition was dependent on exogenous Barx2 expression level in OAW42.

Figure 6- 19 Matrigel Invasion Assay for Comparing Control with Clone Derived from OAW42 Transfected with Barx2



Matrigel invasion of OAW 42 parent and vector transfected control (42H 7.5) and 2 Barx2 transfected clonal lines. Barx2 transfected lines 42B 1.2 and 42B1.7 are suppressed for matrigel invasion.

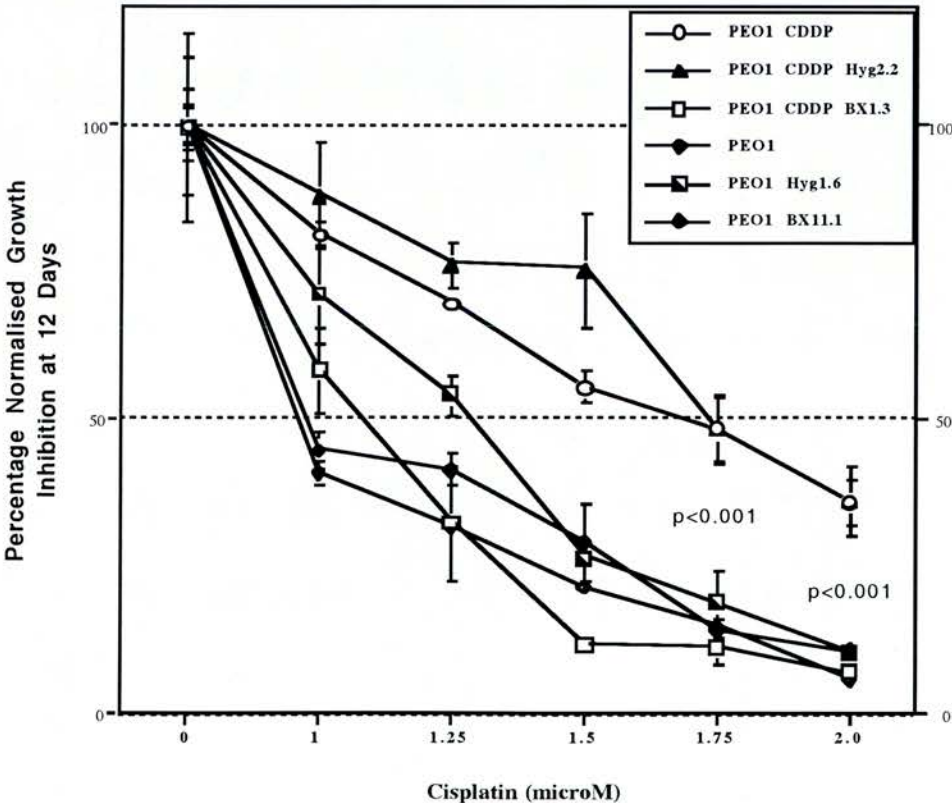
6.3.7 Barx2 and platinum resistance

As stated previously (section 5), ovarian cancer cell lines derived from ascites before and following relapse after successful cisplatin chemotherapy for two patients showed strong downregulation of Barx2 by Northern blotting. PEO1cDDP, a cell line derived from PEO1 by *in-vitro* cisplatin exposure had about half of the Barx2 expression level of PEO1 relative to actin. These two closely related cell lines were transfected with the

Barx2 expression construct. Growth inhibition assays were performed to study the effect of Barx2 expression on cisplatin resistance.

pBABE-Barx2 and control vectors were transfected into PEO1 (platinum sensitive) and PEO1cDDP (platinum resistant) derivative cell lines. The sensitivity of these cell lines to varying concentrations of cisplatin was then directly compared in inhibition assays. A total three separate experiments assaying growth inhibition have been performed. Transfection of Barx2 into PEO1cDDP resulted in highly significant ($p < 0.001$) growth inhibition at 1.75 and 2.0 μM cisplatin compared with vector control and untransfected PEO1cDDP. (Figure 6-20) This growth inhibition curve was essentially super-imposable over the Barx2-transfected, vector-transfected, and untransfected PEO1 (platinum sensitive) growth inhibition curves.

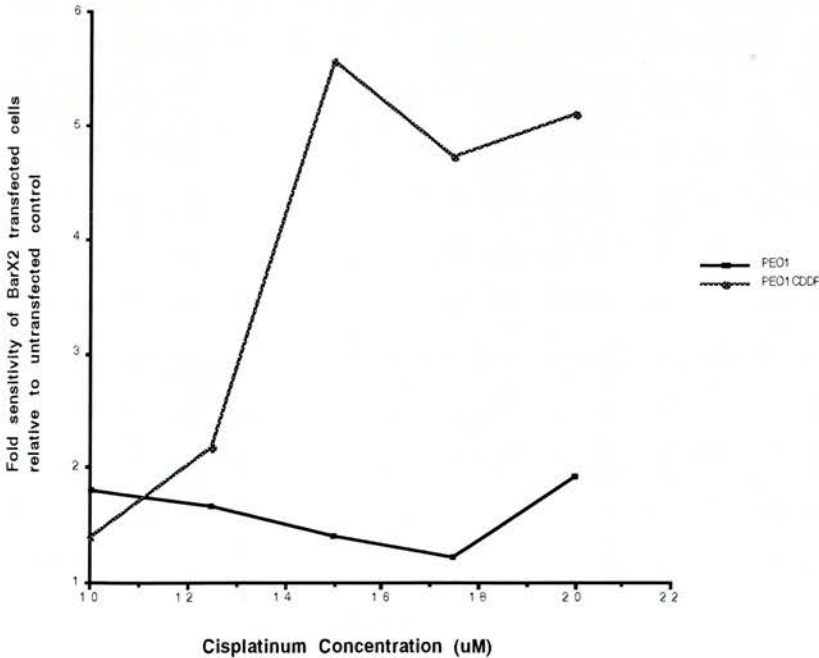
Figure 6- 20 The Growth Inhibition Curve for Cisplatin Sensitivity Comparing in PEO1 and PEO1cDDP



Growth was expressed as a proportional of the no-cisplatin control on day 12 across a cisplatin concentration range of 0 to 2 μ M. Direct comparison of 6 cell lines is made. PEO1 platinum sensitive parent line and representative vector only derivative (PEO1-Hyg1.6) and the Barx2 transfected clone (PEO1-BX11.1) show a similar pattern of cisplatin sensitivity. PEO1cDDP parent line and vector transfected control (PEO1cDDP-Hyg2.2) show a significantly more resistant growth inhibition curve. The Barx2 transfected clonal derivative PEO1cDDP-BX1.3 shows a growth inhibition curve identical to PEO1 and its derivatives, and significantly different from PEO1cDDP control lines

These data were further analyzed for IC₅₀ in these cell lines. The IC₅₀ for barx2 transfected PEO1cDDP was decreased from 1.75 μ M to 1.25 μ M cisplatin, the same range as for PEO1 platinum sensitive parent.

Figure 6- 21 Fold Sensitivity of Barx2 Transfected Cells Relative to Untransfected Control



A different way of presenting the same data is shown in Figure 6-21. In this figure data are presented for PEO1 and PEO1cDDP as the fold sensitivity of Barx2 transfected cells relative to the appropriate untransfected control at each cisplatin concentration. The fold sensitivity of PEO1 to cisplatin following Barx2 transfection remains essentially unchanged over a 1.0 to 2.0 μM concentration range. In contrast, the fold sensitivity of PEO1cDDP to cisplatin following Barx2 is increased over this range, with a maximum 5-fold increase at 1.5 μM cisplatin.

In conclusion, Barx2 transfection predominantly reversed platinum resistance acquired in PEO1cDDP without enhancing the intrinsic platinum sensitivity of PEO1.

