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AN INTEGRATED POSITIONAL AND FUNCTIONAL APPROACH FOR IDENTIFYING OVARIAN CANCER TUMOUR SUPPRESSOR GENES ON CHROMOSOME 11p.

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I declare that this thesis has been composed entirely by myself and that it is my own work except for where I have acknowledged the work of others.

Cherie Blenkiron

December 2002

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Collaborations

The following people have all been involved in this work:

From the Cancer Research UK Labs, Edinburgh;

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Abstract

Ovarian cancer represents the most lethal gynaecological malignancy in the UK. Numerous tumour suppressor genes (TSG) are postulated to be involved in the aetiology of epithelial ovarian cancer (EOC). Cytogenetic analyses of cancer cells by methods such as LOH and CGH, have identified regions of genomic aberration. Allele loss on chromosome 11p has frequently been implicated in ovarian cancers, suggesting the presence of TSGs in these regions.

Ovarian cancer cell line OVCAR3 has lost a whole copy of chromosome 11. The remaining copy is fragmented, rearranged and duplicated. Transfer of normal chromosome 11 into OVCAR3 by Microcell Mediated Chromosome Transfer (MMCT) produced microcell hybrids that display suppression of growth and cellular migration *in vitro* and inhibition of tumour growth *in vivo*. Analysis of revertant clones was unable to further minimise regions harbouring candidate TSGs.

Subsequently, mRNA populations from OHN, a clonal derivative of the OVCAR3 parent line, and from 11OH2.1, a growth suppressed microcell hybrid, were used for expression difference analysis by Differential Display RT-PCR (DDRT-PCR), cDNA-Representational Difference Analysis (cDNA-RDA) and cDNA high density filter array (HDFA). In all, these techniques identified 159 up and 162 down regulated genes with respect to growth suppression.

Quantitative real time RT-PCR was used to validate expression differences in 178 transcripts. We identified, in total, 12 validated upregulated products and 4 validated downregulated products.

Of the 12 upregulated products associated with growth suppression, 4 were localised on chromosome 11, three at 11p15. These were cathepsin D (CTSD), proteasome subunit PSMD13, ribosomal subunit RPL27A on 11p15 and αB crystallin (CRYAB) on 11q23. All were shown to have decreased expression in several ovarian cancer cell lines and primary tumours. Furthermore, a tight correlation was observed between the expression of PSMD13 and RPL27A in cell lines and primary ovarian tumours. Low expression of CTSD and CRYAB were associated with adverse survival in patients with ovarian cancers.

The genes downregulated in association with growth suppression, and therefore of potentially oncogenic function, were RALDH2, IGFBP2 and 2 novel cDNAs. When examined on cell line and primary tumour panels, these genes did not however appear to demonstrate a global increase in expression over that of normal OSE.

An extensive LOH analysis of 87 ovarian tumours and their matched normal samples was then performed. Thirty-nine microsatellite markers spanning 19.8Mb on 11p15 were used in the most comprehensive analysis in ovarian cancer to date. Loss of the complete region was common (24%) and peaks of high LOH (>35%) were seen for 12 markers. Six microsatellite markers showed an association with one or more clinicopathological variables ($p<0.01$). Nine minimal regions of LOH were found.

PSMD13 and CTSD were both found within these regions of LOH as characterised by the markers D11S2071 and D11S922. RPL27a resides on 11p15.4 near the marker D11S932 which was not located within a minimal region of loss but LOH of that marker was significantly associated with advanced FIGO stage ($p=0.0001$).

This approach has demonstrated that the integration of functional and positional molecular genetic techniques can co-operate in the identification of candidate ovarian cancer TSGs.

LIST OF ABBREVIATIONS

Amp	ampicillin
BLAST	Basic Local Alignment Search Tool
BMI	body mass index
bp	Base pairs
BSO	bilateral salpingo-oophrectomy
CA125	cancer antigen 125
CDDP	Cis-di-amino-dichloro-platinum
cDNA	copy DNA
CGAP	Cancer Genome Anatomy Project
CGH	competitive genomic hybridisation
Chr	chromosome
cM	CentiMorgan
CT	computer tomograph
CTSD	Cathepsin D
DD-RT-PCR	differential display RT-PCR
DMEM	Dulbecco's modified minimal essential medium
DMF	Dimethyl formamide
DMSO	Dimethyl sulphoxide
DNA	deoxyribonucleic acid
DOTMA	dioleyloxypropyl-trimethyl-ammonium chloride
DOPE	dioleoylphosphatidylethan- olamine
DP	Difference product
DPX	Distyrene, plasticizer, and xylene
ECM	extra-cellular matrix
EDTA	Ethylene di-amine tetra-acetic acid
EGF	epidermal growth factor
EOC	epithelial ovarian cancer
FCS	Foetal calf serum
FIGO	International Federation of Obstetrics and Gynaecology
FISH	Fluorescent in situ hybridisation
FSH	follicle stimulating hormone
G418	geneticin
HDFA	high density filter array
HNPCC	hereditary non-polyposis colon cancer
HOSE	human ovarian surface epithelium
HOV	human ovarian tumour
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
HRT	Hormone replacement therapy
HTGS	High throughput genome sequencing
IGFBP	insulin-like growth factor binding protein
IHC	immunohistochemistry
IPTG	isopropyl-beta-D-thiogalactopyranoside
Kb	kilobases
KDa	kilo-Daltons
KRT	keratin
L-Broth	Luria Broth
L-agar	Luria agar

Lac	lactose
LH	luteinizing hormone
LMP	low malignant potential
LOH	loss of heterozygosity
LOI	loss of imprinting
Mb	Mega bases
MBq	Mega bequerell
min	minute
ml	millilitres
MMCT	microcell mediated chromosome transfer
MMLV	Mouse Moloney Leukaemia Virus
MMP	Matrix metalloprotease
NCBI	National Center for Biotechnology Information
NER	nucleotide excision repair
OC	Oral contraceptive
OSE	Ovarian surface epithelium
PAR	population attributable risks
PBS	Phosphate buffered saline
PCA	principal component analysis
PCR	polymerase chain reaction
PR	progesterone receptor
RALDH2	Retinaldehyde dehydrogenase 2
RDA	Representational Difference Analysis
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT	reverse transcription
SAGE	Serial Analysis of Gene Expression
SDS	sodium dodecyl sulphate
SRO	shortest region of overlap
TAH	total abdominal hysterectomy
TBS	Tris buffered saline
TIMP	Tissue inhibitor of matrix metalloproteases
TSG	tumour suppressor gene
UCSC	University of California, Santa Cruz
UV	Ultra violet

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1. INTRODUCTION

1.1 Cancer overview

In the UK, cancer, heart disease and stroke are the three biggest killers. The five most common female malignancies in western countries are, in order of prevalence, breast, lung, colon, stomach and ovarian. The total number of new cases of cancers in women appears to have stabilised but the common types of cancer have altered, with lung cancer cases on the increase and breast and cervical tumour incidence declining. An improvement in diet is believed to have helped reduce the incidence of many other common cancers such as bowel and colon. Another factor involved in declining cancer incidence and mortality has been the improvement of screening methods, particularly for cervical and breast cancers.

World-wide, there are over ten million new cases of cancer diagnosed annually and even though Europe and North America accommodate only 11.5% of the global population, they account for 41.2% of these cases (Parkin *et al.*, 2001). The incidence per person is therefore much higher in these areas.

1.2 Epithelial Ovarian Cancer: Global distribution and causation

1.2.1 Epidemiology

Ovarian cancer is the 6th most common malignancy and the 6th leading cause of cancer related deaths, globally, in women (Parkin *et al.*, 2001). It is the third most common but the leading cause of mortality from a gynaecological malignancy in the western world.

In 1990, worldwide, there were 165,000 new cases of ovarian cancer representing 4.4% of all female cancers (Parkin, 1998). Latest studies estimate 191,000 new cases annually (Parkin *et al.*, 2001). The frequency of ovarian cancer is greatest in the developed world with highest incidence and mortality rates in Europe, particularly northern (UK and Scandinavia) and North America (Parkin, 1998). Japan conversely has a greater than 2 fold lower rate of incidence (Parkin, 1998).

Cancer Research UK statistics for the year 2000 show ovarian cancer affected 6884 women and was responsible for 4,430 deaths. This represents 1.5% of all women affected by cancer and is the fourth (6%) most common cause of all female cancer related deaths. Five-year survival rates are the fourth worst for cancers at 29.2% in

1991-1993 compared to the average five-year survival rate of 43% for all cancers in females.

Information and statistics division (ISD), Scotland, have reported that, as for the whole of the UK, ovarian cancer was the fourth most frequently diagnosed cancer and in 1998 represented 4.7% of all new cancer incidences in females. Over the past ~25 years incidence and mortality rates have increased 1.5- and 1.25-fold respectively but there is, however, an associated increase in the five year survival of 3.9% to 35.9% as adjusted for age.

1.2.2 Cause and aetiology

The cause of ovarian cancer is widely debated with many environmental, endocrine and genetic factors having been researched. Numerous protective and risk associated factors have been identified or suggested. These include;

- Menstrual factors; age of menarche/menopause (Chiaffarino *et al.*, 2001), irregularity of cycles.
- Reproductive factors; pregnancy, breast feeding, tubal ligation/hysterectomy, oral contraceptive use (Schildkraut *et al.*, 2002), Hormone replacement therapy (HRT), (Lacey *et al.*, 2002a) and fertility treatment.
- Dietary factors; Fat/protein consumption, vegetable consumption (McCann *et al.*, 2001), caffeine/coffee and alcohol intake (Tavani *et al.*, 2001), body mass index (BMI) (Lukanova *et al.*, 2002), lactose and cholesterol intake.
- Other factors; Family history, physical exercise (Bertone *et al.*, 2002), talc use, smoking (Modugno *et al.*, 2002), aspirin use (Fairfield *et al.*, 2002), radiation, psychotropic drug intake, hair dye, asbestos exposure (Heller *et al.*, 1996) and race (Barnholtz-Sloan *et al.*, 2002).

Multiple studies have shown that oral contraceptive use, parity, tubal ligation and hysterectomy, and breast-feeding can protect from ovarian cancer (Holschneider and Berek, 2000; Runnebaum and Stickeler, 2001; Titus-Ernstoff *et al.*, 2001). Increased risk has been associated with infertility, nulliparity and family history.

A case control study in Italy showed that population attributable risks (PAR) accounted for 51% of cases. That is, 51% of cases could have been avoided if the exposure had not been present in the population. PAR were 5% for nulliparity, 12%

for no OC use, 4% for family history, 16% for late age at menopause, 24% for low intake of vegetables and 7% for high fat intake (Parazzini *et al.*, 2000). Three of these factors are modifiable so it was thought that these could therefore be suitable methods of prevention, which could be implemented within this and other populations. The remaining 49% must be due to other non-inherited factors and exposures that have not been analysed in this paper. The latter three risks examined however have yet to be fully recognised as ovarian cancer risk factors.

There are multiple theories to the causation of ovarian cancer. The earliest was suggested by Fathalla in 1971 and called the incessant ovulation theory (Fathalla, 1971). He proposed that chronic repetitive ovulation without interruption from pregnancy or OC use could induce neoplasia of the ovarian surface epithelium (OSE). The OSE undergoes rapid proliferation for 24 hours post ovulation and invaginations of the epithelium are more prevalent at this time so clefts and cysts are common. There is some support that these cysts may be associated with neoplasia and tubal metaplasia and so sites of potential carcinomas (Resta *et al.*, 1993). Epithelial cancers of the ovary arise most frequently within these inclusion cysts. Repeated proliferation of the OSE may also lead to mutations in epithelial cells, making them susceptible to transformation.

Casagrande added to this hypothesis by suggesting that periods of anovulation could decrease cancer risk by providing 'protected time' (Casagrande *et al.*, 1979). Ovulation also exposes some OSE to stromal estrogens, through the production of inclusion cysts, which may further contribute to transformation. Support for the theory comes from numerous studies into the protective effects of OC, parity and breast-feeding as all of these methods lead to anovulation. There are however, discrepancies to the hypothesis. La Vecchia suggested that, assuming ovulation occurs for 20 years of a woman's life and if 1 pregnancy halts ovulation for one year then that accounts for 5% of all ovulation (La Vecchia *et al.*, 1983). The risk reduction per pregnancy however is approx. 14%. This discrepancy between numbers suggests there may be a parallel mechanism that induces ovarian transformation. Also, as ovarian cancers appear to arise more frequently from inclusions cysts, which themselves are not constantly affected by repetitive

proliferation from ovulation and repair of the OSE, it is thought there are other environmental factors involved in progression (Godwin *et al.*, 1992).

A number of alternative or perhaps corresponding theories study the involvement of hormones with neoplastic progression. The main idea is called the 'gonadotrophin hypothesis'. Excessive gonadotrophin secretion is not associated directly with ovulation and surface epithelium damage but causes consequent estrogen stimulation which is believed to result in proliferation and malignant transformation of the OSE. This theory is supported by the protective effects associated with parity and OC use as both of these mechanisms decrease levels of basal and peak gonadotrophins (FSH and LH) (Risch, 1998). Levels of FSH and LH are known to rise peri/post menopausally, due to loss of feedback inhibition on the pituitary. This suggests an association between these increased hormone levels and onset of ovarian cancers, as they are associated with age (Risch, 1998). However, LH and FSH do not stimulate estrogen post-menopausally and levels decrease. Gonadotrophins may therefore act through a non-estrogen related mechanism. Increased LH is also known to prevent apoptosis and increase angiogenesis, which may promote cancer formation in these post-menopausal women.

Another hormone to be potentially associated with ovarian cancer risk is estrogen. Estrogen levels in a normal ovary peak at rupture of the OSE at ovulation. During menstrual cycles, the OSE proliferates most when estradiol levels are high. Estrogen may therefore have a proliferative role that could increase mutation risk in the exposed OSE cells. Support for the role of estrogens in ovarian carcinoma initiation is seen from the effects of breast-feeding, OC use and HRT. The former two suppress LH and subsequently decrease levels of estrogen so inhibit proliferation. Estrogen-only HRT causes a serum increase in estradiol and recent studies have associated this therapy with increased cancer risk (Lacey *et al.*, 2002b). As for many hypotheses however, there is evidence against this idea. For example, pregnancy causes a 100-fold increase in estrogen levels but confers protection (Risch, 1998). This suggests that there may be yet another/other hormone(s) involved in neoplasia.

Androgens cause increased proliferation and cell death in OSE cells (Edmondson *et al.*, 2002). Inclusion cysts, themselves formed from OSE, are exposed to high levels of androgens in the ovarian cortex. A study looking at levels of androgens in ovarian

cancers discovered 50% higher levels of androstenedione and dehydropiandrosterone in both pre and postmenopausal cases as compared to normal controls (Helzlsouer *et al.*, 1995). The postmenopausal ovary sees a 15-fold increase in levels of the androgen testosterone correlating with the incidence of EOC in postmenopausal women. Testosterone has also been detected at 30-65% of normal levels in OC users who have a decreased lifetime risk of developing ovarian cancer (Judd *et al.*, 1974). The in vitro effects of estrogen and testosterone were studied in human epithelial cells and both showed a positive effect upon cell growth after three weeks, supporting their role in increasing OSE proliferation (Karlan *et al.*, 1995).

Progesterone may have a protective role in ovarian cancer as a 10-fold increase in levels is found in pregnant women and risk of EOC decreases with parity. It is suggested that high progesterone levels and 'protective time' from anovulation during pregnancy may be additive to close the gap in risk reduction between postulated and actual risk (La Vecchia *et al.*, 1983). High levels of physical activity may decrease progesterone levels and increase androgen levels that may cause the inflated risk of EOC found in a few very active women (Bertone *et al.*, 2001).

Parmley and Woodruff suggested that EOC might arise from the transformation of OSE cells exposed to pelvic contaminants and carcinogens (Parmley and Woodruff, 1974). This theory is linked to the idea that inflammation may be involved in EOC. Inflammation produces toxic oxidants that may cause direct damage to DNA and proteins, leading to disruption of normal gene expression. Chronic inflammation is also known to increase cellular division. Evidence that contaminants may travel up the fallopian tubes was found when traces of asbestos fibre were noticed in the ovaries of cancer patients who had been exposed (Heller *et al.*, 1996). It is believed that blocking the upper genital tract disallows exposure to proinflammatory substances and support for this hypothesis is seen from strong data that tubal ligation and hysterectomy are protective for EOC (Runnebaum and Stickeler, 2001).

Aspirin, an anti-inflammatory, has in some cases been found to decrease cancer risk if used three or more times a week (Akhmedkhanov *et al.*, 2001). The protection appears to reduce over time with more recent users being at less risk. Aspirin may also however act by inhibiting both angiogenesis and, importantly, the activity of the

cox-2 enzyme that indirectly induces cell proliferation. Cox-2 inhibition induces apoptosis, so may protect against cancerous induction from damaged progenitor cells by undergoing programmed cell death (Rodriguez-Burford *et al.*, 2002). Aspirin is known to inhibit proliferation of ovarian cancer cells by blocking the effects of the HER-2/neu/ErbB-2 receptor, which may also confer protective effects in normal OSE cells (Drake and Becker, 2002). The most recent case study in America however, suggests that aspirin does not decrease risk but there was a 40% reduction of risk of ovarian cancer occurrence with other non-steroidal anti-inflammatory usage (Fairfield *et al.*, 2002).

1.3 Ovarian Histopathology

1.3.1 Normal Ovarian development

The ovaries are ovoid, paired organs. They consist of a thick peripheral cortex that surrounds the medulla, and are covered in a continuous sheet of cuboidal epithelial cells that lie on a basement membrane. This in turn lies on a dense layer of connective tissue called the tunica albuginea. Ovarian epithelial cells are attached lightly to the basement membrane by desmosomes and tight junctions. The surface of the ovary is contoured with invaginations of the epithelia which, with age, internalise into the inner stroma to form inclusion cysts.

The OSE is derived from embryonic coelomic epithelium that arises from enlarged mesoderm upon gastrulation. Foetal development of the ovaries involves invagination of the coelomic epithelium to form the Müllerian duct. The duct then differentiates to form the uterus, cervix, fallopian tubes and upper vagina. Coelomic epithelium persists to line the ovaries as the OSE. These cells, importantly, retain the potential for Müllerian differentiation to display metaplasia and neoplastic progression.

The normal involvement of the OSE is in cyclical ovulatory rupture and repair. Ovarian epithelial cells are believed to produce proteolytic enzymes important for release of the ovum and they also have the potential to proliferate for repair of the wound.

The work of Auersperg documents the development and roles of the OSE in great detail. The biology, endocrinology and pathology of the OSE are documented in a great review from 2001 (Auersperg *et al.*, 2001).

1.3.2 Ovarian Cancer histopathology

Ovarian cancers arise from all cell types within the ovary. The oocytes give rise to various germ cell tumours and cancers may also arise from sex cord/stromal origin. These tumours generally have a good prognosis and can be treated successfully.

Most importantly, tumours can arise from the ovarian surface epithelium (OSE). Ninety percent of all malignant neoplasia are believed to originate from the surface epithelium. OSE cells, when neoplastic, retain the potential for Müllerian differentiation giving rise to tumours with different pathological characteristics. The most common are serous, endometrioid, mucinous, and clear cell tumours. Rare Brenner tumours are formed when the tumour develops along wolffian lines to resemble uroepithelium.

Ovarian surface epithelium tumours may be benign, borderline (low malignant potential LMP), or malignant. It is the latter which are the frank epithelial ovarian carcinomas (EOC). The spread of an EOC is seen in Figure 1.1.

Benign tumours generally have a very good prognosis. They are characterised by a single layer of columnar cells with lack of atypia and a normal nucleocytoplasmic ratio. They undergo few mitoses and display no invasion into the underlying stroma. They tend to be of serous or mucinous origin and large and cystic. The most common are mucinous cystadenomas which are characterised by large, smooth multilocular cysts, and are usually unilateral and filled with gelatinous fluid. Serous cystadenomas are less common, being thin-walled unilocular cysts which are filled with watery fluid and are well differentiated. The cells resemble epithelia of the fallopian tube. Endometrioid cystadenomas are rare.

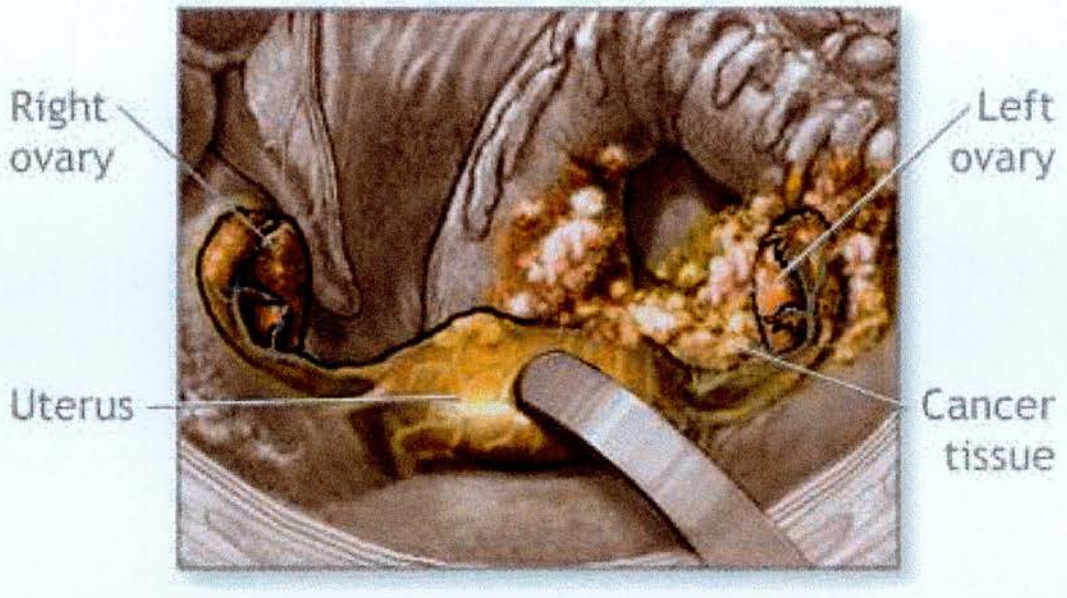


Figure 1.1 The spread of EOC through the intraperitoneal cavity.

Borderline tumours (LMP) are multilayered with irregular budding and cellular atypia. They resemble malignant neoplasms but are distinguished by the lack of stromal invasion. Median age of diagnosis is 40 years and prognosis is good with 95% five-year survival. Most LMP tumours behave in a benign fashion and few become malignant. Serous borderline tumours are the most common histological type.

Malignant tumours tend to be aggressive with a very poor prognosis if disseminated from the site of origin. Primary tumours often have regions of solid tissue which is haemorrhagic and necrotic as well as being destructively invasive. They infiltrate locally into pelvic tissue and seed tumour implants into the peritoneum, including the fatty tissue structure, called the omentum. They, however, rarely metastasise to distant sites. Malignant cells are characterised by cellular atypia, high nucleocytoplasmic ratio with frequent mitoses. These tumours are rare before 20 years of age and incidence increases linearly after 55 years old. Tumours are classified, as already mentioned, according to histological similarities to epithelial components distinctive of different Müllerian structures. They are also classified by

grade of differentiation. Well-differentiated tumours give the best prognosis with little cellular atypia and few mitoses. Moderate and poorly differentiated cells show increasing cellular atypia, more frequent mitoses and enhanced aggressiveness. Undifferentiated tumours are rapid growing and aggressive with very poor outcome. Serous ovarian carcinomas, the commonest type of ovarian cancer, are usually cystic with numerous papillary ingrowths and are often solid in areas. These represent about half of all EOC. Approximately 65% are bilateral with poor prognosis (dependent upon stage) of 20-30% five-year survival. They are most likely to arise de novo as carcinomas as opposed to development from benign lesion.

Twelve percent of EOC are mucinous cystadenocarcinomas. They are cystic, multilocular and often well differentiated. They often progress from mucinous cystadenomas. At the cellular level they are irregular structures lined by abnormal, endocervical-like epithelium.

Endometrioid cancers are rarely benign or borderline and represent 15% of all EOC. They are solid with areas of haemorrhage and tend to be less cystic than other histological sub-types. Endometrioid tumours are characterised by glands lined with columnar cells containing a large nucleus and little cytoplasm. Twenty-five percent of cases are associated with endometrial carcinoma and a minority arise from foci of pre-existing endometriosis. Five-year survival is better than for serous tumours at about 51%.

Clear cell or mesonephroid tumours are thought to be a morphological variant of endometrioid neoplasms. They are invariably malignant and are characterised by large polyhedral cells containing abundant clear cytoplasm. They predominantly affect post-menopausal women and, as for endometrioid carcinoma, may co-exist with endometriosis. Clear cell carcinoma has a worse prognosis, stage for stage, than serous carcinomas.

Brenner tumours are the most rare EOC as this histology is most commonly associated with benign tumour type. They are characterised by rounded islands of transitional type epithelia of wolffian origin embedded in a dense fibrous stroma. Five-year survival is approximately 50%. Other malignant sub-types are mixed Müllerian and undifferentiated tumours. The latter have a very poor five-year survival of 16% due to the aggressive nature of the neoplasm.

1.3.3 Origins and Progression of EOC

The origins and pathway of progression of ovarian carcinoma, although well studied, are still undefined. There are two possible hypotheses for occurrence. The first follows the progression theory, similar to that suggested for colon cancer by Fearon and Vogelstein, in which, through the acquisition of defined genetic changes, a benign tumour will progress to an aggressive carcinoma (Fearon *et al.*, 1987). The second, and most widely held view, is that the tumours arise de novo from direct transformation of the OSE within inclusion cysts. There is also the potential that both pathways of progression occur.

Colorectal cancer progresses from the collection of defined genetic lesions in oncogenes and tumour suppressors. This multistep process ultimately produces the neoplastic phenotype from the original normal colon epithelial cells. This progression theory is rarely applied to ovarian cancers due to the lack of evidence for early genetic lesions. A study by Zanetta looking at the progression of borderline tumours to EOC showed only 2% progress, of both mucinous and serous histologies (Zanetta *et al.*, 2001). This suggests that the vast majority (98%) of EOCs do not progress from borderline tumours. Molecular studies analysing expression of the oncogene K-RAS in mucinous and serous tumours suggest that progression from borderline and benign lesions may occur in only a subset of mucinous tumours (Haas *et al.*, 1999; Mandai *et al.*, 1998). From these analyses, they determined that serous tumours are unlikely to have progressed from borderline tumours or even from well-differentiated carcinomas (Haas *et al.*, 1999). A more recent study of K-RAS mutations in serous tumours found 50% of borderline tumours and low-grade invasive micropapillary serous carcinomas had K-RAS mutations (Singer *et al.*, 2002). None of the 23 high-grade serous carcinomas carried the mutation. They also noted that 95% of bilateral ovaries from non-invasive serous carcinomas had discordant patterns of genetic lesions. They found that the majority of the high-grade serous carcinomas showed clonal allelic imbalances, which supports the notion that they arise de novo. This study suggests two pathways of tumorigenesis. Firstly, a low-grade invasive serous carcinoma may arise in a step-wise fashion from a borderline tumour. In the second pathway, high-grade carcinomas develop de novo by transformation of the ovarian surface epithelium. Singer does note however that

other pathways of progression may exist. More support for the de novo theory is that benign tumours appear phenotypically stable over long periods of time showing no biological changes towards a more aggressive tumour-type.

In a study looking for a genetic lesion, loss of Chr 11p15, common to the high-grade tumour phenotype, Zheng showed that in those high-grade tumours which lie adjacent to seemingly low-grade or benign sections of tumour, that the whole clonal tumour has the lesion (Zheng *et al.*, 1993). This suggests that the carcinomas are heterogeneous in appearance with differing degrees of maturation and also lends support to the idea that low-grade tumours may progress to higher grade lesions or that the high-grade lesions may differentiate into benign-looking structures.

A review of the multistep pathway of EOC progression is seen in Figure 1.2.

There has also been debate about the origins of ovarian carcinomas of differing histological sub-types and the suggestion that these may represent distinct disease entities. Genetic lesions within a tumour sub-type differ. For example K-RAS mutations are more frequent in mucinous tumours and p53 mutations are more common in serous tumours. A recent study of gene expression analysis using oligonucleotide microarrays showed mucinous and clear-cell EOCs can be readily distinguished from serous ovarian cancers, regardless of tumour stage and grade (Schwartz *et al.*, 2002). This wide genetic heterogeneity may further separate ovarian carcinomas into biologically and, importantly, clinically meaningful subgroups.

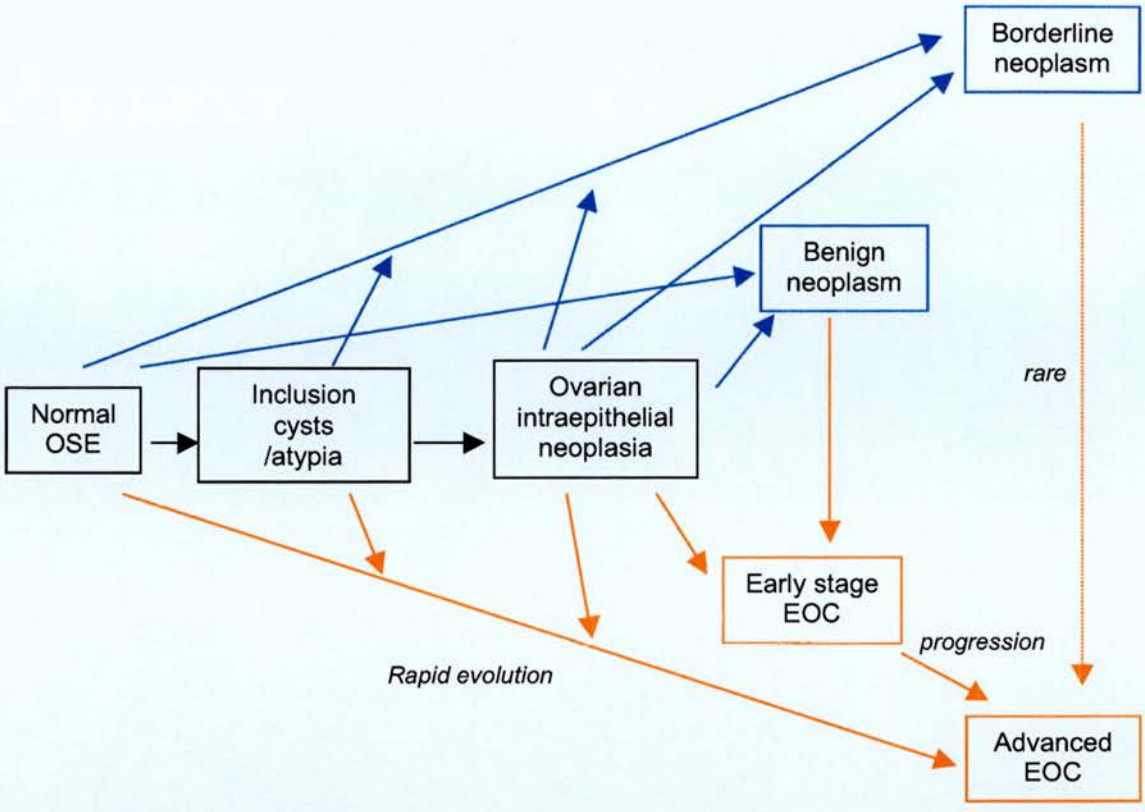


Figure 1.2 Multistep pathway of EOC progression. Proposed pathways of EOC generation from precursor lesions. Red pathways are the progression to EOC, blue pathways are the progression to a benign or borderline histology.