7 DISCUSSION

7.1 Preamble

Ovarian cancer is a disease that affects approximately 1% of European and American women during their lifetime, and remains the number one cause of mortality from gynecological malignancies (Landis SH, et al. 1998). Its high mortality is related to its late presentation. By the time of diagnosis, more than 70% of these cancers will have disseminated beyond the ovary (Lynch HT, et al. 1998), thereby compromising the patient's survival. However, we know relatively little about how it develops at the molecular level, and much work is still required to provide us with a comprehensive grasp of its molecular pathophysiology. The prospect of fresh conceptual insights from a functional and molecular genetic approach leading to novel therapeutic strategies is a powerful motivating factor for any researcher. The study of tumour suppressor genes (TSG) in malignant neoplasia has been an interesting one in the field of molecular pathophysiology. The study of TSGs has mainly followed two general strategies; (1) Identifying the possible location where tumour suppressor genes may reside in the cancer cell genome and (2) determining TSG function in tumours (Auersperg N, et al. 1998).

The most popular approach for identifying where tumour suppressor genes may reside in the cancer cell genome is by examination for loss of heterozygosity (LOH). The basis for these strategies rests in the mechanism by which these recessively acting genes are inactivated. According to Knudson's two hit hypotheses, inactivation of a tumour suppressor gene involves two events. The first hit usually involves a mutation in one of the alleles of the gene while the second hit, which leads to loss of function of the remaining normal copy of the gene, may occur by a variety of mechanisms including mutation or deletion (Knudson AG Jr. 1986; Knudson AG Jr, et al. 1995). This latter mechanism appears to be most common and from it derives the way to determine where an unknown TSG may reside or if the locus for a known TSG is altered. Polymorphic markers distributed at high density throughout the genome can be used to distinguish the paternal and maternal contribution of DNA in normal tissues and tumour from the same individual. Loss of DNA from one chromosome in tumour cells is associated with failure of the polymorphic marker to detect the heterozygosity characteristic of the individual's normal DNA at that locus, hence LOH. When a series of polymorphic markers that cover sites

throughout the genome are used on a series of tumours and related constitutive DNA, the frequency of LOH in a given region of the genome can be determined. It is often stated that a frequency of LOH of approximately 30 % suggests that this region of the genome may contain a tumour suppressor gene targeted for inactivation by deletion (Godwin AR, et al. 1994; Schultz DC, et al, 1996). Many regions that contain a high frequency of LOH have been described in epithelial ovarian cancer, indicating that multiple genes may be inactivated during the initiation or progression of the disease.

Our work is based on previous studies of the role of chromosome 11 disruption in the development and progression of malignant neoplasm of the human ovarian surface epithelium.

To investigate new TSG regions, Dr Hani Gabra and colleagues used PCR based polymorphic microsatellites to define regions of frequent loss of heterozygosity throughout chromosome 11 especially at 11 q24 in DNA blood/ tumour pairs from patients with ovarian cancer. The results showed that LOH at 11 q24 was significantly associated with advanced stage and adverse actuarial survival. Refinement of the 11 q24 region revealed an 8.5 Mb LOH region between *D11S934* and *D11S1320* retaining the above survival association (Gabra H, et al. 1996). In order to analyse further the causality of the LOH observation, microcell mediated chromosome transfer was used to introduce chromosome 11 into ovarian cancer cell line NIH:OVCAR3. The analysis for microcell hybrid clones (MHCs) showed that chromosome 11 MHCs remained immortalised and demonstrated significant in-vitro growth inhibition. But this inhibition was not due to cell cycle block or apoptosis. MHCs were significantly reduced in their ability to invade matrigel in the transwell invasion assay. MHCs retaining whole chromosome 11 showed significant reduction in invasiveness compared with MHCs taking up all chromosome 11 as far as 11q22. Further analysis of the inhibition of invasiveness associated with the introduction of chromosome 11q revealed that components of this include specific inhibition of attachment and spreading to laminin coated plastic, and abrogation of the stimulation of tumour cell migration in response to a collagen IV or fibronectin mediated haptotactic signal. These studies suggested that the putative TSG from this region act as a suppressor of ovarian cancer progression.

Since then, investigation has focused on the distal 11q region, and candidate tumour suppressor genes within this region were investigated. The Barx2 gene is one such candidate. (Hjalt T, et al.1999; Krasner A, et al. 2000). The Human Barx2 gene encodes a 254 amino-acid homeodomain transcription factor (Krasner A, et al. 2000) homologous to the Drosophila Bar class of homeobox-domain containing proteins.

The function of the Barx2 gene is still unclear, but some lines of evidence have revealed the role of the barx2 .1) It has a developmental transcription profile at the site of epithelio-mesenchymal transition and similarity to other homeodomain regulators of mesodermal cell fate (Jones FS, et al. 1997; Gawantka V, et al. 1995). 2) Barx2 plays a bifunctional role in regulation of specific cell adhesion molecules (CAMs) (Edelman D, et al. 2000). It has been demonstrated that epithelial tumour cells lose cell-cell and cell-matrix contact in the later stages of tumorigenesis, in association with down-regulation of cell adhesion molecules (Takeichi M. 1995). 3) Barx2 is also identified as a regulatory element in the ras/raf pathway (Krasner A, et al. 2000). Therefore, these findings suggest that the Barx2 gene may function as a candidate tumour suppressor gene in ovarian cancer.

The objective of my work was to investigate whether or not the Barx2 gene acts as tumour suppressor gene in ovarian cancer and if so, its mechanisms of inactivation and action.

7.2 Loss of Heterozygosity at Chromosome 11 q between D11S1894 to D11S1390 Region in Colorectal Cancer

Three independent lines of analysis suggested that a putative tumour suppressor gene was located at chromosome 11q22-q24 and could be involved in the tumorigenesis of several solid tumours of diverse cell types, such as tumours of the breast (Carter SL, et al.1994; Tomlinson IP, et al. 1996), stomach (Baffa R, et al.1996) and so on. The involvement of this chromosomal region in the tumorigenesis of colorectal cancer is still unclear, with cytogenetic (Konstantinova LN. 1991; Keldysh PL, et al. 1993) and some molecular studies (Tomlinson IP and Bodmer WF, et al.1996) indicating losses in this region but with other molecular studies reporting no significant losses (Koreth J, et al. 1997). Previous LOH data from our laboratory suggested that disruption of chromosome 11q24 frequently occurs in ovarian cancer and colorectal cancer, and is associated with poor prognosis in epithelial

ovarian cancer patients (Gabra H, et al; Connolly KC, et al. 1999). It is worth noting that these studies employed four or fewer loci in this region, an insufficient number to establish clearly the regions of chromosome 11q deletion in colorectal carcinogenesis but the two studies did suggest identical regions.

In order to confirm these results, to narrow this region (4.5Mb) further by using more polymorphic microsatellite markers, and to identify whether there is relationship between loss of this region in tumour and the localisation of Barx2 gene, we performed a comprehensive genetic analysis of chromosome 11q24 in 39 blood/tumour pairs with colorectal cancer using 14 highly polymorphic microsatellite markers across the 4.5 Mb region in chromosome 11 q24.

This study describes a detailed deletion mapping exercise of the chromosome 11q24 region in colorectal carcinoma. The region of LOH on chromosome 11q24 has been refined further. From 39 cases of colorectal cancer that could be analysed, 28 cases (71.18 %) had evidence of LOH involving at least one locus in this region. 10 cases (25.6%) had LOH at all markers, 18 cases (46.2%) had partial LOH and 11 cases (28.2%) showed no evidence of LOH in this region. These results are lower than but consistent with previous findings (44/50 cases, 88% of LOH at microsatellite marker between D11S1351 to D11S969) (Connolly, et al.1999). The highest LOH frequencies were observed at D11S1894 (58.33%), at D11S874 (50.8 %) and at D11S1320 (50 %). Previous microsatellite analyses of the chromosomal region 11q24 –q25 in colorectal cancer utilising only four loci have demonstrated that there was a higher frequency of LOH in this region. Connolly et al (1999) reported high frequencies of LOH of 70% at D11S912, 68% at D11S1320 and 39.1 % at D11S969. Our finding of high frequencies of LOH provides further evidence that the chromosomeal region 11q24 is indeed lost during colorectal cancer.

A study by Korth et al. (1997) concluded that distal loss on chromosome 11q was not frequent in colorectal cancer, in contradistinction to a parallel series of breast cancer cases reported in the same study. In particular, the telomeric marker D11S969 was subject to a low LOH rate (15%; 3 of 20). This variation in the frequencies of LOH between studies of the same tumour type and using the same microsatellite marker may be due to the different cut-off criteria used for the assessment of LOH or due to methodological differences.

Koreth et al used $r > 0.5$ as cut-off for the r-value and used $\gamma^{32}\text{P}$ -end-labeled PCR autoradiography to detect LOH, whereas $r < 0.7$ was used as the cut-off for automated laser fluorescence (ALF)-PCR in this study. Our study, clearly demonstrated a region of frequent LOH lying just centromeric to D11S969 in colorectal cancer using >0.7 r value cut-off; however, D11S969 was lost at a much higher rate (41.67%) in this study. It is also consistent with the previous finding of Connolly *et al* (39 %) using the same cut-off criteria (Connolly, et al.1999). We therefore re-analysed our data using the >0.5 r value cut-off used by Koreth et al (1997) and found that the LOH rate at D11S969 dropped to 25 %. It is therefore apparent that wide discrepancies in LOH rate can be generated by differences in well-established methodologies.

In this colorectal study, we found no associations with clinicopathological factors, although a trend towards significance was noted between LOH at D11S4131 and differentiation grade ($p=0.0561$). These data are consistent with the findings of Keldysh et al (1993) as well as of Connolly et al (1999). which did not associate LOH on 11q with adverse clinicopathological features. Evidence has been published that the 11q23.3-24.2 region may contain a late-acting suppressor gene that is prognostically important for ovarian cancer (Gabra H, et al. 1996; Launonen V, et al. 1998). However, this evidence is not unequivocal because another ovarian cancer study (Davis M, et al. 1996) found a relatively high rate of LOH in this region in early stage tumours, suggesting that LOH in this region may represent an early rather than a late event. The findings in our colorectal cancer study appear to concur with latter view.

The centromeric deletion unit described by Connolly et al was frequently subject to LOH but was much larger (4.5Mb) (Connolly KC, et al. 1999). Compared with this study (see Figure 7-1), three shortest region of overlap (SRO) was found to correspond to three consensus regions of deletion/allele imbalance at D11S912 to D114150, at GAT72A01 to D11S4131 and at D11S1320 to D11S4085. These three minimal region not only overlapped completely with others that have been defined previously in colorectal and ovarian cancer (Gabra H, et al. 1996; Davis M, et al. 1996; Launomen V, et al. 1998; Launomen V, et al. 2000), but also further narrowed this region from 4.5 Mb to approximately less than 200Kb between D11S912 and D11S4150, 200kb between GATA72A01 and D11S4131 and 2Mb

D11S1320 and D11S4085. This study has therefore more precisely defined the regions of chromosome 11q likely to contain tumour suppressor genes.

Two papers by launonen et al (1998 and 2000) described regions of LOH on chromosome 11q which partially overlap the three minimal region described here. However, Launonen did not analyse any markers which lie more distally on 11q, and therefore the regions of LOH he describes may extend further distally and fully encompass the three minimal regions that we have defined. (see Figure 7-1)

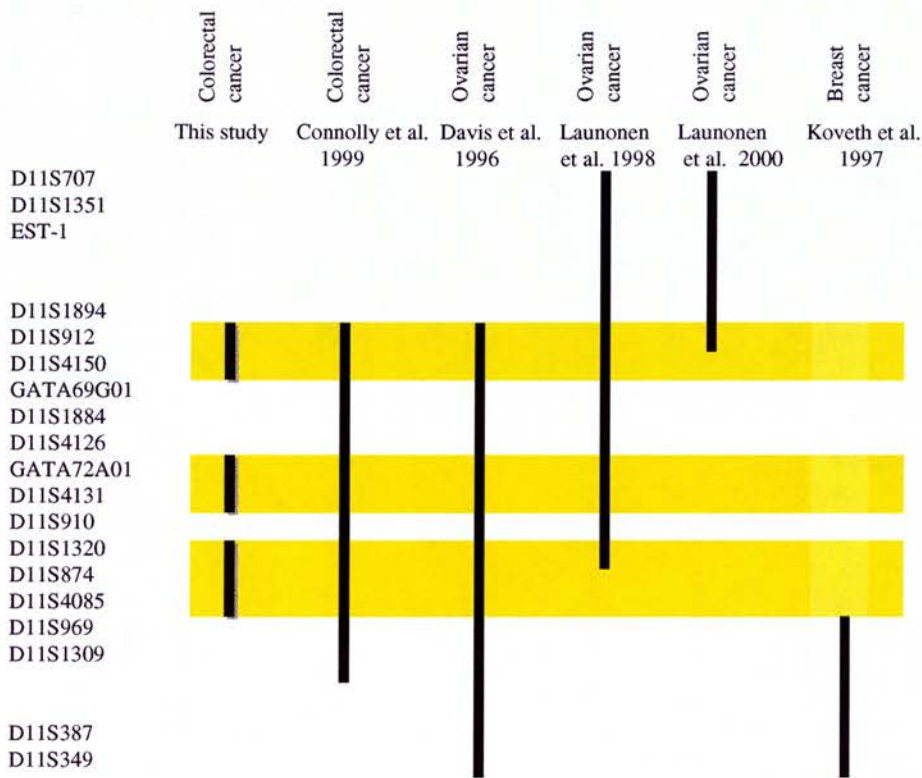


Figure 7-1 Consensus deletion pattern for 11q24 between the microsatellite marker D11S1894 and D11S1309 from studies of different tumour types. Numbers on left, ordering of polymorphic microsatellites is from publications shown and the Genome Database.

Quoted publications in top. Black vertical bars regions of deletion published previously. Black shadow vertical bars, regions of deletion defined by this study. yellow open boxes, consensus regions of deletion.

This study not only confirmed the previous studies but also has already contributed to the cloning effort by reducing further the minimum size of the most telomeric region. The accumulating evidence for frequent LOH in the distal deletion unit in many tumour types suggests that the proposed tumour-suppressor gene from this region may be fundamental to the process of neoplasia.

To date, although some genes have been identified in this region such as PIG8 (p53-target gene)(Gu Z, et al.2000), APLP2 (encoding a human sperm membrane protein) (Leach R, et al. 1999), KCNJ1 (encoding various isoforms of the human potassium channel) (Bock JH, et al. 1997), SNC19 (Zhang Y, et al. 1998) and Barx2 gene, they have not yet linked directly to carcinogenesis.

The Barx2 gene is located at chromosome 11q between marker D11S912 to D11S1320. Our LOH data showed that 10 cases (25.6%) had LOH at all loci, 18 cases (46.2%) had partial deletion within this region. Combined with previous LOH data (Gabra H, et al. 1996; Davis M, et al. 1996; Launomen V, et al. 1998; Launomen V, et al. 2000) in same region for ovarian cancer, the data from this study suggest that the Barx2 gene was a promising putative tumour suppressor gene involved especially in ovarian cancer progression.