1.4 Clinical management

1.4.1 Clinical prognostic factors

Clinical prognostic factors for survival from ovarian carcinoma include age, stage, histological subtype, volume of disease pre and post operatively, differentiation status, performance status and presence of ascites. It has been found that women of a younger age at diagnosis generally have a better outcome. Five-year survival for women aged 15-45 years is 65% compared to the over 75s survival of just 18%, although younger women tend to have earlier stage, so less aggressive disease. The histological subtypes of mucinous and clear cell carcinomas have a worse prognosis than serous carcinomas. Grade of differentiation of the tumour is also important to assess. Undifferentiated, high-grade, tumours are highly aggressive and have a poorer prognosis. Lesser residual disease post-operatively is favourable and masses under 1cm give a survival of 50% compared with those 2+cm of 13%. Stage is also a

prognostic factor with the five-year survival rates of stages of disease seen in Table 1.1.

STAGE	FIVE-YEAR SURVIVAL (STAGE A)	FIVE-YEAR SURVIVAL (STAGE B)	FIVE-YEAR SURVIVAL (STAGE C)
I	92%	85%	82%
II	67%	56%	51%
III	39%	26%	17%
IV	12%		

Table 1.1 Five-year survival rates of patients with differing stages of EOC. Subgroups A-C represent increased spread of the cancer.

1.4.2 Clinical diagnosis

Early stage ovarian cancer is generally asymptomatic. The most common symptoms, such as nausea and abdominal fullness and discomfort, are often related to the later spread of the disease outside of the pelvis. Abnormality is often detected by pelvic examination followed by transvaginal ultrasound and analysis of serum CA125 levels. CA125 is a large glycoprotein that is anchored to the surface epithelium. It is secreted by way of cleavage in tumours. For the last 20 years CA125 has been used as a tumour marker in ovarian cancers and which, although not only limited to expression from ovarian carcinomas, is elevated in 80% of patients with advanced stage ovarian cancer. Only 50% of early stage carcinomas, however, have elevated levels of the antigen. Other less used diagnostic tests are CT scan or biopsy via a laparoscopy. The latter method has however caused concern for spread of the cancer due potential cyst rupture.

1.4.3 Staging and surgery

In the absence of extra-abdominal metastatic disease, staging of ovarian carcinomas is done during exploratory laparotomy. Designated by FIGO, the stages represent the extent of spread of the tumours (Table 1.2). Staging is important to determine follow-up treatment although is often suboptimal. The laparotomy is often required to diagnose the cancer. Once confirmed, surgery is performed; a total abdominal hysterectomy (TAH), a bilateral salpingo-oophrectomy (BSO) and omentectomy. Examination of all serosal surfaces is carried out and ascites or peritoneal washings

are collected for cytogenetic analysis and histological diagnosis. A biopsy may also be taken of peritoneal surfaces. Gross disease is then maximally debulked and residual implants are noted for size. Debulking surgery is often stabilising for low stage and grade disease and these patients have a five-year survival of 91-98%. Optimal debulking is dependent upon the biology of the tumour and the ability of the surgeon.

STAGE	CHARACTERISTICS
Ia	Growth limited to one ovary; no ascites. No tumour on the external surfaces; capsule intact.
Ib	Growth limited to both ovaries; no ascites. No tumour on the external surfaces; capsule intact.
Ic	Tumour either stage Ia/Ib but with tumour on surface of one or both ovaries; or with capsule ruptured; or with ascites present.
IIa	Extension and/or metastasis to the uterus and/or tubes.
IIb	Extension to other pelvic tissues.
IIc	Tumour either stage IIa/IIb but with tumour on surface of one/both ovaries; or with capsule ruptured; or with ascites present.
IIIa	Tumour grossly limited to the true pelvis with negative nodes but with histologically confirmed microscopic seeding of the peritoneal surfaces.
IIIb	Tumour involving one or both ovaries with histologically confirmed implants of abdominal peritoneal surfaces. Nodes- none exceeding 2cm diameter and negative.
IIIc	Abdominal implants >2cm diameter and/or positive nodes
IV	Growth involving one or both ovaries with distant metastases. Positive cytology.

Table 1.2 FIGO classification of EOC. Stages I-IV represent increased spread of the disease with generally poorer prognosis.

1.4.4 Chemotherapy

Combination chemotherapy is the standard postoperative therapy for patients with residual disease or those at high risk of recurrence. Patients with well-differentiated, stage IA/B disease have a good five-year survival after surgery, which is not improved by adjuvant therapy. Those however with stage IC or I- high grade, stages II-IV or clear cell carcinoma (any stage) should receive chemotherapy. It is for this reason that accurate staging and pathological review is essential.

Carboplatin/paclitaxel combination therapy is currently classed as the gold standard in treatment of the disease. External abdomino-pelvic radiotherapy is also an option, but rarely used.

Chemotherapy after second-look surgery has no standard regimen. Options are to stop treatment, continue same chemotherapy, use other chemotherapy or intensification.

1.5 Genetic Epidemiology of ovarian cancer

1.5.1 Familial epithelial ovarian cancer.

Malignant transformation is believed to be a multistep process and data has suggested that a series of 5 or 6 genetic alterations is rate limiting for cancer development (Boyd and Rubin, 1997). Most ovarian cancers are sporadic, only 5-10% are familial. Aside from age, family history is the strongest predictor of ovarian cancer risk with one or two affected primary relatives with ovarian cancer conferring a 5% and 7% risk respectively over the general population (Kristensen and Trope, 1997). There are distinct hereditary patterns for ovarian cancers that have high penetrance of autosomal dominant genes.

Site-specific ovarian cancers account for 10-15% of all hereditary cases. Virtually all are linked to mutations in the BRCA1 tumour suppressor gene on chromosome 17q21 (Boyd and Rubin, 1997). BRCA1 mutation carriers have a cumulative risk of developing ovarian cancer, by age 70, of 63% (Boyd and Rubin, 1997; Elit, 2001; Kristensen and Trope, 1997).

Breast/ovarian cancer syndrome accounts for 65-70% of all familial cases. Patients with 5+ primary or secondary relatives with breast/ovarian cancer or, 3 cases early-onset breast/ovarian cancer are categorised with the syndrome. It has been suggested that site-specific ovarian cancer may be part of the same syndrome, but in which early onset breast cancer has not occurred. BRCA1 mutations account for 75% of all cases and most others are linked to BRCA2 on chromosome 13q12-13 (Boyd and Rubin, 1997).

The second distinct syndrome, HNPCC, accounts for 10-15% of all hereditary cases (Bewtra *et al.*, 1992). It is a cancer susceptibility syndrome that confers an increased risk of colon, gastric, endometrial, small bowel and ovarian cancers. Patients are

classified with HNPCC if they have 3+ primary relatives with either endometrial or colon cancers and 2 were diagnosed with the latter earlier than age 50. HNPCC confers a 3.5 fold increased risk of ovarian cancer over the general population and is linked to four mismatch repair genes MSH2, MLH1, PMS1, and PMS2 (Boyd and Rubin, 1997). Ovarian cancer is detected in 10% of HNPCC patients with mutations of one of these four genes. Greater than 90% of HNPCC/ovarian cancer mutations are in MLH1 (Chr 3p) and MSH2 (Chr 2p) (Boyd and Rubin, 1997). These mismatch repair pathway genes are often silenced by loss of function mutations associated with genetic instability.

1.5.2 Sporadic epithelial ovarian cancer

Sporadic ovarian carcinomas arise by clonal selection and accumulation of somatic mutations in critical genes. Genetic changes such as amplification, altered expression and mutations in oncogenes and tumour suppressor genes contribute to the development of sporadic ovarian neoplasms. Those genetic lesions that confer a selective advantage will predominate through a heterogeneous tumour.

Genes commonly over expressed or amplified in ovarian cancer include c-myc, c-fos, CSF-1, ErbB2, PIK3CA and AKT. CSF-1 and its receptor, encoded by oncogene c-fms, are over expressed in 78-89% of ovarian tumours and mostly co-expressed (Baiocchi *et al.*, 1991). They are involved in autocrine control of proliferation in ovarian cancer cells.

Amplification or over expression of the oncogene c-myc is often associated with the more common serous EOC histology. It is amplified in 50% of serous tumours and over expression appears to be related to increased stage and aggressiveness (Bian *et al.*, 1995).

ErbB2/HER-2/neu gene, which encodes an EGF receptor, is amplified in 24-31% of ovarian carcinomas and is commonly associated with poor survival and advanced stage (Bian *et al.*, 1995; Fan *et al.*, 1994). More recently, a study in serous carcinomas confirmed the association of amplification with late stage, showing 71% amplification in stage III tumours as compared with only 22% amplification in stage I tumours (Afify *et al.*, 1999). EGFR/HER-1/ErbB1 is another EGF receptor, which is expressed in 70-100% of ovarian tumours (Baekelandt *et al.*, 1999a). This over

expression is believed to play a role in enhancing the invasive phenotype of the tumour. Increasing expression correlates with increasing FIGO stage.

Hormone receptors ER and PR have been reported as prognostic factors in ovarian cancer. The estrogen receptors ERα and ERß are co-expressed in normal OSE. Over expression of ERα relative to ERß may be a marker of ovarian carcinogenesis (Pujol *et al.*, 1998). PR expression is commonly decreased in ovarian cancers and can be used as a significant independent prognostic variable of progression-free survival in advanced tumours (Hempling *et al.*, 1998).

Recently, amplification of signalling molecules PIK3CA and AKT1/2 has been reported. CGH detected that PIK3CA, a subunit of PI3K, is amplified in 40% of tumours. AKT1/AKT2 are downstream molecules of the PI3K signalling pathway. AKT1 has increased kinase activity in 39% of ovarian tumours with 73% of these being of high grade and stage. AKT2 is amplified in 12% of ovarian tumours and may be associated with high grade aggressive tumours (Bellacosa *et al.*, 1995).

K-RAS, as mentioned previously, is commonly mutated in 71-75% of mucinous tumours as compared to only 13-20% serous (Enomoto *et al.*, 1991; Fujita *et al.*, 1994).

Finally, CDKN2A/MTS1 is a chromosome 9p21 cell cycle regulator that has been reported as both a TSG and an oncogene. LOH at 9p21 is 45-65% but the gene is not frequently mutated or methylated (Niederacher *et al.*, 1999; Shih *et al.*, 1997). A paper by Dong suggests that increased expression of CDKN2A is related to progression and unfavourable prognosis with 89% of tumours overexpressing the gene (Dong *et al.*, 1997).

Tumour suppressor genes are inactivated by mutation, LOH and epigenetic mechanisms, such as methylation and acetylation. The most frequently reported TSG, for all carcinomas, is p53. In ovarian tumours it is mutated in 50% of late stage tumours (Berchuck *et al.*, 1992). Mutations are also more prevalent in high-grade serous tumours than other histological subtypes (Milner *et al.*, 1993). Abnormal p53 expression is detected in 50% malignant tumours and over expression is correlated highly with the presence of mutations in the p53 gene (Marks *et al.*, 1991).

BRCA1 and BRCA2 have already been discussed as tumour suppressors in familial cancer but they are also implicated in the progression of sporadic cancers. Reduced

or absent BRCA1 protein and RNA expression has been detected in 90% of sporadic epithelial ovarian tumours (Russell *et al.*, 2000). LOH at the BRCA1 locus is reported at 44% although somatic mutation rate is low at 3.5% (Russell *et al.*, 2000). BRCA1 is also methylated in 15% of sporadic ovarian tumours (Baldwin *et al.*, 2000). BRCA2 however is not methylated and rarely mutated but LOH of 43% at 13q is reported and is more frequent in serous tumours, with an LOH rate of 75% in these (Gras *et al.*, 2001).

Other candidate ovarian cancer TSGs include PTEN, Dab2/DOC2, and the more recently described NOEY2/ARH1, LOT1/ZAC, OVCA1, SPARC and WWOX.

PTEN is a PI3K inhibitor, so antagonistically associated with the oncogene PIK3CA, and is often allelically deleted and mutated in endometrioid EOC. LOH of 43% and mutation in 21% of the endometrioid subtype has been reported (Obata *et al.*, 1998). These mutations appear to be implicated with early stage and low grade disease. Loss of PTEN expression is also linked to increased phosphorylation of oncogene AKT, probably via the resulting lack of PI3K inhibition (Kurose *et al.*, 2001).

Dab2/DOC2, first described in ovarian cancer by a differential DNA fingerprinting technique comparing OSE cells and ovarian carcinomas, is down regulated particularly in serous ovarian tumours (Mok *et al.*, 1998; Mok *et al.*, 1994). It is an essential suppressor of the RAS mediated signalling cascade and loss has been implicated in the initiation of ovarian tumorigenicity (Yang *et al.*, 2002).

NOEY2/ARH1 is an imprinted gene discovered by DD-RT-PCR with high homology to RAS and RAP (Yu *et al.*, 1999). Imprinting of the gene means that it is expressed from either a maternal or paternal allele, as regulated by methylation. There is 40% LOH at the ARH1 locus on 1p31 in breast and ovarian cancers and the gene is aberrantly methylated in breast cancer cells (Luo *et al.*, 2001; Yu *et al.*, 1999).

LOT1/ZAC, first described in 1997 from loss of expression in transformed rat ovarian surface epithelial cells, is also imprinted (Abdollahi *et al.*, 1997). It lies in a region of common LOH at 6q25 and is a nuclear protein that induces growth arrest and apoptosis so is an ideal TSG candidate (Abdollahi *et al.*, 1999; Piras *et al.*, 2000). LOT1 also exhibits maternal imprinting and demethylation experiments in breast cancers have shown re-expression of the gene (Bilanges *et al.*, 1999).

OVCA1/DPH2L1 was identified by LOH and positional cloning on 17p13.3 and is expressed in normal OSE but transcription is undetectable/reduced in 92% of ovarian tumours/cell lines (Schultz *et al.*, 1996). Reexpression of OVCA1 in ovarian cell line A2780 caused an increase of cells in G1 of the cell cycle due to increased degradation of cyclin D1. The reduction of this gene to hemizygosity by LOH is thought to be enough to deregulate the cell cycle and promote tumorigenesis in ovarian cancer cells (Bruening *et al.*, 1999).

SPARC, a calcium-binding extracellular matrix glycoprotein also identified by DD-RT-PCR, is expressed at lower levels in ovarian cancer cells/cell lines than in normal OSE. Functional studies by transfection of SPARC into ovarian cancer cell line SKOV3 showed it induced slower growth and suppressed tumorigenicity (Paley *et al.*, 2000). SPARC has also recently been reported to induce apoptosis in ovarian cancer cells (Yiu *et al.*, 2001).

Finally, WWOX has been reported as a tumour suppressor in multiple tissue types. Initially identified by Bednarek through breast cancer mapping, the WWOX protein has 2 WW domains and a short chain reductase region (Bednarek *et al.*, 2000). The gene itself spans about 750Kb and is situated at the fragile site FRA16D. It is believed that WWOX may play a role in apoptosis. Paige *et al* showed that in some ovarian cell-lines, WWOX has homozygous deletion of exons as well as finding missense mutations (Paige *et al.*, 2001).