7.3 Alteration of Expression of Barx2 in Ovarian Cancer

The human Barx2 gene, a homeobox gene, has been identified as a transcription factors (Jones FS, et al.1997). In my analysis by RT-PCR, the Barx2 gene was strongly expressed in 10 cases of normal ovarian epithelium samples. This is consistent with the previous study that Barx2 is expressed at the sites of epithelio-mesenchymal interaction during murine development (Jones FS, et al.1997). The multi-tissue northern blot analysis of adult human tissue (Clontech) also showed that Barx2 gene was expressed in the normal prostate, testis and colon, with a band of 1.9-2.2 kb, but very weak expressed in ovary (Sellar GC, et

al. unpublished data). This paradox is because ovarian surface epithelium is very small proportion of the whole ovary. The Barx2 gene therefore has normal physiological functions in ovarian epithelium. Compared with normal ovarian epithelium, the expression of Barx2 F1/R1, F2/R2 and F3/R3 were demonstrated in 52.0%, 60.6% and 68.7% of ovarian cancer cases respectively. Although there were no statistically significant association, the expression level of Barx2 in early clinical stage (I – II) seemed higher than that in late clinical stage (III-IV) patients with ovarian cancer. Analysis of relationship between Barx2 expression level and survival of ovarian cancer patients also showed that the expression of Barx2 F1/R1 was significantly associated with survival of ovarian cancer patients ($p = 0.0048$). These data suggested that the Barx2 gene could be linked to ovarian carcinogenesis. The Barx2 gene has been identified as a transcription factor that mediates ras/raf dependent transcription of the calcitonin gene via a ras/raf responsive transcriptional element within the calcitonin gene promoter (Krasner A, et al. 2000). Clinically, down-regulated expression of the calcitonin gene correlates with the poor differentiation of thyroid carcinoma cells and also with adverse patient survival (Ball, DW. 1996). Therefore, loss of the Barx2 gene expression may be involved in tumour progression.

The expression of Barx2 F1/R3 was found to be significantly lower for endometrioid (EM) and clear cell (CC) variants of ovarian cancer compared with other histological subtypes ($p=0.03$). Mean Barx2/actin ratio was 17.5 for endometrioid and clear cell ovarian cancer but mean Barx2/actin ratio was 46.2 for serous and mixed mesodermal tumour histologic types. EM/CC histological subtypes are regarded as being derived from a common cell type, and are associated with endometriosis. Although epithelial ovarian cancer is generally a chemosensitive disease at presentation, endometrioid ovarian cancer has been shown in a single large study to be significantly associated with platinum chemotherapy non-response (Voest EE, et al. 1989). Clear cell carcinoma has also been associated with poor response to platinum chemotherapy and adverse patient survival. In a series of patients with clear cell ovarian carcinoma, the response rate to platinum chemotherapy was only 11% compared with 70% in matched group of serious ovarian cancer (Behbakht K, et al. 1998; Goff BA, et al. 1996; Recio FO, et al. 1996; Sugiyama T, et al. 2000).

Northern Blot analysis revealed strong expression of a broad 1.9-2.2Kb Barx2 band in the ovarian cancer cell lines PEO1, PEO14 and OVCAR3, but downregulation/absence in most other ovarian cancer cell lines. It is interesting that PEO1 and PEO14 represent platinum sensitive ovarian cancer lines derived from two patients early in the course of their disease and show abundant Barx2 expression. PEO4/PEO6 and PEO23 are platinum resistant cell lines derived from the same two patients respectively following relapse after cisplatin chemotherapy. PEO1cDDP was derived from PEO1 *in-vitro* cisplatin exposure. Comparing the expression of Barx2 gene in PEO1 with its derivative cell lines, PEO1 cDDP, PEO4 and PEO6, have only 57.7%, 14.6% and 9.5% of the Barx2 expression level of PEO1 relative to actin respectively. These findings raised the possibility that Barx2 plays a role not only in tumour progression, but also in the development of resistance to platinum chemotherapy (Figure 7-2).

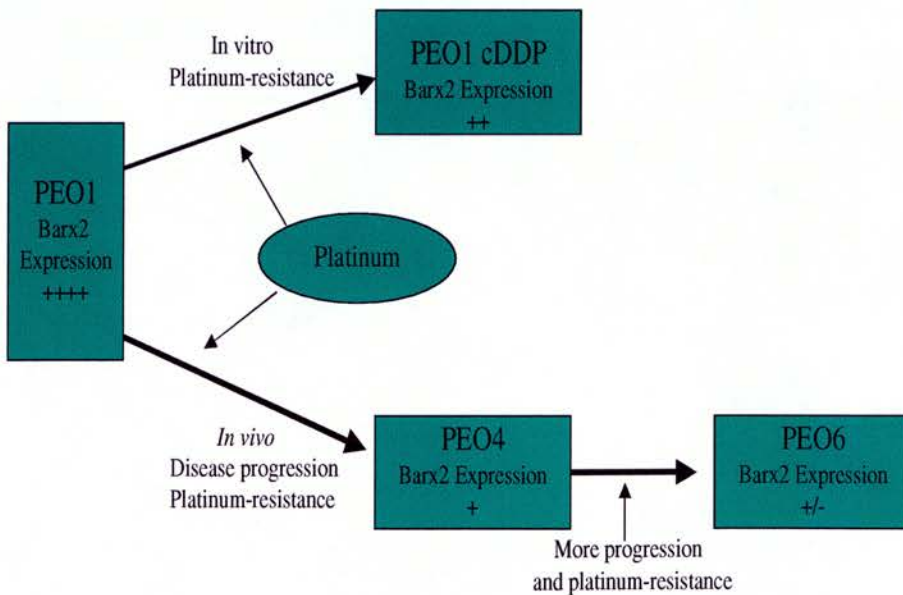


Figure 7-2 A model incorporating cisplatin resistance and expression of the Barx2 in the PEO1 ovarian cancer series

7.4 RNA Isoforms of Barx2 Expression in Ovarian Cancer

RT-PCR and Northern blotting suggested that there were two isoforms of Barx2 expression in the ovarian cancer cell lines, an upper broad band and a narrow lower band. Removal of the polyA tails from the transcript population by RnaseH digestion resolved this broad band into three distinct products, suggesting at least three Barx2 isoforms. Sequencing of RT-PCR products identified two transcript forms: full length, and a minus exon 2 variant. The two Barx2 transcript isoforms identified by Northern blotting are estimated to differ by approximately 300bp. As this estimated difference approximates to the size of exon2. It can be speculated that the lower transcript isoform corresponds to the exon 2 minus variant identifiable sequencing of PCR-generated transcript. The shorter isoform has different relative abundance in different cell lines, but in general is quite abundant in both northern blot and RT-PCR analysis. Translation of the variant transcript isoforms resulted in a frameshift, with the result that a premature termination codon is brought into frame downstream of the splice junction. Translation of these isoforms created by using the exon-intron boundaries reported by Krasner et al (Krasner A, et al. 2000) verified these findings. These proteins would therefore be potentially transcriptionally functionless, but retain the leucine zipper and could therefore bind to the cyclic AMP response element (CRE)-binding protein (CREB) family and differently regulate its function without homeodomain DNA binding activity. The function of these isoforms in ovarian cancer is unknown. Multi-tissue northern blot analysis of adult human tissue (Clontech) also showed that there were two isoforms of Barx2 expression in some normal adult human tissue. Therefore, these isoforms are not specific for ovarian cancer

7.5 Barx2 Is Not Inactivated by Somatic Mutation in Ovarian Cancer

Downregulation/absence of Barx2 gene expression could be caused by alteration of the structure of the gene. To explore this possibility, Southern blot and Pulsed-Field Gel Electrophoresis were first used to analyse whether structural alteration of Barx2 had occurred. Barx2 gene was not rearranged or deleted in ovarian cancer cell lines.

However, tumours may arise through the accumulation of somatic mutation or through the inheritance of a mutation through the germ line, followed by the acquisition of additional somatic mutations (Bishop JM. 1991). While alterations in gene expression also contribute to the malignant phenotype, the sequential mutation of cancer-related genes leading to outgrowth of a clonal population of cells is an important determinant of whether a cancer develops (Tashiro H, et al.1992; Vogelstein B and Kinzler KW. 1993; Weinberg RA. 1989). Although some sequence alterations of Barx2 exon2 were identified in few cancer cell lines, we were unable to demonstrate evidence of somatic inactivating mutations of Barx2 in ovarian cancer.