1.6 Genomic analysis of ovarian tumours.

Genomic analysis of ovarian tumours has been important in the definition of regions that potentially harbour genes associated with tumorigenesis and cancer progression. It is thought that 30% of a solid tumour genome may be abnormal in copy number. Methods used to identify these abnormalities include Fluorescent in Situ Hybridisation (FISH), Competitive genomic hybridisation (CGH), and Representational Difference Analysis (RDA).

The first involves hybridisation of probes targeted to specific chromosomes or subregions to detect losses, gains or rearrangements but is complicated to interpret for complex tumour karyotypes. CGH examines genome-wide DNA copy number gains and losses rather than looking at focussed regions by FISH analysis. RDA is a

subtractive method, again detecting copy number differences between two genomes, comparing a normal reference sample and a tumour genome.
Loss of Heterozygosity (LOH) is a method which detects allelic loss at regions within a cancer genome utilising polymorphic microsatellite markers. Significant levels of allele imbalance may suggest the presence of a TSG(s) within the regions of LOH identified as one of the two 'hits' required for knockout of the gene.

Fluorescence in situ hybridisation (FISH) of metaphase ovarian cancer cells has shown frequent chromosomal abnormalities at one or more loci. Jenkins found 80% of ovarian tumour cells had genomic abnormalities, with common gain of chromosomes +1, +2, +3, +6, +7, +9, +12 and loss of –X, -4, -8, -11, -13, -15, -17 (Jenkins *et al.*, 1993). Structural abnormalities involved regions 1p36, 1q32, 1q42, 3p13-p26, 3q26-q29, 7p22, 9q34, 11p13-p15, 17q21-q23, 19p13.3 and 19q13.3. Other, but more specific, chromosome hybridisations have shown loss of chromosomes 17 and X and gains of chromosomes 12 and 8 (Persons *et al.*, 1993).

Comparative genome hybridisation (CGH) compares the whole genome structure of one cell type to another. Multiple CGH experiments have identified a number of regions frequently associated with ovarian cancers. Chromosome regions 3q25-26 and 20q13 were associated with low-grade tumours where as 8q24 gains are frequent in high-grade tumours (Iwabuchi *et al.*, 1995). An analysis of 24 non-aggressive low-grade tumours showed common gains at chromosomes +1q, +2p, +7q, +8q, +17q and losses at –8p, -9p, and -13q (Tapper *et al.*, 1997). Two recent CGH studies implicated gain at +8q, and +20q and loss at –13q, -18q and -4 with ovarian tumour progression (Kiechle *et al.*, 2001; Watanabe *et al.*, 2001). Kiechle also reported gains at +19p, +3q and +1q and correlated high-grade tumours with loss at -11p and -13q and gains at +8p and +7p. Low-grade tumours were associated with loss at -12p and gains at +18p (Kiechle *et al.*, 2001). Watanabe also reported loss at 11p14-15 and 9p and gains at 5p15, 7q32-36 and 20p (Watanabe *et al.*, 2001). Cisplatin resistance has been associated with, via CGH, gains of chromosome regions +2q14.1-q33, +4p15.2-p13, +4q22-q25, +4q31.1-q43, +6q13-q16 and +8q12-q21.1 and loss of regions –Xp22.2-q21, -7p21-p14 and –11cen-p14 (Wasenius *et al.*, 1997).

RDA is a little used method that identifies specific regions of loss on a chromosome associated with a certain phenotype. Homozygous deletions in ovarian cancer have been identified by the method at 9p21, 16q23.2 and at 22q13 (Lin *et al.*, 2000; Paige *et al.*, 2000; Watson *et al.*, 1999). The last has been dismissed as a potential TSG harbouring region whereas recently a candidate has successfully been identified at 16q23.2 (Paige *et al.*, 2001).

LOH or 'allelic imbalance' analysis is used to hunt for regions of heterozygous loss. According to Knudson's two hit hypothesis LOH would be classed as one 'hit' so one further hit is needed at that locus for gene inactivation. Potential TSGs may therefore lie in these regions. Regions of LOH in ovarian cancer have been commonly identified at 5q21, 11p15, 13q, 17p, and 17q. Launonen associated loss at 11p15.1, 11q23.3 and 16q24.2 with late stage disease and loss at 17p13.1 with early disease (Launonen *et al.*, 2000). Weitzel and Saretzki both showed LOH at 11p15 (50%), 5q21 (30.4-50%) and at 9p (53%) (Saretzki *et al.*, 1997; Weitzel *et al.*, 1994). The CGH and LOH data is summarised in Table 1.3, with loses and gains highlighted on each chromosome arm. Those studies chosen were mainly genome wide assays of chromosome amplification and loss. There are many papers that investigate LOH on individual chromosomes but these are excluded from this figure. LOH analyses of specific chromosome arms are common and have been used to identify many candidate ovarian cancer TSGs including BRCA1 and p53. An overview of these analyses is seen in Table 1.4.

These above genomic techniques have successfully pinpointed regions of potential tumour suppressor genes and oncogenes. For example, the common regions of loss on chromosome 17p and q correlate with p53 and BRCA1 respectively. Amplification at 1q may correlate to the oncogene SK at 1q22-24 and at 8q24.12-q24.13, another common region of amplification, lies c-myc.

There are however regions which have been implicated in ovarian cancer but no corresponding genes have been identified. For example, 3q is commonly over represented in advanced tumours but as yet no confirmed oncogenes have been

identified in this region. Loss at 11p15 is also often seen in ovarian cancers by LOH and CGH analyses but no confirmed tumour associated suppressor genes have been found to date.

	Author	1p	1q	2p	2q	3p	3q	4p	4q	5p	5q	6p	6q	7p
CGH	Hauptmann *et al.*, 2002						gain		loss			gain	loss	gain
	Tapper *et al.*, 1997		q22-q32	p15-p22			qcen-q23					p21-22		
	Arnold *et al.*, 1996	p35-pter	gain		gain		q26-qter	loss				p21-22		
	Sonoda *et al.*, 1997		q32				q26-qter		q26-q31		q21			
	Kiechle *et al.*, 2001		gain				gain		loss					gain
	Watanabe *et al.*, 2001							p11-p14		p15				
LOH	Gallion *et al.*, 1992												*	
	Cliby *et al.*, 1993 (all)										loss	loss	loss	loss
	Dodson *et al.*, 1993 (all)											loss		
	Weitzel *et al.*, 1994	*	*							*	*q21	*	*	
	Saretzki *et al.*, 1997				*q21-22	* loss					*q21	*	*	
	Launonen *et al.*, 2000					* p14.2							*	

	Author	7q	8p	8q	9p	9q	11p	11q	12p	12q	13p	13q	14q	15q
CGH	Hauptmann *et al.*, 2002	gain		gain						loss		loss		
	Tapper *et al.*, 1997	gain	loss	gain	loss							loss		
	Arnold *et al.*, 1996			q23-qter					p12			q21		
	Sonoda *et al.*, 1997	q36		q24	p21-pter	loss			gain					
	Kiechle *et al.*, 2001			gain			loss					loss		
	Watanabe *et al.*, 200)	q32-q36		q23-24	loss		p14-p15		p11-12			q22-24		
LOH	Gallion *et al.*, 1992						* loss					*loss		
	Cliby *et al.*, 1993 (all)		loss			loss						loss	loss	loss
	Dodson *et al.*, 1993 (all)											loss		loss
	Weitzel *et al.*, 1994				* loss	*	* p15	* q13			*	*		
	Saretzki *et al.*, 1997			*	p21			*						
	Launonen *et al.*, 2000		* p12				* p15.5	* q23.3						

	Author	16q	17p	17q	18p	18q	19p	19q	20p	20q	21q	22q	Xp	Xq
CGH	Hauptmann *et al.*, 2002	loss							gain					
	Tapper *et al.*, 1997			gain										
	Arnold *et al.*, 1996	q23-qter				q22-qter		gain		gain			loss	
	Sonoda *et al.*, 1997	loss	loss	q25				q13	gain	q13-qter		loss		
	Kiechle *et al.*, 2001					loss	gain			gain				
	Watanabe *et al.*, 2001			q21-23		q22-q23			gain	q12-13				
LOH	Gallion *et al.*, 1992	*	* loss											
	Cliby *et al.*, 1993 (all)		loss	loss		loss					loss	loss		
	Dodson *et al.*, 1993 (all)		loss	loss								loss		
	Weitzel *et al.*, 1994						* p11	*q22-24						
	Saretzki *et al.*, 1997		*	* q21		*						*		
	Launonen *et al.*, 2000	* q24.3	*p13.1											

Table 1.3. Summary of chromosome losses and gains in ovarian cancers as identified by genome-wide studies using CGH and LOH. Amplifications are highlighted in red and losses in blue. First authors of papers shown. LOH studies are genome-wide unless * shown which represent those chromosome arms assayed. LOH ≥35%.

Chr arm	Region	Notes	Authors
2q	2q21-q22	-	Saretzki *et al.*, 1997
3p	3p25-p26, 3p24-p25, 3p14.2, 3p12-p13, 3p22-p25, 3p21.3, 3p25	Advanced stage Familial associated gene? RASSF1A Me in 40% tumours Associated with Chr 17 LOH	Lounis *et al.*, 1998, Fullwood *et al.*, 1999, Agathanggelou *et al.*, 2001, Sekine *et al.*, 2001, Yoon *et al.*, 2001, Zhang and Xu, 2002, Manderson *et al.*, 2002.
5q	5q13-q21	APC 5q21-q22?, Associated with p53 mutation, Early stage.	Allan *et al.*, 1994, Tavassoli *et al.*, 1996, Wang *et al.*, 2001b.
6q	6q22.3-pter, 6q25.1-25.2, 6q24-q27, 6q27, 6q21-q23, 6q25.1-q27	Poor grade ESR candidate	Foulkes *et al.*, 1993b, Cheng *et al.*, 1996, Colitti *et al.*, 1998, Otis *et al.*, 2000, Suzuki *et al.*, 2000, Wang *et al.*, 2001b.
7p	7p	Early stage	Watson *et al.*, 1998
7q	7q31.1 7q21-31	Advanced stage, fragile site FRA7G, TES candidate Associated with Chr 13 LOH	Zenklusen *et al.*, 1995, Kerr *et al.*, 1996, Koike *et al.*, 1997, Edelson *et al.*, 1997, Huang *et al.*, 1999, Neville *et al.*, 2001, Tobias *et al.*, 2001.
8p	8p12-p21, 8p21.1, 8p22-p23.1, 8p23.1	Advanced stage, associated with Chr 9p LOH, serous histology, poor grade, FEZ1, GATA4 candidates.	Wright *et al.*, 1998, Ishii *et al.*, 1999, Brown *et al.*, 1999, Lassus *et al.*, 2001a, Pribill *et al.*, 2001.
9p	9p21-p22	CDKN2A candidate, serous histology, early stage	Rodabaugh *et al.*, 1995, Campbell *et al.*, 1995, Watson *et al.*, 1998,Niederacher *et al.*, 1999.
11p	11p15.5-15.3, 11p15.1, 11p13	Adverse survival, poor grade, advanced stage, nonmucinous histology.	Eccles *et al.*, 1992a, Viel *et al.*, 1992, Kiechle-Schwarz *et al.*, 1994, Lu *et al.*, 1997.
11q	11q22-23, 11q23.2-q24.3	Adverse survival, early stage, serous histology, Loss of PR protein, ATM candidate	Foulkes *et al.*, 1993b, Watson *et al.*, 1998, Gabra *et al.*, 1995, Koike *et al.*, 1999.
13q	13q12, 13q14.1, 13q33-q34	Early event, Chr 17q LOH, BRCA2, MCJ, RB candidates	Yang-Feng *et al.*, 199), Yang-Feng *et al.*, 1993, Liu *et al.*, 1994, Foster *et al.*, 1996, Shridhar *et al.*, 2001a, Gras *et al.*, 2001.
16q	16q23.1-q23.2, 16q23.3-q24.1, 16q24.2, 24.3	Poor grade, CDH13 candidate, advanced stage	Iwabuchi *et al.*, 1995, Kawakami *et al.*, 1999.
17p	17p13.1, 17p13.3	Serous histology, advanced stage, poor grade, aberrant p53 expression, p53, OVCA1/OVCA2 candidates	Eccles *et al.*, 1992b, Gallion *et al.*, 1992, Foulkes *et al.*, 1993a, Yang-Feng *et al.*, 1993, Godwin *et al.*, 1994, Wertheim *et al.*, 1996, Otis *et al.*, 2000, Manderson *et al.*, 2002.
17q	17q23-qter, 17q12-q21, 17q22-q25	Poor grade, advanced stage, serous histology, BRCA1, septin candidates	Eccles *et al.*, 1992b, Foulkes *et al.*, 1993a, Tavassoli *et al.*, 1993, Nagai *et al.*, 1994, Godwin *et al.*, 1994, Chen *et al.*, 1995, Wertheim *et al.*, 1996, Otis *et al.*, 2000, Dion *et al.*, 2000, Russell *et al.*, 2000.
18q	18q21, 18q22-q23	Serous histology, advanced stage, SMAD4/SMAD2, DCC candidates	Zborovskaya *et al.*, 1999, Lassus *et al.*, 2001b.
19p	19p13.3	STK11 candidate	Wang *et al.*, 1999b.
19q	19q13.2-13.4	ERCC1/ERCC2 candidates	Bicher *et al.*, 1997.
22q	22q12	-	Englefield *et al.*, 1994, Bryan *et al.*, 2000.
Xp	Xp21.1-p11.4	-	Yang-Feng *et al.*, 1992.
Xq	Xq25-26.1, Xq11.2-q12, Xp22.2-22.3	Advanced stage, poor grade, Chr 17p LOH, Androgen receptor candidate, BRCA1 associated.	Choi *et al.*, 1997, Edelson *et al.*, 1998, Buekers *et al.*, 2000.

Table 1.4 Individual analyses of LOH in ovarian cancers. These papers analyse only regions or just a few chromosome arms. Regions of loss, histopathological correlations and candidate TSGs within those regions are shown.

1.7 Chromosome 11p And Ovarian Cancer

Numerous LOH and cytogenetic studies have implicated Chr 11 in ovarian cancer. The earliest cytogenetic study showing Chr 11 loss in ovarian neoplasia was published in 1989 and found structural loss of 11p13-11pter in 55% of high grade serous carcinomas (Pejovic *et al.*, 1989). Pejovic later reported frequent loss of genetic material at 11p13-p15 (Pejovic *et al.*, 1992).

The H-RAS locus on 11p15.5 has been analysed in multiple LOH studies. Viel reported LOH at H-RAS in 4 of 7 cases of ovarian cancer but mutation analysis of the gene suggested that H-RAS is not a TSG candidate (Viel *et al.*, 1991). Lee also detected LOH at this locus in 46% of ovarian cancers (Lee *et al.*, 1990). Another study of H-RAS showed LOH in 53% of invasive tumours and associated loss with an advanced stage in progression (Gallion *et al.*, 1992). It was thought, from these studies, that a TSG might lie nearby this gene marker.

Further LOH mapping suggested the presence of two candidate TSG loci at 11p15.5 and 11p13 (Viel *et al.*, 1992). LOH analyses of chromosome 11p by Lu et al narrowed down the regions of LOH to an 11cM region spanning from D11S2071 to D11S988 at 11p15.5 and a 4cM region surrounding the marker D11S1310 at 11p15.1 (Lu *et al.*, 1997). These regions correlated with LOH rates of 43% and 32% respectively. They also reported that 52% of tumours had allelic deletion in both regions and that these losses are associated with high-grade non-mucinous epithelial ovarian carcinoma (Lu *et al.*, 1997).

Using 4 polymorphic microsatellite markers, Kiechle-Schwarz detected 48% LOH along 11p15.5-11p15.1 (Kiechle-Schwarz *et al.*, 1994). A significant association was seen between loss at 11p15 and late stage and high grade tumours. A correlation was seen between the rate of LOH and grade of differentiation, further indicating that 11p15 loss plays an important role in ovarian cancer development.

An early study using RFLP analysis of markers on 11p showed 33% LOH at 11p15.4 and LOH of only 18% at the H-RAS locus at 11p15.5 (Eccles *et al.*, 1992a). Loss at one or both of these loci was associated with significantly poorer survival of patients. A recent clinicopathological study associated LOH at 11p15.5 (D11S1318) with high tumour stage, metastasis, residual tumour after surgery and reduced patient survival. This again supported a role for loss in this region at a late point in disease

progression (Launonen *et al.*, 2000). LOH at 11p15.5 also correlated with loss at 6q27 (Launonen *et al.*, 2000). Zborovskaya however reported loss at D11S922 (11p15.5), adjacent to D11S1318, in benign and borderline malignancies (Zborovskaya *et al.*, 1999). The majority of studies however support the association of 11p allelic deletion with high grade aggressive tumours.

Chromosome 11p15 is a very gene dense region containing >100 genes. Despite the extensive LOH and CGH mapping that has been performed, no candidates within this region have been identified in ovarian cancers. The region has however been minimised substantially by the efforts. Prior to this work being performed, the minimal candidate TSG-containing regions were an 11cM region spanning D11S2071-D11S988 and a 4cM region centred on the marker D11S1310, bordered by D11S926 and D11S899, from the LOH analysis by Lu et al (Lu *et al*, 1997).

LOH mapping in other cancers has been more successful in minimising candidate regions. For example in Lung cancer, two regions, labelled LOH11A and LOH11B, through fine mapping of the region have been minimised to D11S1758-D11S12 and H-RAS-D11S1363 respectively (O'Briant and Bepler, 1997). These regions span approximately 2Kb and 1Kb. In breast cancers, LOH is again detected in two regions, between D11S1318- D11S4088 and D11S1338-D11S1323 (Karnik *et al.*, 1998a). These regions overlap with others detected in Wilm's tumour, lung cancer and rhabdomyosarcoma. Although this mapping is substantial only one candidate TSG has been investigated. The gene is SRBC and lies in breast cancer region 2 (Xu *et al.*, 2001). SRBC transcription is affected by frameshift and truncating mutations in a few lung and ovarian cancer cell-lines. Methylation of the promoter CpG island was also detected in breast and lung cancers and SRBC was re-expressed upon treatment with demethylating agents 5'azacytidine and Trichostatin A (Xu *et al.*, 2001).

Further mapping in ovarian cancers is required to minimise interesting regions of high LOH along 11p15 in the eventual aim of identifying EOC-associated TSGs.

1.8 Microcell Mediated Chromosome Transfer

As discussed previously, cytogenetic and LOH studies have implicated loss of normal Chr 11 in tumour suppression. Evidence for regional loss has been reported

in numerous tumour types including EOC. This data however does not prove that the chromosome disruptions are functionally important in cancer. Functional evidence can be obtained by introducing an intact copy of a TSG into a cancer cell that lacks a fully functional copy. A method was developed by which single specific chromosomes were transferred into cancer cells to generate clones with suppressed tumour phenotypes, so suggesting that a functional TSG(s) lies on that chromosome (Harris *et al.*, 1969). This method is called microcell-mediated chromosome transfer (MMCT). It is used to transfer selectable-tagged chromosomes into cancer cells that have previously been shown to have deletions at a particular locus based upon cytogenetic studies or by LOH. The first study to use this technique transferred an HPRT- tagged Chr 11 from mouse A9 x t(X;11) hybrid cells into tumorigenic HeLa cells (Saxon *et al.*, 1986). They reported complete suppression of HeLa cell tumorigenicity caused by the transfer.

The method originates from a technique used by Stanbridge in which he cell-fused HeLa cells and normal diploid fibroblasts (Stanbridge *et al.*, 1981). These fused hybrids were found to be completely suppressed for the tumorigenic phenotype. Only after prolonged passage did rare tumorigenic segregants arise. Karyotype analysis of the segregants showed a correlation between loss of a single copy of chromosomes 11 and 14 and their reversion back to the tumorigenic phenotype. A later study by Klinger confirmed this result and Kaelbling reported that it was the normal fibroblast Chr 11 that was being lost in the tumorigenic segregants, rather than the HeLa derived copy (Kaelbling and Klinger, 1986; Klinger, 1982).

Since these early studies, normal chromosome 11 has been reported to suppress tumorigenicity when transferred into numerous carcinoma cell-lines including Wilm's tumour, cervical cancer, rhabdomyosarcoma, uterine endometrial cancer, breast cancer, lung adenocarcinoma and ovarian carcinoma (Cao *et al.*, 2001; Negrini *et al.*, 1992; O'Briant *et al.*, 1997; Oshimura *et al.*, 1990; Weissman *et al.*, 1987; Yamada *et al.*, 1990).

Microcell-mediated chromosome transfer itself is a powerful tool used to locate senescence, metastasis and tumour suppressors, in the mapping of disease genes such as Fanconi anaemia group D and Leigh syndrome and in the physical mapping of chromosomes. It has been useful in both locating and identifying genes as well as

narrowing interesting regions by the transfer of chromosome sub-fragments. Examples of genes identified by MMCT are KAI1, a metastasis suppressor in prostate cancer at 11p11.2-13, Ribosomal Protein L14, a candidate TSG at 3p21.3 in lung carcinomas and PTEN at 10q23 in gliomas (Dong *et al.*, 1995; Satoh *et al.*, 1993; Shriver *et al.*, 1998) (Steck *et al.*, 1999). Potential ovarian cancer related TSG containing regions which have been narrowed by the method are a 2 cM locus at D6S1637 to D6S1564, and a region at chromosome 22q11-q12 lost in SKOV3 cells (Wan *et al.*, 1999; Kruzelock *et al.*, 2000). In prostate cancer, MMCT has minimised a 1.2 Mb region spanning D10S1172- D10S226 (Fukuhara *et al.*, 2001).

The success of MMCT makes it a good method to hunt for potential TSGs in ovarian cancer. Chromosomes 3, 6, 10, 11,12, 14, 17 and 22 have successfully been transferred into the ovarian cancer cell-lines SKOV3 and/or HEY (Cao *et al.*, 2001; Kruzelock *et al.*, 2000; Rimessi *et al.*, 1994; Sandhu *et al.*, 1996; Wan *et al.*, 1999).

Rimessi introduced, using MMCT, normal chromosomes 3, 6, 11 and 17 into HEY ovarian cancer cells (Rimessi *et al.*, 1994). Chr 3, when transferred, caused gradual growth arrest and decreased tumorigenicity of cells in nude mice. Chr 11 produced no morphological differences although in vitro growth rate and clonogenic activity in agar was reduced. They noted that tumorigenicity of clones was varied and concluded that chromosome 11 was not involved in the phenotype. HEY cells, however, only have loss at 11q21 so the addition of chromosome 11 into the cells may only complement this region. Transfer of chromosomes 6 and 17 are not discussed in the paper.

Sandhu reported that transfer of chromosome 6 or 6q into human and rat ovarian tumour cells restored senescence but no similar effect was seen with transfer of either chromosome 10 or 14 (Sandhu *et al.*, 1996). Revertant rat microcell clones revealed a deletion at 6q14-21 suggesting this is the locus of a senescence gene, confirmed by transfer of the 6q13-21 region into human and rat cells. They named the senescence-related gene, which lies within this region, SEN 6A. A second transfer of chromosome 6 but into ovarian lines HEY and SKOV3 by Wan abolished tumorigenicity (Wan *et al.*, 1999). Revertants were subsequently isolated from the suppressed clones and microsatellite mapped to determine extent of chromosome 6 in the cells. They identified a 2 cM region potentially harbouring a TSG at 6q26-27.

As a control, a fragment of distal chromosome 11q was also transferred but clones were not suppressed in growth or tumorigenicity.

Kruzelock reported the transfer of chromosome 22 by MMCT again into SKOV3 (Kruzelock *et al.*, 2000). Complete suppression of tumorigenicity and cell growth was seen in 89% of clones. Again, the region was successfully minimised to 22q11-q12, characterised by the marker D22S429.

Chromosomes 11 and 17 are the most recently reported to be transferred into the ovarian cancer SKOV3 cell-line (Cao *et al.*, 2001). Chr 17 hybrids showed an increased latency period when injected into nude mice. Tumour volume was also significantly decreased but there was little change in in vitro doubling times, so, tumorigenicity was not completely suppressed in these hybrids. Introduction of Chr 11 into SKOV3 produced 5 independent clones, all of which contained informative microsatellite markers, so transfer of the chromosome. FISH analysis revealed that all hybrids had gained one copy of chromosome 11. Four of the five had prolonged in vivo tumour latency of >100 days as compared to normal SKOV3 latency of just 7 days. Tumour volume on average was about half of that of SKOV3 parental cell-line and in vitro doubling times were longer than normal meaning the cells were growing more slowly. The contradiction of these results of chromosome 11 transfer compared to transfer into HEY cells has been explained by the difference in subtype of ovarian cancers. Cao noticed that one of the five hybrids, even though it contained all 4 microsatellite markers, grew like the parental SKOV3 line. This may be due to incomplete transfer of chromosome 11 and the clone having lost the TSG at a region out with the microsatellite markers. Markers D11S1984, D11S1999, WT1 and D11S2000 were used located at 11p15.5, 11p15.3, 11p13 and 11q22 respectively. Further mapping would be required to narrow the region of a potential TSG.

None of the studies that transferred chromosome 11 in an ovarian cancer cell-line have successfully minimised the area of interest by microsatellite mapping. Further revertant studies or repeat of the chromosome 11 transfer is needed in order to refine the TSG containing region. It should be noted however, that with the advent of the Human Genome sequencing project, MMCT is becoming redundant in its use due to the huge gene positioning effort that has occurred.

1.9 Differential gene expression analysis

1.9.1 Gene expression analysis - techniques

Cancer genetics over the past 60 years has focussed on the identification and analysis of genes that are differentially expressed between normal and cancerous phenotypes. It is thought that there are 20,000 genes expressed at once in a typical cell and 1% of those may be involved in cancer. These cancer genes are associated with apoptosis, angiogenesis, cell cycle control, signalling, adhesion, migration, and DNA repair among other processes (Hanahan and Weinberg, 2000). It is the study of such genes that will give us an insight into the progression of cancers. Identified candidate genes may translate to a clinical setting for use in prevention, detection, diagnosis or therapy. Currently, however, only a small subset of genes has been identified as playing a role in ovarian cancer.

Techniques applied to ovarian cancer-related gene discovery in recent years have included differential display RT-PCR (DD-RT-PCR), cDNA representational difference analysis (cDNA-RDA), and serial analysis of gene expression (SAGE). All have been successful in identifying cancer-implicated genes. Even more recently, microarray analyses have been applied to the problem; hunting for genes that are differentially expressed between either differing histological subtypes of ovarian cancer or between normal ovarian surface epithelial cells and pooled tumour sources.

Differential display RT-PCR dates back to 1992 when first described by Welsh and then further developed by Liang and Pardee (Liang *et al.*, 1993; Welsh *et al.*, 1992). The method is based upon arbitrarily primed PCR fingerprinting and detects differences in gene expression between two RNA populations. The general strategy is to compare patterns of radiolabelled cDNA sequences that are amplified by PCR and separated on a sequencing gel. The use of several different primer sets allows analysis of a large number of genes. Each mRNA is represented as a single band, which may or may not be altered in expression between the sample populations, and differentially expressed bands are excised, cloned and sequenced for identification. Differential display is a simple technique that can be performed in a standard molecular biology lab, requiring minimal bioinformatics ability. DD-RT-PCR can identify known and novel genes. The disadvantages are that the method is limited in

sensitivity so that not all differences are detected by using a single primer. The main drawback of DD-RT-PCR however is the high level of false positives. 50% false positives have previously been reported (Martin *et al.*, 2000).

Hubank and Schatz adapted cDNA-RDA from genomic RDA, a method used to identify regions of deletion between two genomes (Hubank and Schatz, 1994; Lisitsyn and Wigler, 1993). Unlike DD-RT-PCR, cDNA-RDA couples PCR amplification with subtractive hybridisation so in theory the method should identify only actual differences between the two populations. The cDNA populations are restriction enzyme digested to generate representations, containing fragments ranging from 50bp to 1Kb, called the tester and driver. cDNA-RDA requires less than 5% of the mRNA amount required for DD-RT-PCR, so is suitable for analyses with limited samples. The representations are ligated to adapter sequences and PCR amplified with primers complementary to these. The driver has linkers removed and then is mixed in excess with the tester population. Kinetic enrichment amplifies only those tester fragments that do not have a counterpart in the driver pool. The RDA procedure is usually reiterated twice or more to produce sequential, increasingly stringent 'difference products' and the cDNA fragments within these are identified by sub-cloning and sequencing. The method requires validation of products to establish the actual expression difference. The identification of differential genes relies on the relative difference in expression levels between sample populations. Those genes with a greater expression difference appear to be amplified preferentially so are more abundant within the difference products. Although sensitive, cDNA-RDA is technically demanding and, as for DD-RT-PCR, is prone to a high rate of false positives (Hubank and Schatz, 1999)

SAGE is a more recent difference analysis method (Velculescu *et al.*, 1995) and based upon sequence analysis of short regions, close to the 3' end, of every cDNA in a sample. Sequence tags of 10-14 bp from the 3' most *NlaIII* restriction sites of genes are ligated together, separated by a distinctive linker sequence. These concatamers are sequenced and results compiled to form a distribution showing the frequency of various gene- associated tags. The frequency of tags is related directly to the

expression of the gene associated with the tag. This method requires specialised computer software to count the abundance of thousands of tags simultaneously. SAGE data is then compared between different cDNA samples, with a gene with differential expression represented by more tags in one population as compared to the other. The downside to the method is the need for detailed computer analysis and also the extensive sequencing strategies required for a high level of sensitivity.

In comparison to the above open systems, that is methods that are capable of identifying all- including novel- differentially expressed genes, cDNA array technology assays a set of fixed known genes for expression. cDNA arrays are formed by amplification of 0.6-2.4 Kb DNA segments by PCR then mechanical spotting of these at a high density onto a glass or membrane 'chip'. The DNA is chemically fixed to the surface and heat denatured prior to hybridisation. Chips are then simultaneously probed with fluorescent-tagged cDNA representations of total RNA from tester and reference samples. Cy3 and Cy5 dUTP tags are commonly used. A confocal laser scanner measures fluorescence to determine intensity of hybridised sequences.

cDNA arrays are sensitive detecting a single transcript in 100,000 or less of total RNA (Lockhart and Winzeler, 2000). They are also practical for application on a large scale. The major drawbacks to the method are cost and the technical expertise required to carry out the experiment and the subsequent data analysis. cDNA arrays also need a large amount of RNA (50-200μg) although this may be decreased if RNA amplification is used. They do not have such a high level of false positives as the previous methods but a single hybridisation may still have a misclassification rate of up to 9% (Lee *et al.*, 2000). The reliability and accuracy of results increase with repeats of analysis with 3 replicates giving a final error rate of just 0.7%. It has also been recommended that validation of products are done in using RT-PCR or Northern blotting to confirm the findings of a microarray. This is important when single genes are being analysed as candidates.

cDNA arrays, even though they have limitations, are one of the most widely used methods for profiling gene expression, often with the an aim of identifying gene signatures of certain cancer types.