7.6 Barx2 Is Not Somatically Methylated in Ovarian Cancer

Numerous studies have confirmed that dysregulation of the DNA methylation machinery is a feature of many different types of neoplasms, manifest by general genomic hypomethylation, as well as regional hypermethylation and increased expression of a 5-methyl transferase (5-MT) encoding gene (Baylin SB, et al. 1991; Kay PH, et al. 1997). Methylation may induce silencing of tumour suppressor genes such as Rb (Simpson DJ, et al. 2000) and p16 (Merlo A, et al. 1995), which are usually hypomethylated. Such genes are critical in control of the cell cycle and cellular differentiation (Kay PH, et al. 1997). Methylation southern blotting and methylation-specific PCR were used to detect whether there was methylation of the Barx2 gene in ovarian cancer. Although southern blot analysis showed that Barx2 5'CpG island was methylated in some cancer cell line and evidence of progressive methylation was observed in cell lines downregulated for Barx2, methylation-specific PCR, a novel and highly sensitive technology for detection of genes methylation (Herman JG, et al. 1996), did not show any evidence for methylation in 80 epithelial ovarian normal/tumour pairs and 38 matched ovarian cancer biopsies prior to and following

relapse after cisplatin chemotherapy as well as cancer cell lines, except for HeLa cell line. Therefore, the Barx2 gene is not methylated in ovarian cancer. The Southern Blot result was probably caused by an artefact of inadequate digestion with restriction enzymes in DNA.

Since we were unable to detect evidence of somatic methylation in the CpG island or inactivating mutations in the coding region of the Barx2 gene in ovarian cancer, we therefore reasoned that the existence of promoter mutations or alterations in trans acting factors are likely to be responsible for the downregulation/absence of Barx2 expression that we observed. Further investigation will be necessary to confirm this hypothesis. The use of reporter gene constructs would make it possible to elucidate which of these mechanisms are responsible. Reporter gene constructs containing the luciferase gene and varying lengths of the 5' Barx2 flanking sequence could be constructed. Transfection of a construct containing the full-length 5' sequence would be performed initially into a cell line that does not express Barx2. Demonstration of the presence or absence of luminescence in a cell that does not express Barx2 would identify whether or not the necessary trans acting factors were present to stimulate gene transcription. The absence of luminescence would suggest the presence of dysregulated trans acting factors. Presence of luminescence would suggest that there could be a mutation within the promoter and sequencing would identify this. Transfection of constructs containing shorter lengths of 5' sequence would allow identification of the minimal critical region that is required for gene transcription (see Figure 7-3). This could be a cloned and sequenced and putative binding factor identified.

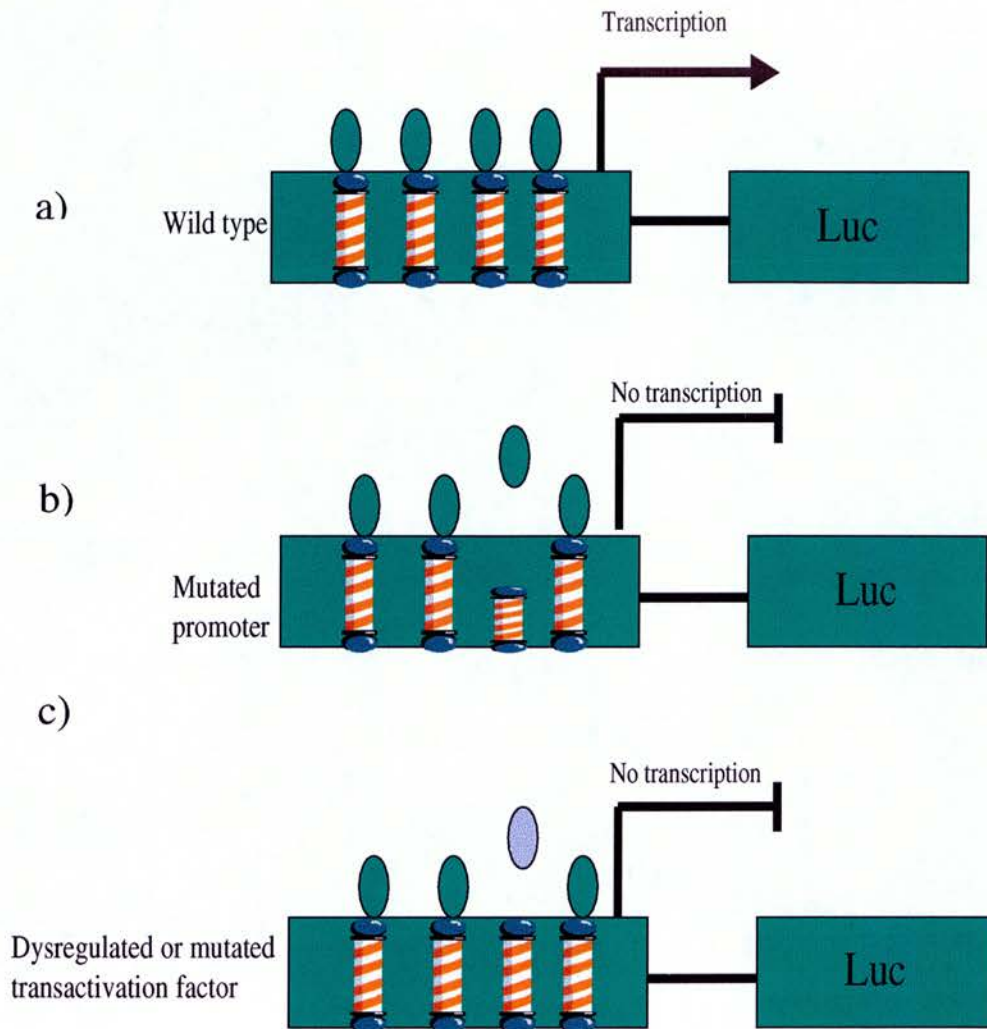


Figure 7-3 A model for reporter gene constructs to elucidate promoter mutation or alteration in trans acting factors.

Green box = 5' Barx2 flanking sequence. Luc = luciferase gene.

Green ellipse = transactivation factor.

Blue ellipse = dysregulated or mutated transactivation factor

Reporter gene constructs expressing the luciferase gene under the control of the Barx2 promoter. a) Transactivating factors may be required to bind to the Barx2 promoter to cause transcription. b) Mutations within the Barx2 promoter may disrupt this binding and

result in no transcription. c) Similarly, dysregulation, or mutation, of the transactivating factors may also lead to no transcription.

7.7 The Barx2 Gene Acts as Suppressor of Ovarian Cell Line Growth *in vitro*

In order to understand the role of the Barx2 gene in ovarian cancer, gene transfection and functional analysis were performed in this study.

The OVCAR3 cell line was first chosen for transfection with Barx2 gene because condition of transfection for this cell line was well-established in our lab. OAW42 and A2780 are non-expressors of Barx2. They were also suitable recipients for transfecting with Barx2. Also, data above has shown that falling expression of Barx2 was associated with disease progression and cisplatin-resistance in PEO1 series. Therefore, the PEO1 series were also selected for to transfection with Barx2 construct. In molecular analysis of the clonal lines derived from parent cell lines transfected with the Barx2 gene, the expression of Barx2 full-length sequence in Barx2 transfected clonal derivatives OAW42BX 1.2, 1.3 and 1.7 was determined by Northern blot and RT-PCR. Because Barx2 full length sequence is expressed in PEO1 and PEO1 cDDP, It is difficult to distinguish the difference between expression of Barx2 transfected insert and expression of Barx2 originally in cell lines. However, the Barx2 F1/R1 expression was negative in PEO1 cDDP. Barx2 F1/R1 RT-PCR was used to detect whether Barx2 F1/R1 is expressed in Barx2 transfected clonal derivatives PEO1 cDDPBX series.