1.9.2 DD-RT-PCR and epithelial ovarian cancer.

DD-RT-PCR was the first published method to be used in the gene expression analysis of ovarian tumours in comparison the their normal counterpart.

Mok et al successfully used DD-RT-PCR to isolate genes differentially expressed between normal OSE cells and ovarian tumours (Mok *et al.*, 1994). Two cDNA fragments were found to be absent in all of the ovarian cancer cell lines used in DD-RT-PCR. These were named DOC-1 and DOC-2. They also identified a fragment, LF4.0, which is over expressed in most tumour cell lines in comparison to normal OSE. Results were confirmed by Northern blotting (Mok *et al.*, 1994). DOC-2 has been further characterised as a candidate TSG in multiple tumour types through functional studies (Fulop *et al.*, 1998; Tseng *et al.*, 1999; Wang *et al.*, 2001a).

A later DD-RT-PCR study, in 1999, identified NOEY2/ARH1 as a RAS and rap homologue expressed only in normal breast and ovary epithelial cells (Yu *et al.*, 1999). The gene was confirmed as differentially expressed by Northern blotting and identified as being maternally imprinted. This gene has subsequently been characterised as aberrantly methylated in breast cancer cells (Luo *et al.*, 2001).

Candidate oncogene Op18/stalthmin was identified by DD-RT-PCR and shown to be over expressed in 12 malignant ovarian cancers (Price *et al.*, 2000). Protein over expression was also determined using IHC. This cell cycle protein had been previously identified by 2D-gel electrophoresis as over expressed in ovarian tumours (Alaiya *et al.*, 1997).

DD-RT-PCR has been used in a number of studies to identify chemotherapy resistance related genes in ovarian cancers (Duan *et al.*, 1999; Shridhar *et al.*, 2001a; Yamamoto *et al.*, 2001a; Yamamoto *et al.*, 2001b).

The comparison of cisplatin (CDDP) sensitive and CDDP resistant cell lines 2008 and 2008/C13*5.25 discovered heat shock proteins HSP75, HSP27 and HSP70 were associated with the resistant phenotype (Yamamoto *et al.*, 2001a). A novel gene CRR9 (CDDP resistance related gene 9) was also identified by DD-RT-PCR highlighting its ability to discover uncharacterised transcripts (Yamamoto *et al.*, 2001b). Another novel gene TRAG-3 was seen to be associated with taxol resistance (Duan *et al.*, 1999).

Shridhar reported the association of a DNAJ protein family member, MCJ, with chemotherapy resistance (Shridhar *et al.*, 2001a). Expression of MCJ (Methylation containing J Protein) was absent or reduced in a majority of primary and ovarian cancer cell lines. MCJ nonexpressing cell lines showed resistance to paclitaxel, topotecan and CDDP.

1.9.3 cDNA-RDA and ovarian cancer.

cDNA-RDA has in the literature been described only once for ovarian cancer. Its use with other tumour types has proven it as a successful method of gene identification (Chang *et al.*, 1998; Gress *et al.*, 1997; Lang and Schuller, 2001; Wallrapp *et al.*, 1999).

Ismail described the comparison of gene expression profiles of a HOSE sample and two pooled tumours to identify candidate cancer genes using cDNA-RDA (Ismail *et al.*, 2000). Instead of using the method of cDNA-RDA alone, they coupled it with array analysis for identification of genes that are aberrantly expressed in a large number of ovarian tumours.

After 2 rounds of cDNA-RDA subtractive hybridisation, 255 differentially expressed genes were identified, 160 upregulated and 95 down regulated with respect to HOSE cell expression. These candidates were then spotted onto filters to create the cDNA array. Pooled cDNA representations from 5 HOSE and 10 serous ovarian tumours respectively were hybridised onto the filters. Sixty genes were subsequently identified as having >2.5-fold difference in expression. The majority of genes identified by cDNA–RDA were be differentially expressed by <2.5- fold on the array suggesting a high false positive rate for the cDNA-RDA. They found that variability in gene expression within tumour and HOSE samples was leading to elimination of previously published cancer associated genes such as DOC-1. The array analysis was thought to have been effective in identifying genes that display a constant pattern of expression differences in a large number of tumour samples. The ability of arrays to comparatively analyse multiple samples is one advantage of not using simply cDNA-RDA alone. The coupling of these two techniques has been further supported in Ewing's sarcoma (Welford *et al.*, 1998).

A method called Suppression Subtraction Hybridisation (SSH), which is similar to cDNA-RDA has also been used on ovarian cancer samples (Shridhar *et al.*, 2002). SSH selectively amplifies target cDNA fragments whilst simultaneously suppressing non-target DNA amplification in a population, generating a library of differentially expressed sequences. Unlike cDNA-RDA however, it does not need several rounds of subtraction. Shridhar generated down regulated cDNA libraries from two early and two late stage ovarian cancer cDNA libraries subtracted against pool of 20 normal OSE cell brushings. Forty-five genes were identified as decreased in expression in all four tumours. These included ovarian cancer associated genes such as NOEY2, IGFBP5, Caveolin 1 and SPARC. These genes were then chromosomally organised and many were found to map to regions of known deletions and LOH in ovarian cancers, suggesting that the method has successfully identified ovarian cancer related genes which may act as TSGs.

1.9.4 SAGE and ovarian cancer

As for cDNA-RDA, SAGE has been used in the analysis of multiple tumour types and has successfully identified cancer-associated genes.

Recently SAGE databases such as that funded by Cancer Genome Anatomy Project (CGAP) have been established for public use (www.ncbi.nlm.nih.gov/SAGE/). These databases contain more than 3 million tags from 88 different cancer associated libraries.

SAGE studies have been performed on patient material from colon, lung, ovarian and breast cancers to name but a few (Polyak and Riggins, 2001).

Hough carried out the most substantial work, using SAGE, in ovarian cancer (Hough *et al.*, 2000). They compared normal tissues in the form of OSE cultures, immortalised OSE and cystadenoma cells with malignant tissues composed of pooled cell lines and individual primary serous ovarian cancers using SAGE. A huge sequencing effort identified >56,000 individual genes in 10 different libraries from ovarian tissues, 3 normal and 7 cancer derived. They found 45 genes over expressed >10-fold in all three primary ovarian tumours analysed compared to OSE cells, and 9 downregulated >10-fold. Up regulated genes included secreted proteins, tight junction proteins and other cell surface proteins. Those genes down regulated in

normal OSE generally play roles in proliferation and cellular architecture and adhesion. Over expressed genes Ep-CAM, Apo J, claudin-3 and –4 were all validated at the protein level by IHC (Hough *et al.*, 2000).

A subsequent study of 13 ovarian cancer associated genes, many identified by the original SAGE analysis, was done using real-time quantitative PCR (Hough *et al.*, 2001). Expression of each was analysed in 39 microdissected ovarian tumours of varying histology, stage and grade. All genes analysed showed various levels of upregulation in the majority of the tumours. Induction levels were higher when validated than previously determined by SAGE, possibly due to removal of contaminating normal stroma by microdissection. They identified overexpression of three genes not previously implicated in ovarian cancer progression being S100A2, STAT1 and MGP. Confirmed ovarian cancer associated genes include Ep-CAM, Folate receptor, SLPI, Apo E, Apo J, Kop, ceruloplasmin, IGFBP2, TIMP3 and GPX3. The paper shows the importance of validation of genes identified from difference analysis methods.

1.9.5 cDNA arrays and ovarian cancer

Microarrays, due to their ability to analyse a large number of genes simultaneously, are the most published method of gene expression analysis in ovarian cancers. There have been more than 10 papers published in this field ranging from the comparison of cell lines with normal tissues to the comparison of ovarian cancers with other tumour types.

Ono et al identified 55 up and 48 down regulated genes when 9 ovarian tumours were sampled (Ono *et al.*, 2000). Using a glass 9121 cDNA array, they compared 9 serous/mucinous ovarian tumours with their corresponding normal ovary samples. Using cut-off values they identified 55 genes up in 6 or more tumours and 48 genes down in 8 or more tumours. Examples of up regulated genes include Protein disulphide isomerase related protein, RBP1, HE4, RpnAO, EIF4δ, 14-3-3σ, calreticulin and keratin-17, and –18. Genes down regulated in more than 8 tumours include Golgi SNARE, TGFßIIRα, KIAA0851, calcineurin A2, calmodulin, CLIM1, MOV34 and DAP5. They also compared microarray profiles of 5 serous to 4 mucinous tumours and identified 115 genes as differentially expressed. Protein

kinase PKX1, RPL8, complement B and a regulator of G protein signalling, RGS12, were all down regulated in mucinous compared to serous tumours. They found that creatine kinase B and myosin heavy polypeptide 11 were down regulated in serous tumours. These differences in gene expression between histologies suggest different pathways of progression.

RT-PCR was used to validate the differential expression of 8 genes standardised against G3PDH. KSA, claudin-10, complement factor B, RhoA, TSG101 and DOC1 were all confirmed as down regulated in tumours of both histologies. Two genes, haptoglobin and secretory protein P1.B, were both confirmed as up regulated by RT-PCR. In this study they also identified genes, such as HE4, 14-3-3σ, and DOC-1, which have been previously implicated in tumour progression. Both HE4 and DOC-1 were identified by other difference analysis methods as being over expressed in ovarian tumours (Hough *et al.*, 2000; Mok *et al.*, 1994).

Wang used a 5766 clone cDNA array to compare gene expression between RNA extracted from frozen ovarian tumour tissue blocks and normal whole ovary RNA (Wang *et al.*, 1999a). Arrays were hybridised independently with 7 different tumour specimens representing all histologies. They found that 30% of cDNAs had a >2-fold difference in expression and 9% altered >3-fold. In total 726 clones were identified to have 3-fold difference in expression between tumour and normal samples. These cDNAs consisted of 94 ribosomal protein-encoding genes, 149 mitochondrial gene transcripts and 248 other genes including 12 uncharacterised genes. All mitochondrial transcripts were over expressed and all but 1 (RPS6) ribosomal protein genes were down regulated in tumours. Other down regulated genes included FOS, vimentin, glutathione S transferase, JUN, protease nexin and TR3 orphan receptor. Upregulated candidate oncogenes included cofilin, α enolase, HepG2, CD9, mesothelin, HE4, MMP7 and cytokeratin 8. Again, many of these genes have been previously implicated in cancers. RT-PCR was used to validate the expression levels of 15 putative cancer markers on a panel of normal tissues and ovarian cancers. Overexpression of mesothelin, HE4 and MMP7 are all confirmed in this paper. Interestingly, HE4 is once again identified as over expressed in multiple tumours.

The comparison of ovarian carcinomas with benign adenomas on a 588-gene microarray again identified numerous up and down regulated genes (Tapper *et al.*, 2001). Using an Atlas Human Cancer cDNA array, Tapper *et al* compared expression between firstly carcinomas and benign tumours, then local highly differentiated tumours to benign tumours and, finally, advanced and moderate/poorly differentiated tumours with local, highly differentiated tumours.

Six serous adenocarcinomas, of varying grade and stage, were compared in expression to a benign serous cystadenoma. This identified 38 up and 19 down regulated genes in the tumour samples. Those up regulated include Rho family genes, MMP9, ERBB3, Cad6 and MDM2s. Down regulated genes included cell cycle regulators, such as cyclins D3 and G1 and CDK1, invasion regulators, MMP11 and TIMP2, and receptors PDGFA/B and EGFR.

When three local and highly differentiated adenocarcinomas were compared to a benign tumour, 49 genes were found to be differentially expressed. Up regulated genes include, of the 23, apoptosis regulators AKT2, caspases and TNFA, cell interaction genes, Cad6 and integrin ß8, and others including MMP7 and ERBB3. Downregulated genes included thrombospondin 1, collagens, TIMP2, MMP2, IGFBP4 and SKY.

Fifty-eight genes were found to be differentially expressed between generally poor prognosis advanced tumours and local highly differentiated tumours. Twenty-one genes were up and 12 down regulated in all advanced carcinomas. Those genes highly expressed in tumours included motility related genes; collagens, fibronectin and semaphorin, and oncogenes/tumour suppressor genes; Met, cFOS, RBQ1 and STAT1. Down regulated genes again included apoptosis related genes, DNA damage repair genes and various others. These genes may be involved in the progression of low grade tumours to a more aggressive poorly differentiated phenotype. Using semi-quantitative RT-PCR, upregulation of two genes, RHOGD12 and COL3A1 was confirmed in at least 4 tumours. Tapper *et al* have successfully identified genes which may play a role in the progression of cancers to the more advanced phenotype.

Welsh reported the comparison of gene expression in individual serous tumours and cell lines with 4 un-matched normal whole ovary samples (Welsh *et al.*, 2001). Their technique used an 'array of arrays' suitable for analysing multiple RNA samples in a

single experiment so removing experiment to experiment variability (Zarrinkar *et al.*, 2001). Affymetrix HuGeneFL arrays, carrying >6,000 human genes were hybridised individually with RNA samples. Results were analysed by hierarchical cluster analysis. A cluster of approx. 100 genes were highly expressed in normal tissue and under expressed in the majority of tumours. These included many immediately early genes such as cFOS, Jun B and EGR1 and others including IGFBP5, ZFP-36, Transcription factor ETR101, CL138 protein tyrosine phosphatase and 3 nuclear receptor family members. A second cluster showed genes that are underexpressed in normal tissue as compared to ovarian tumours and cell lines. This cluster included HE4, PRAME, COX8, SMARC A4 and glutathione s-transferase P1 (GSTP1). Candidate oncogenes HE4 and PRAME were highly expressed respectively in 51% and 55% of the tumours. Three other gene clusters are represented as proliferative, ribosomal and stromal. It is noted that generally ribosomal genes are over expressed except for in a group of poorly differentiated and rapidly growing tumours in which there was a relative under expression of these genes.

Thirty transcripts were designated as having high expression in 24 ovarian tumours and cell lines. These genes had low expression in normal tissue and high expression in neoplastic tissues so may be candidate molecular markers for ovarian cancer. These genes include CD24 and -9, Keratins –7, –8, -18 and -19, mucin1, HE4, ENO1, MEIS1, and GA733-2 (TACSTD1). Increased expression of the genes CD24, HE4 and LU was confirmed on a panel of four normal and 11 tumour samples by RT-PCR. Again many of these genes, such as CD24, CD9 and HE4, have been previously reported as over expressed in ovarian cancers using microarray profiling, supporting Welsh's' data.

Tonin reported the comparison of gene expression between ovarian cancer cell lines and a primary culture of normal ovarian surface epithelium (Tonin *et al.*, 2001). They used cell lines to avoid problems with tumour heterogeneity, stromal contamination and simply because they can generate sufficient RNA for the studies. Cell lines have, however, a drawback in that their gene expression compared to tumour gene expression may have altered through repeated passaging in culture so that the cell lines do not wholly represent true ovarian cancers. Four long-term cell

lines, derived from malignant tumours, and one solid tumour were compared in gene expression to normal OSE cells using a Hs6000 gene chip from Affymetrix. The chip contains probes for 6416 genes. A threefold difference was deemed significant and two way comparisons between samples were carried out to determine the number of 3- 5- and 10-fold differences between them. A six-way comparison of all cell-lines/tumour to normal OSE was also performed. They found that the majority of genes only varied in expression in 1 carcinoma sample (280), and only 1 gene varied in all 5 cell-lines/tumour. Furthermore, the expression profiles of >80% of genes did not differ greater than 3-fold in comparison to normal OSE. Differentially expressed genes were then assigned to respective chromosomes using Genemap'99. Chromosomes 1 and 19 appeared to harbour a lot of the genes that were over expressed.

Although the paper discusses genome-wide profile similarities between samples, only specific differentially expressed genes (other than those on Chr 6) are highlighted. This was due to their previous identification as over expressed in ovarian cancers. Genes discussed for example were Jun D, PAI 1, ILB1 which have all been confirmed as differentially expressed at the protein level by IHC. Genes previously identified as over expressed include CD24, HE4, CD9 and Mucin 1, Keratins –8, -9, -10, -18, and –19, MACMARKS and adenylosuccinate synthase.

Tonin was able to conclude that all cell lines are more similar to one another than to normal OSE.

Arnold, like Tonin, reported the comparison of ovarian cancer cell lines and human OSE cells (Arnold *et al.*, 2001). Using a Clontech Atlas array carrying 588 cDNA fragments they individually hybridised RNA from three ovarian cancer cell lines and 1 OSE sample. A visual comparison of arrays identified 17 genes expressed at a higher level and 10 and a lower level in OSE cells as compared to cell-line expression. Visual rather than computational analysis of data however, may have missed out vital low expressed candidate genes. OSE associated genes include Id3, TAF131, ICAM1, CD44, and neural cadherin. EAR-1 and –2, RAF, CLK, ETR103, ETR101, Integrin-α1 and -7ß were all highly expressed in the three ovarian cancer cell lines. Six potential tumour suppressor genes were validated using RT-PCR on a

panel of HOSE and ovarian cancer cell lines. Only three of these genes were however validated as having lost or decreased expression in multiple ovarian cancer cell lines, showing the importance of using further gene difference validation techniques. The gene Id3 was taken forward for further studies.

Interestingly, Tapper and Arnold used the same Clontech Atlas array but the genes identified were completely different in both directions of expression (Arnold *et al.*, 2001; Tapper *et al.*, 2001). Tapper compared tumour samples with benign cystadenoma where as Arnold compared cell lines with human OSE cells. The differences between these two papers show the variation between primary samples and those that have been cultured. The choice of 'normal' RNA may also be important. Most groups have chosen to use OSE cells because all frank ovarian carcinomas are believed have progressed from these cells. However, a few groups have used benign ovarian tumours but there is little evidence that these actually progress to epithelial ovarian cancer so they may not be suitable for use as 'normal' controls.

The MICROMAX™ cDNA microarray system was used by Wong in the comparison of three pooled human OSE samples with three pooled ovarian cancer cell lines with the aim of identifying tumour markers (Wong *et al.*, 2001). The array system contains 2400 known human cDNAs. The system was chosen as it uses 20-100 times less RNA than a conventional microarray technique due to the use of an efficient amplification system. This lower RNA usage makes the method valuable in analysing precious RNA samples. Thirty different genes were identified as over expressed in the ovarian cancer cell lines. Those over expressed by >10-fold include antigen GA733-2, prostasin, CD24, ATF3, proteasome subunit HC8, Creatine kinase B, thymosin ß 10 and kinase myt 1. To validate this data they used real time quantitative RT-PCR on 5 genes that are known cell surface antigens or secreted proteins which would be easily detectable if used as tumour markers. Six ovarian cancer cell lines were examined and overexpression of only one gene, GA733-2, was detected in all of these compared to OSE cells. Overexpression of prostasin and Creatine kinase B was detected in five samples and only three of six samples showed elevated osteopontin and KOC transcripts.

Wong discusses that pooling RNA samples allows identification of genes that may be over expressed in only a proportion of cancer cell lines so would be useful in molecular classification. Their validation again highlights the need to use a complementary follow up technique with microarray analysis so false positives that are not representative of ovarian tumours are ruled out. A later paper further studies the overexpression of prostasin and identified protein overexpression in a majority of tumour samples supporting its potential role as a tumour marker (Mok *et al.*, 2001).

Schummer used a large 21,500 clone cDNA array in the individual comparison of 10 tumour samples to OSE and other normal tissue samples (Schummer *et al.*, 1999). The high density array allowed analysis of a large number of genes in one hybridisation so saving time and experimental variation. It was noted that tumour samples are often contaminated with normal cells and that cultured OSE will probably have some differences in expression patterns to *in vivo* OSE cells. This was overcome by setting suitable limits of detection. As for Wong, Schummer et al only studied up regulated genes, which may be of use as molecular markers. They found that, in the comparison of tumour tissues to OSE, 7%, 0.9%, and 0.5% of genes exhibited tumour-to-OSE ratios of 2.5- 5.0- and 10-fold respectively.

Sixteen cDNA clones with overexpression in 6 or more of the 10 ovarian cancers were identified but 14 of these were also expressed in non-ovarian tissues. To eliminate those non-specific genes that are expressed in non-ovarian tissues, selection criteria were introduced. Ratios of >2.5-fold in at least five tumours and below 2.5 in other tissues as compared to OSE were used. Using these values they identified 134 clones which consisted of 26 mitochondrial and ribosomal genes, 37 other known genes, 47 EST's and 24 Novel sequences. Focussing on the 37 characterised genes, 10 had previously been identified as expressed in epithelial tissues including 14-3-3σ, CD44, HE4, mucin1, COL1A2 and Rho A. Thirty-five percent of genes had been reported as over expressed in various cancers including those genes previously mentioned as well as BA46, E16, IGFBP3, ß-ACTIN and MDC15 metalloprotease. The fact that a lot of the genes identified have been previously reported as up regulated shows that analysis of a large number of genes is capable of narrowing down to a few cancer related transcripts.

Eight genes were validated using RT-PCR. Semi-quantitative RT-PCR analysis of 14-3-3σ varied widely from the array tumour-to-OSE ratio, supporting the need for sensitive validation. Other genes did show a general concordance between microarray and RT-PCR ratios. The largest difference between the two methods was however seen for HE4, which for the array showed 11-fold overexpression but only 4-fold by RT-PCR. They attribute this difference to a better signal-to-noise ratio in RT-PCR or simply due to the use of different tumour samples. HE4 gene expression was further analysed by Northern blotting, as this gene was the only one with clear tumour restricted expression. It was found that of four patient tumour samples, two over expressed HE4 as compared to normal paired ovarian tissues. HE4 is suggested to be a suitable candidate marker protein and this is supported by multiple microarray studies, as previously mentioned.

Wong and Schummer both analysed a set of similar transcripts (Schummer *et al.*, 1999; Wong *et al.*, 2001). Schummer identified 32 genes upregulated in ovarian tumour samples compared to human OSE RNA. The MICROMAX™ array used by Wong, carries 14 of these although only 5 were up more than three-fold in their study. Differences between cell lines and primary tumour samples may be responsible for these discrepancies. Tonin suggested that due to this tumour heterogeneity, analysis of cell lines, primary cultures and tumours needs to be extended before signature expression profiles and biologically important candidates in ovarian cancer can be fully established (Tonin *et al.*, 2001).

Gene expression in seven early and seven late ovarian tumours and normal OSE brushings pooled from 20 patients was examined using a ~25,000 clone cDNA microarray (Shridhar *et al.*, 2001b). All tumours were poorly differentiated but of varying histology. It was found that gene expression in all tumour types tended to be more often down rather than up regulated as compared to normal OSE expression. Genes up regulated >5-fold in both early and late stage tumours include HE4, properdin, lipocalin 2, keratin-13, mucin1, MMP7, ceruloplasmin and claudin 4. Those genes down regulated by >5-fold include c-type lectin, E25 protein, CD36, amphiregulin, PEG3 and Glutathione-S-transferase A3. Genes which showed expression changes were clustered into four categories: cell-cell interactions;

intermediate filament markers; cell cycle and growth regulators and genes involved in invasion and metastasis. These categories include keratins, cadherins, matrix metalloproteases and cyclins.

Semi-quantitative RT-PCR was used to validate these results standardised against GAPDH. A set of genes was tested for expression on a panel of seven ovarian cancer cell lines, and 20 early and 16 late stage ovarian tumours. The number of genes that were validated by RT-PCR is not mentioned but loss of expression of PAI1, FGF7 and Decorin in the majority of tumours is reported. Upregulation of genes PUMP1 (MMP7) and HE4 was also confirmed. A set of five genes was also analysed by real-time RT-PCR and results were said to be similar as for semi-quantitative RT-PCR data.

Although the paper is titled 'Genetic analysis of Early- *versus* Late-stage Ovarian Tumours', this comparison is not clearly seen. Early and late stage tumours are pooled in their analysis and there is no direct comparison of the two stages. Unlike the paper by Tapper, Shridhar et al do not report any specific gene differences between the early and late tumour stages. The only genes discussed are those which are altered in expression in all tumours regardless of stage. Of those identified, again many had previously been reported as differentially expressed between normal and cancerous tissues.

In an interesting paper by Giordano the molecular profiles of 57 lung, 51 colon and 46 ovarian adenocarcinomas are compared (Giordano *et al.*, 2001). RNA from each of these tumours was individually hybridised onto 7129-clone oligonucleotide HuGeneFL arrays. Using principle component analysis (PCA), a method that identifies those two-way comparisons with the greatest amount of discrimination, four 'views' were generated. These views each contained 25% of all transcripts, with increasing abundance. For example the first view contains the 25% of transcripts with the least average abundance and so on. All views showed substantial differences in gene expression between the three tumour types that clustered individually within the region. They revealed that ovarian tumours showed the greatest heterogeneity. A set of markers for each tumour type was next identified. These genes had much higher expression in one cancer type as compared to either of the other two. Thirty-

one unique genes were identified as ovarian cancer specific. These include PEG3, WT1, somatostatin, cyclinA1, SLPI, MEIS1, StAR, PRAME, CLU and IGFBP2. Of these genes, many have been associated with ovarian cancers such as MEIS1 and PRAME, both reported previously as over expressed in tumours by microarray analyses (Welsh *et al.*, 2001).

This study did not aim to identify genes that were differentially expressed between tumours and their normal counterparts, it was instead attempting to group the individual tumour types using gene expression patterns. The genes identified cannot therefore be compared directly to other published data. For example the genes StAR and PAI have previously been identified as down regulated in tumours but in this system their expression is seemingly increased.

Most recently, in a follow on from their successful SAGE work, Sawiris et al have generated a 516 cDNA chip which is a specialised ovarian cancer array (Hough *et al.*, 2000; Sawiris *et al.*, 2002). The Ovachip contains genes chosen from their previous SAGE and cDNA array data, some of which have been validated by real-time RT-PCR (Hough *et al.*, 2001). The aim of such a specialised chip is to reduce noise from irrelevant genes during data analysis. The Ovachip was used to analyse 11 late stage ovarian tumours, four colon tumours and various cell-lines. They found that the array data could distinguish clearly the ovarian from the colon tumours.

In a comparison of ovarian tumours with non-malignant OSE cells using the Ovachip, 25 genes were up and 11 genes down regulated. Those up regulated genes included IGF2, HSP90, RAB1, BRF1, SLPI, IGFBP2 and SPRR1B. Candidate TSGs, which are down in tumours, included the previously characterised BRCA1 as well as other novel cancer genes such as ß-tubulin, LDHA, BTF3, RPL7A and MYLK.

Four genes were validated for gene expression by real time RT-PCR and it was found that although results from the array and the RT-PCR were not exact, they followed the same trends. Quantitative RT-PCR, which is both sensitive and accurate, is an essential complement to arrays, as was shown in these studies. The chip has identified two clusters of co-altered genes that have been named the IGF

and CAK clusters, which contain up and down regulated genes respectively. These clusters are being studied further for their roles in cancer progression.

As discussed, many gene expression analysis techniques have been used in ovarian cancer for the identification of candidate TSGs and oncogene ovarian cancer markers. These studies have linked numerous genes with ovarian cancer progression although some genes, such as HE4, are reported by numerous groups as differentially expressed between normal and tumour samples. Those genes identified by two or more papers as differentially expressed are listed in Figure 1.3.

Although gene expression analysis techniques have only been available for ten years, many groups have applied the technologies to many aspects of ovarian cancer studies. The multitude of papers published in the area and the number of individual genes shown to be associated with ovarian cancer progression has proven the power of such investigations, and the complexity of apparently differentially expressed ovarian cancer genes.

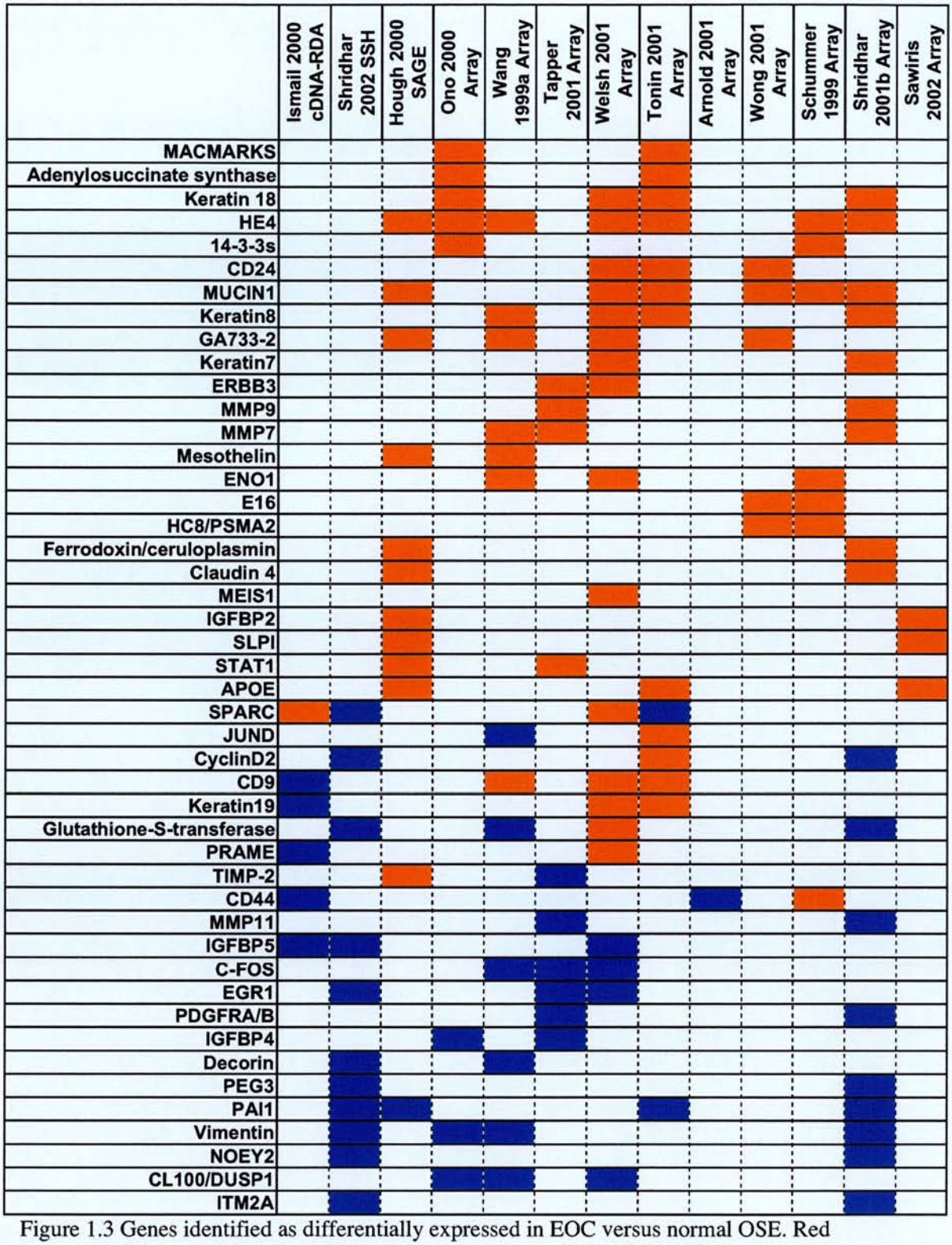


Figure 1.3 Genes identified as differentially expressed in EOC versus normal OSE. Red boxes= up in tumours (oncogenic) blue= down in tumours (TSGs). First Authors shown along top with year and analysis method.