Growth assays for 4 Barx2 transfected clonal cell lines compared with each of the parent cell lines and Hygromycin transfected control clonal cell lines showed growth inhibition *in vitro* for these Barx2 transfected clonal cell lines. This inhibition was independent of the cell lines' endogenous Barx2 expression level. For example, in OVCAR3 series, BX 3.2 was found to be a Barx2 transfection revertant that had lost the Barx2 insert and it was compared with Hygromycin transfected series derived from control vector transfected OVCAR3, there was no significant difference in cell growth. Transfection of OVCAR3 with Barx2 also yielded several lines with variable expression of Barx2 BX4.2 and BX3.6, the highest expressed clones, were strongly suppressed for cell growth compared with

controls ($p < 0.01$). BX3.4, a clone with lower Barx2 expression, was also suppressed for cell growth compared with controls, but much less than the highest Barx2 expressed clones. This suggested that the extent of cell growth-inhibition was related to the exogenous Barx2 expression level in ovarian cancer cell line. Further investigation of cell cycle distribution and apoptosis by FACS- analysis for Barx2 transfected clonal derivatives OAW42BX series revealed that transfection of Barx2 into OAW42 appeared to cause clustering in S-phase. There was no evidence of either increased cell death or apoptosis in this assay to explain the observed phenotypes, suggesting that although the Barx2 gene acts as suppressor of ovarian cell line growth, these effects were not obviously due to non-specific cytotoxicity.

7.8 Barx2 inhibits invasion, migration, adhesion of ovarian cancer

Cell lines *in vitro*

In this study, ovarian cancer cell lines OVCAR3 and OAW42 transfected with vector controls and Barx2 construct were compared in the matrigel invasion assay. Introduction of Barx2 into OVCAR3 and OAW42 produced a highly significant ($p < 0.001$) and profound inhibition of matrigel invasion and the extent of matrigel invasion-inhibition depends on exogenous Barx2 expression level in these clonal cell lines.

Two Barx2-transfected clones were directly compared with two vector-only controls for cellular migration and cellular adhesion functional assays. Introduction and expression of Barx2 in OAW42 was significantly ($p < 0.001$) associated with inhibition of transwell cellular migration to a collagen IV haptotactic signal. Again, the higher expressing Barx2 clone had greater inhibition of cellular migration. Introduction of Barx2 in OAW42 was also significantly associated with inhibition of attachment to collagen IV coated plastic. No such effect was observed on plastic alone. Furthermore, the higher expressing Barx2 clone had greater inhibition of adhesion to collagen IV (40 % of control, $p < 0.001$). These data suggest that the function of the Barx2 may be an inhibitor of invasion, migration, adhesion and, by analogy peritoneal dissemination of ovarian cancer. These findings are consistent with recent thinking regarding molecular mechanisms of ovarian cancer progression. Firstly, LOH studies have demonstrated that chromosome 11q24 deletion region within

which Barx2 is located is associated with disease progression in ovarian cancer. The frequency of LOH in late stages is higher than it in early stages of ovarian cancer, combining with locoregional dissemination and disease progression (Gabra H, et al. 1996; Launonen V, et al. 2000). Secondly, previous studies by Dr Hani Gabra in our lab demonstrated that introducing chromosome 11 containing the q24 region into an ovarian cancer cell line (OVCAR3) reduced the ability of cancer cell line in to invade matrigel, cellular migration and attachment compared with a micocell hybrid containing chromosome 11 without q22-ter. Thirdly, contiguous YACs overlapping most of the chromosome 11q25 deleted region were used for transfection into murine fibrosarcoma cell and caused significant suppression of tumorigenicity compared to the control (Koreth J, et al. 1999).

The Barx2 gene has been identified as regulator of specific cell adhesion molecules (CAMs) (Edelman D, et al. 2000). In sheep, Barx2 is expressed in the epithelial component of the developing follicle and in the outer root sheath of the adult follicle in a pattern that is similar to E-cadherin. This suggests that Barx2 may have a general function in controlling adhesive processes in keratinising epithelia (Sander G, et al.2000). Alteration of normal cell to cell adhesion is an important event in the development of invasive and metastatic disease. The role of CAMs in epithelial-mesenchymal transition is well documented in cancer progression. They have demonstrated their utility as prognostic markers in a variety of neoplasms. Dysfunction of cadherins has a major impact in the progression of epithelial tumours (Shiozaki H, et al. 1991). Loss or abnormal expression of cadherins in tumours can lead to tumour invasion and disease progression, as demonstrated for E-cadherin expression in prostate (Umbas R, et al. 1994), stomach (Shimoyama Y, et al. 1991), bladder (Bringuier PP, et al.1993), breast (Rasbridge SA, et al. 1993), and cervical cancer (Vessey CJ, et al. 1995). Similar results have been reported for P-cadherin, for example in breast cancer (Rasbridge SA, et al. 1993), gingival carcinomas (Sakaki T, et al. 1994), and gastric neoplasia (Shimoyama Y, et al. 1991). Furthermore, treatment of madin darby canine kidney cells with an antibody against the extracellular domain of E-cadherin increases the invasiveness of these cells *in-vitro* (Behrens J, et al. 1989).

It was recently demonstrated that Barx2 interacts with two leucine zipper containing proteins of the CREB family of transcription factors, CREB1 and ATF2 (Edelman SH, et

al. 2000). CREB and its associated proteins have been proposed to act as survival factors for human melanoma cells. Transfection of a dominant negative CREB (KCREB) into melanoma cells inhibits the invasiveness and tumorigenic and metastatic potential of these cells (Jean D, et al.1998). This is a similar phenotype to that observed with transfection of Barx2 into ovarian cancer cell lines.

More recently, a study by Dr Grant C. Sellar in our lab demonstrated that cadherin-6 (K-cadherin, CDH6) is a likely target of the human Barx2 gene. Cadherin-6 may function to maintain epithelial-epithelial cell interactions (Stewart DB, et al. 2000). In formation of the adherens junction complex, E-cadherin interacts with and stabilises β -catenin to establish cell adhesion. α -catenin also binds to β -catenin, connecting cell membrane to the actin cyto-skeleton. However E-cadherin by sequestering β -catenin from its intracellular free pool antagonises the Wnt signalling pathway (Orsulic S, et al. 1999). In the madin derby canine kidney (MDCK) system, where E-cadherin is identified as the major cadherin, cadherin-6 was identified by microsequencing as a 130kd complex associating with β - and α -catenin. This complex accumulated with cell-cell contact. Cadherin-6 was post-translationally downregulated in the presence of activated mutant β -catenin that does not regulate cadherin levels in MDCK cells. This was achieved by a non-ubiquitinating proteolytic degradation pathway. Cadherin-6 downregulated cells were flatter, formed less compacted colonies, and were more migratory (Stewart DB, et al. 2000), which is consistent with the phenotypes that we have observed in this study. Although the involvement of cadherin-6 in the development or progression of ovarian cancer has not previously been documented, absent expression of cadherin-6 was demonstrated in renal cancers of different histological type and tumour grade with downregulation of expression in a significant subset of renal cell carcinomas consistent with an involvement of this cell adhesion molecule in the progression of renal cell cancer. Importantly, absent expression of CDH6 in renal cancer was associated with poor prognosis (Paul R, et al. 1997; Shimazui T, et al. 1998).

From these data Barx2 appears to act as inhibitor of invasion and dissemination of ovarian cancer, and although no structural alteration of Barx2 was observed, we can hypothesise

that the Barx2 may mediate its role as a suppressor via transcriptional regulation of specific target cell adhesion molecules such as cadherin-6 (see Figure 7-2).

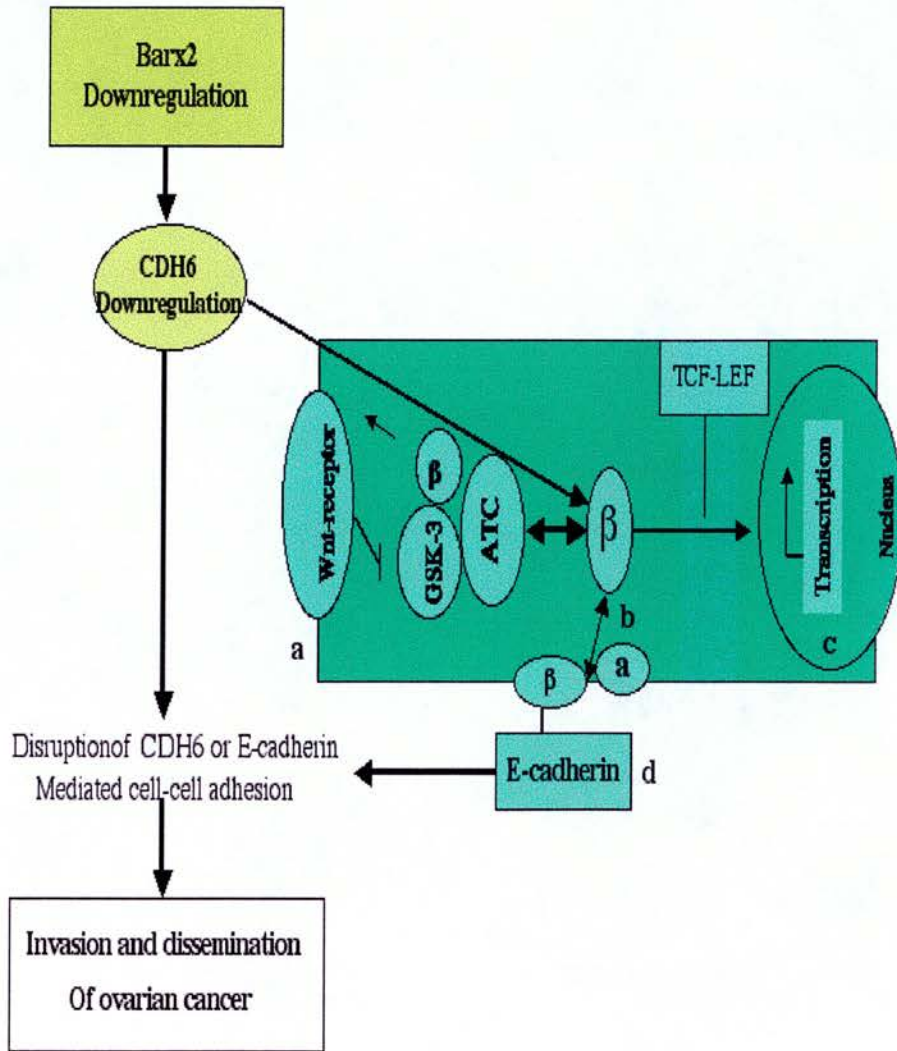


Figure 7-2; A putative model incorporating invasion and dissemination of cancer cells and downregulation of the Barx2 gene in ovarian cancer cell lines. Downregulation of Barx2 may be targeted to CDH6 and would act as a regulator.

Disruption of CDH6 may directly affect invasion and dissemination of cancer cell. CDH6 may be bound with β - and α -catenin. This complex accumulates with cell-cell contact, and would indirectly act with E-cadherin as a regulator (Stewart DB, et al. 2000). E-cadherin not only plays a direct role in suppressing invasion and dissemination of cancer cells but also plays a role as a modifier of the Wnt signalling pathway. Wnt signalling is involved in a large number of developmental processes, including tumorigenesis. a) activation of the Wnt receptor prevents β -catenin being targeted for degradation by glycogen synthase kinase 3 (GSK-3) and the adenomatous polyposis coli (APC) protein. b) β -catenin can then form complexes with the lymphoid enhancer factor (LEF) and T-cell factor (TCF) families of transcription factors, c) initiate the transcription of genes involved in cell proliferation, and d) because E-cadherin sequesters β -catenin into the adhesion complex, downregulation of E-cadherin is likely to result in increased levels of free β -catenin and transcription of the targets of Wnt signalling (Guilford P. 1999).

7.9 The Barx2 Transfection Predominantly Reversed Platinum Resistance Acquired in PEO1 cDDP

As was demonstrated earlier (Figure 4 – 18 and Figure 4-15), downregulation of Barx2 expression is associated with platinum resistance in ovarian cancer cell lines and endometrioid/clear cell primary ovarian cancers that have been shown to have a poor response rate to platinum chemotherapy. These findings raised the possibility that Barx2 plays a role in the development of resistance to platinum chemotherapy. In order to confirm further this possibility, two closely related cell lines were transfected with the pBABE Barx2 expression construct. The growth inhibition and clonogenicity assays were performed to study the effect of Barx2 expression on cisplatin resistance. These two closely related cell lines are PEO1, platinum sensitive and strongly Barx2 expressing cell line, and PEO1cDDP, a cell line derived from PEO1 by *in-vitro* cisplatin exposure with 57.7% expression level of Barx2 compared with PEO1. The sensitivity Barx2-transfected, vector-transfected and non-transfected PEO1 cDDP was directly compared using varying concentrations of cisplatin in growth inhibition assays. Transfection of Barx2 in PEO1cDDP resulted in highly significant increase ($p < 0.001$) in growth inhibition at 1.75

μM and $2.0 \mu\text{M}$ cisplatin compared with vector control and untransfected PEO1cDDP. This growth inhibition curve was essentially super-imposable over the Barx2-transfected, vector-transfected, and non-transfected PEO1 growth inhibition curves. IC_{50} for Barx2-transfected PEO1cDDP was reduced from $1.75 \mu\text{M}$ to $1-1.25 \mu\text{M}$ (the range for PEO1 platinum sensitive parent). No significant difference was observed between the intrinsically more platinum sensitive PEO1 cell line and its Barx2- or vector-transfected derivatives (see Figure 7-4). We conclude therefore, that in this model system, Barx2 transfection predominantly reversed platinum resistance acquired in PEO1 cDDP without enhancing the intrinsic platinum sensitivity of PEO1 in this *in vitro* model.

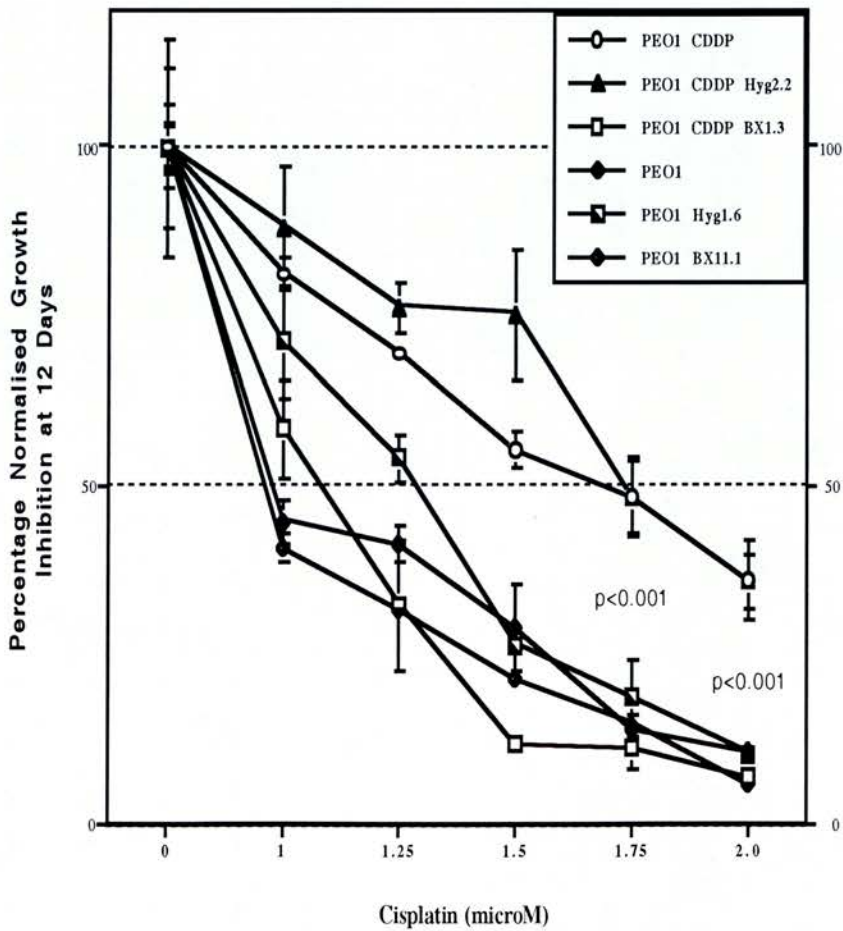


Figure 7.4 The Growth Inhibition Curve for Cisplatin Sensitive Testing in PEO1 and PEO1cDDP

Normalised growth inhibition curve on day 12 across cisplatin concentration range. Direct comparison of 6 cell lines derivative is made. PEO1 platinum sensitive parent line and representative vector only derivative (PEO1-Hyg1.6) and Barx2 transfected (PEO1-BX11.1) clones show a similar pattern of cisplatin sensitivity. PEO1cDDP parent line and vector transfected control (PEO1cDDP-Hyg2.2) show a significantly more resistant growth inhibition curve. The Barx2 transfected clonal derivative PEO1cDDP-BX1.3 shows a growth inhibition curve identical to PEO1 and its derivatives, and significantly different from PEO1cDDP control lines.

To further explore the mechanism of platinum resistance reversal, clonogenic assays were performed by Dr Karen P. Watt in our lab to determine if the observed growth inhibition was due to loss of clonogenic cells (i.e. cell death) or merely slower growth rates. These assays demonstrated reduced clonogenic survival of Barx2 transfected cells compared to controls on exposure to cisplatin and confirmed further that the Barx2 gene plays a role in determining sensitivity to cisplatin (Watt KP, et al. unpublished data).

Although the mechanisms by which the Barx2 gene exerts these effects are at present unclear, we can speculate as to the mechanism by which Barx2 may mediate this sensitivity to cytotoxic agents.

Multiple mechanisms have been proposed for cellular resistance to cisplatin;

a) Increased inactivation by detoxification enzymes such as glutathione-S-transferases (GST), or direct binding to nonprotein thiols such as glutathione (GSH). A wide variety of detoxification pathways exist that enable a cell to inactivate cytotoxic drugs. The mechanisms that have received the most attention in mediating drug resistance in ovarian cancer include increased levels of GSH, GSTs transferases, and metallothioneins. Spontaneous conjugation of GSH with cisplatin has been shown in vitro (IshikawaT, et al. 1993) and increased levels of GSH have been found to be associated with resistance to platinum (Britten RA, et al.1991; Godwin A, et al. 1992; Hosking LK, et al.1990). The GSTs, a multigene family which catalyses the conjugation of GSH with electrophilic drug,

have been implicated in resistance to alkylating agents , anthracyclines, and platinum (Meijer C, et al. 1990; Black SM, et al. 1990).

b) Decreased expression or alteration of a gene or its product may enable a cell to escape the cytotoxic effect of certain chemotherapy. Decreased expression or mutation of the topoisomerase I and topoisomerase II genes has been implicated in resistance to platinum in derived drug resistant models in vitro (Eng WK, et al. 1990; Wang LF, et al. 1997; Hsing Y, et al. 1996).

c) Increased DNA repair activity. Tumour cells may be able to develop DNA repair mechanisms that are more efficient than normal, counteracting the genotoxic effects of cytotoxic chemotherapy, and hence contributing to resistance to DNA damaging drugs. Increased repair of platinum-DNA adducts have been shown to be associated with cisplatin resistance in human ovarian cancer cell lines (Parker R, et al. 1991), and evidence for increased repair of cisplatin interstrand crosslinks in specific genes and nongene regions in cisplatin-resistant cells also have been demonstrated (Johnson S, et al. 1994).

The role of either cell adhesion molecules or transcription factors involved in this process of platinum-resistance is poorly defined. It has been shown, however, that small cell lung cancer cell lines can be protected from chemotherapy-induced apoptosis by interaction with the extra-cellular matrix (ECM) and its components (Fridman R, et al. 1990). Recently, it has been demonstrated that adhesion to extracellular matrix protects cancer cell line from chemotherapy-induced apoptosis by $\beta 1$ integrin-mediated adhesive process (Sethi T, et al. 1999). In a similar vein, maintenance of cadherin-6 may be associated not only with the maintenance of normal tissue integrity but may also play a role in the sensitivity to cytotoxic agents. It has been recently demonstrated that the expression of E-cadherin reduced bcl2 expression and increased sensitivity to etoposide induced apoptosis (Sasaki CY, et al.2000). In this study, we not only found that Barx2 transfection into ovarian cancer cells resulted in suppressed adhesion to collagen IV but also demonstrated that cadherin-6 was a likely target of the Barx2 gene. Therefore, the Barx2 may mediate increased sensitivity to cisplatin through inhibition of adhesion to component of ECM, perhaps by regulation of cadherin-6. In contrast, we did not observe Barx2-mediated enhanced apoptosis in response to cisplatin. This difference may reflect contextual

differences between our system and that reported by other investigator (Xie S, et al. 1997) although, in a similar analysis of CREB2 knockout in melanoma cells, there was a suggestion that CREB2 abrogation sensitised the cells to chemotherapy (Ronai Z, et al. 1998).

As a general principle, it may be that interaction of cancer cells with the extra-cellular matrix or with other cells induces resistance to cytotoxic agents (Kerbel RS, et al. 1994; St Croix B and Kerbel RS. 1997; Green SK, et al. 1999). For ovarian cancer, there is also variation in intrinsic primary platinum sensitivity that not only reflects inter-individual pharmacogenetic and pharmacodynamic differences, but also histology specific differences. It may be that, additionally, Barx2 mediates cisplatin sensitivity via regulation of cell adhesion molecules. Furthermore, the finding of Barx2 downregulation after platinum therapy raises an interesting hypothesis that the embryotoxic effects of cisplatin (Kopf-Maier P, et al.1985) might be specifically mediated by disruption of homeobox gene expression. Alterations in the expression of specific HOX genes have been reported in human embryonal carcinoma cells following exposure to the anti-epileptic drug valproate, and this mechanism may underlie the teratogenic effects of the anti-epileptic drug valproate(Faiella A, et al. 2000).

7.10 In summary

LOH analysis presented within this thesis has confirmed the previous studies of LOH at chromosome 11q between D11S1894 and D11S1309 in colorectal and ovarian cancer and also has reduced further the minimum size of telomeric region from 4.5 Mb to 200kb and 2Mb. Three distinct LOH regions were detected, between D11S912 and D11S4150, GAT72A01 and D11S4131, and D11S1320 and D11S4085, suggesting possible sites for tumour suppressor genes. Therefore, it provides complementary evidence for the ongoing effort to identify the genes involved in these minimal regions, which include some known genes although they do not represent logical candidate TSG based on their known functions.

The Barx2 gene, a homeobox transcription factors located at chromosome 11q25 between the marker D11S912 and D11S1320, was investigated in this study. We present evidence

from *in vitro* expression studies that, following transfection, human Barx2 inhibits matrigel invasion in the ovarian cancer cell lines OAW42 and OVCAR3. OAW42, a Barx2 non-expressing cell line, following transfection, was also inhibited for migration in response to a collagen IV signal and adhesion to collagen IV. In two sequential cell line series derived from patients with ovarian cancer pre- and post-platinum chemotherapy, Barx2 was downregulated in ovarian cancer cells collected after relapse. Transfection of the Barx2 gene into a platinum resistant cell line, PEO1 cDDP derived from PEO1 by *in-vitro* cisplatin exposure and downregulated expression of Barx2, showed that Barx2 reversed cisplatin resistance. We also show that Barx2 expression levels are lower in clear cell/endometrioid ovarian adenocarcinoma histological variants that are less likely to respond to cisplatin. Taken together, our data suggested that the Barx2 gene is a candidate suppressor of tumour progression, a modulator of cisplatin resistance in ovarian cancer. Furthermore, this represents the first report of Barx2 gene expression associated with modulation of cytotoxic drug sensitivity.

Further investigation of Barx2 is necessary for the understanding a mechanism as TSG. The use of reporter gene constructs would make it possible to elucidate whether downregulation/absence of Barx2 expression could be caused by the existence of promoter mutations or alterations in trans acting factors. In addition, although Barx2 has been involved in interacting with CAMs such as CDH6, which have been demonstrated to have associated with invasion, dissemination and drug resistance of cancer cells, the relationship between both is unclear. So further study of the relationship between Barx2 and CAMs would be helpful to reveal the mechanism that Barx2 acts as TSG in ovarian cancer.

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