10 Background

Dr Hani Gabra carried out MMCT of a *neo*-tagged human chromosome 11 from donor mouse cell-line 556.1.5 into a clonal derivative of the ovarian cancer cell-line OVCAR3. These transfers generated independent clones that have been fully phenotypically characterised in relation to the parent OVCAR3.

OVCAR3 is an ovarian cancer cell-line established from malignant ascites of a patient with progressive adenocarcinoma of a poor differentiation grade (Hamilton *et al.*, 1983). Chromosome 11 in OVCAR3 is markedly rearranged and hypotetraploid suggesting a mechanism of whole homologue loss and then reduplication. It is postulated that some genes on chromosome 11 in OVCAR3 are non-functional and therefore potential TSGs.

Parental lines with *neo* tags were generated for use as controls by either transfecting the *hyg* resistant clonal line OH3 with a *neo* containing plasmid (OHN) or by use of ON3 as a microcell donor to transfer *neo* tagged Chr 11 into OH3 (ONOH). Functional studies showed no differences between these two controls. As chromosome 11 is rearranged in OVCAR3, these clonal lines should not carry any functional TSGs along this chromosome.

MMCT of donor 556.1.5 chromosome 11 into OH3 generated two series of independent clones (11OH1 and 11OH2). The cell-line OHX was generated from an OH3 xenograft that was then subjected to MMCT to generate hybrids 11OHX1-3. OHX was created in order to more readily form tumours in mice, requiring a far fewer cell number than OH3.

Microsatellite mapping of these clones determined the extent of chromosome 11 transferred in each case (Figure 1.4). Cell-lines 11OH1.1 and 11OH1.3, generated from the same MMCT, have complete transfer of chromosome 11. Fragmentation of chromosome 11 occurred in cell-lines 11OH2.1-2.4, which lack the region telomeric to marker D11S936 at 11q23. They also appear to be missing a small region at 11p13, defined by the marker D11S935.

Xenograft clones 11OHX1.1 and 11OHX3.1 contain a complete chromosome 11 whereas 11OHX2.1 has only partial transfer, missing 11q23-ter, two regions at 11q13 and D11S935 at 11p13.

The 11OH hybrid cell-lines all exhibited *in vitro* and *in vivo* growth suppression (Figure 1.5). This supported the hypothesis of a tumour suppressor on chromosome 11. Also, as all of the clones showed slowed growth, it is suggested that the suppressor must lie within the regions transferred in the 11OH2 series so outwith11q23-ter and 11p13.

Matrigel invasion studies showed a clear decrease in invasion by the 11OH1 clones. The 11OH2 hybrids did not have this altered phenotype. This suggests that an invasiveness suppressor maps to 11q23-ter, which is supported by morphological studies. Cellular attachment assays showed 11OH1 hybrids were inhibited in attachment to a laminin matrix. This effect was not seen in the11OH2 hybrids.

Apoptosis and cell cycle studies showed no differences in phenotypes between parental and hybrid clones.

Cell-line	*In vitro growth*	*In vivo tumorigenicity*	*Cellular invasion*	*Cellular adhesion*	*Cell cycle*	*Apoptosis*
Parental line OHN	Normal	Normal	Normal	Normal	Normal	Normal
Hybrids 11OH1	Suppressed	Suppressed	Suppressed	Normal	Normal	Normal
Hybrids 11OH2	Suppressed	Suppressed	Normal	Normal	Normal	Normal
Hybrids 11OH3	Suppressed	Suppressed	Normal	Normal	Normal	Normal
Hybrids 11OHX	Suppressed	Suppressed	Normal	Normal	Normal	Normal

Table 1.5 Overview of hybrid characteristics as compared to parental line OHN. Suppressed characteristics are highlighted in red.

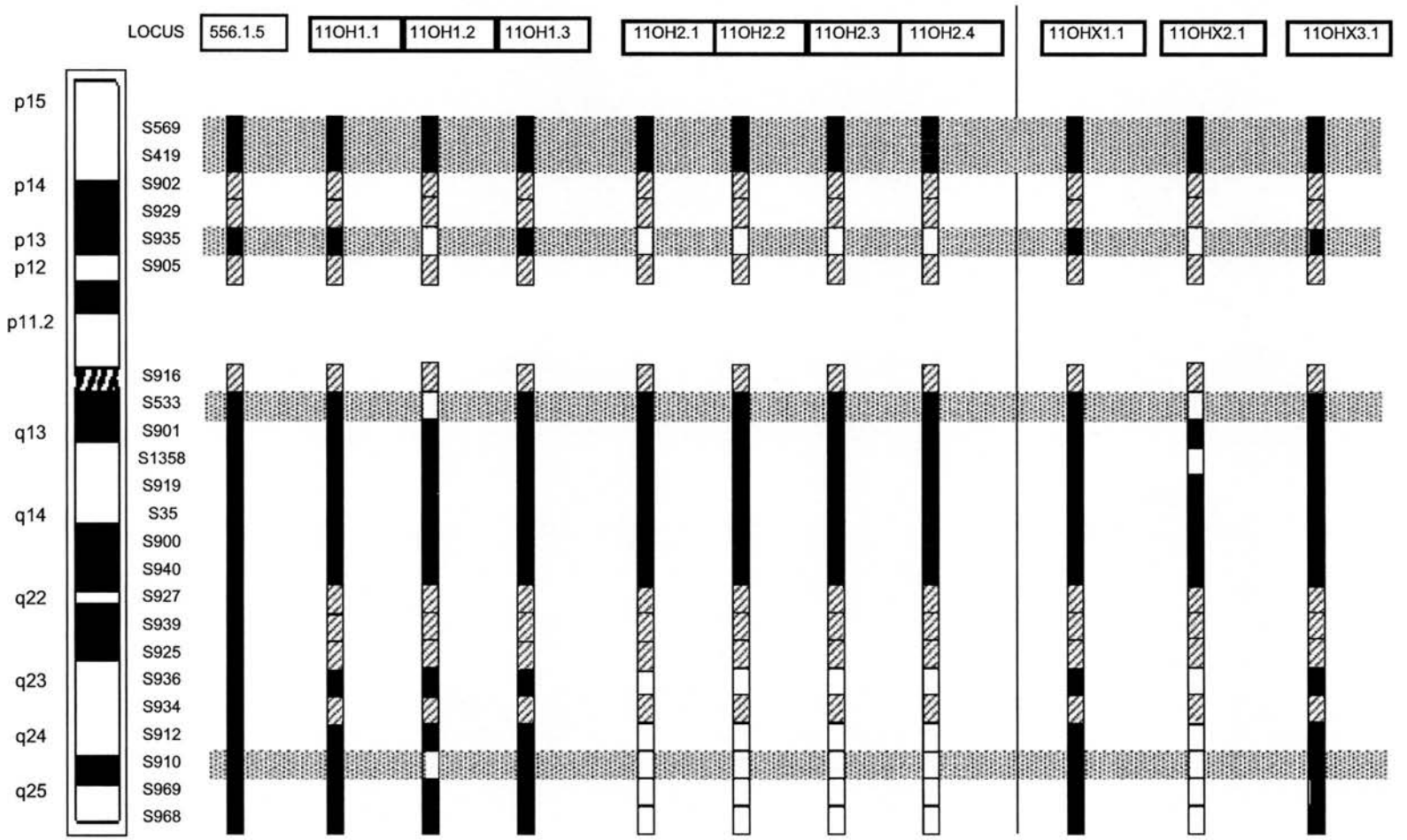


Figure 1.4 Microsatellite map of chromosome 11, transferred by MMCT, into OVCAR3 derivative cell-lines. Loci are shown on the left and positioned relative to the Chr 11 ideogram. Black boxes= transfer, a white box= no transfer and a hashed box= an uninformative locus.

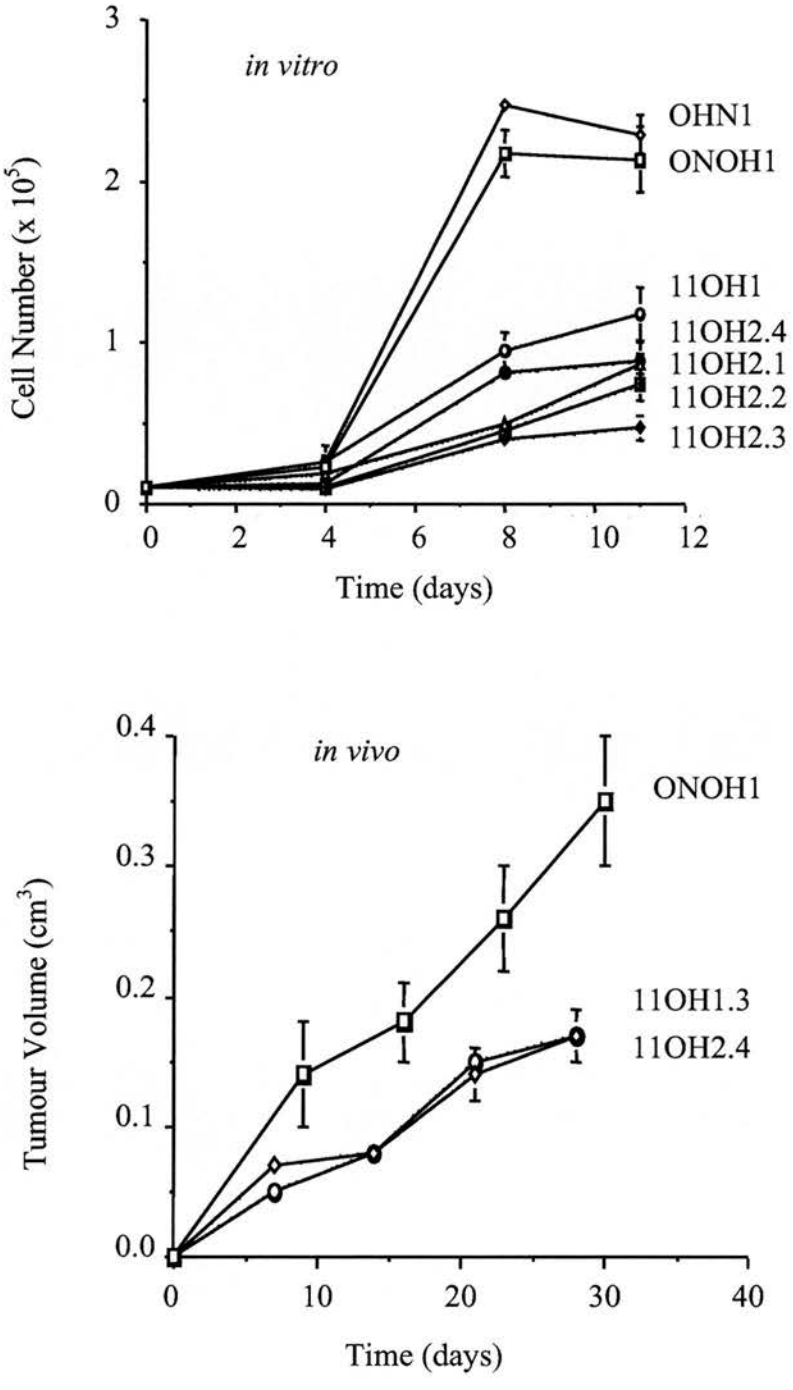


Figure 1.5 *In vitro* growth and *in vivo* tumorigenicity curves of hybrid cell-lines as compared to parental line OHN/ONOH. Error bars represent the standard error for each cell-line.

A traditional approach of revertant mapping to identify those regions harbouring TSGs was conducted. Growth suppression occurred in those clones with deletion of

markers D11S533 and D11S1358 on 11q and at 11p13 suggesting that a growth suppressor may be positioned outwith here. As frequent LOH is detected at 11p15 in ovarian cancers (see introduction- section 1.7), clones were mapped along this region in order to identify areas of loss associated with a change in the growth and tumorigenic phenotypes. 11OH and 11OHX hybrid cell-lines were further passaged with the aim of generating revertant clones to identify regions of chromosome loss by microsatellite mapping.

A more novel approach was to use this hybrid resource in multiple difference analysis techniques to identify genes along chromosome 11 which showed increased expression in the growth suppressed clones to investigate these as candidate tumour suppressor genes.

Using three difference analysis methods, Differential Display RT-PCR, cDNA-Representational Difference Analysis and Clontech High Density Filter Arrays (HDFA), we compared gene expression between Parental OVCAR3 derivative OHN and hybrid line 11OH2.1. This hybrid was chosen with identification of candidate genes responsible for the growth suppression phenotype in mind.

2. MATERIALS AND METHODS

Unless stated, consumables and reagents were purchased from Sigma-Aldrich or Fisher Scientfic Chemicals. Other suppliers are listed in Table 2.1.

Supplier	Methods in which reagents were used
Abcam, Cambridge, UK	Western blotting and IHC
Amersham Biosciences, Chalfont St Giles, UK	Northern blotting, Western blotting
Applied Biosystems (Perkin Elmer), Foster City, USA	ABI310 work and sequencing
BioGenex, San Ramon, USA	Western blotting
Bio-Rad, Hercules, USA	Electrophoresis
BD Biosciences Clontech, Palo Alto, USA	High density filter array
GeneSys Ltd, Farnborough, UK	Light Cycler analysis
Fisher Scientific Chemicals, Loughborough, UK	General
Idaho Technology Inc., BioGene Ltd, Kimbolton, UK	Light Cycler analysis
Invitrogen-Life Technologies, Paisley, UK	RNA work
Millipore, Bedford, USA	Western blotting
New England Biolabs Inc., Beverly, USA	Western Blotting
Novagen, Madison, USA	Sequencing
Pfizer Inc., New York, USA	Northern Blotting
Promega, Madison, USA	Plasmids and transformation
Qiagen, Crawley, UK	RNA/DNA extraction and purification
Roche Applied Science, Basel, Switzerland	cDNA synthesis, Northern blotting, Western blotting
Sigma-Aldrich, St Louis, USA	General
Stratagene, La Jolla, USA	RNA extraction

Table 2.1 Suppliers of reagents and equipment used in my studies.

2.1 DIFFERENCE ANALYSIS METHODS.

2.1.1 Differential display RT-PCR

DD-RT-PCR was carried out by Genevieve Rabiasz and Dr Grant Sellar. DD-RT-PCR was performed according to described protocols using a combination of 3 single base anchored antisense primers and 7 arbitrary sense primers. Antisense primers used were: 5'-AAGCTTTTTTTTTTTA-3'; 5'-AAGCTTTTTTTTTTTC-3'; 5'-AAGCTTTTTTTTTTTG-3'. Sense primers used were: 5'-AAGCTTTCTACCC-3'; 5'-AAGCTTTGGCTCC-3'; 5'-AAGCTTATACAGG-3'; 5'-AAGCTTGTCATAG-3'; 5'-AAGCTTCAAGTCC-3'; 5'-AAGCTTCTGACAC-3'; 5'-AAGCTTCTAACCG-3'. Products identified as being differentially expressed were excised from 6% (w/v) polyacrylamide sequencing gels, re-amplified, subcloned and sequenced (See section 2.1.2). Analysis was performed by me.

2.1.2 cDNA Representational Difference Analysis.

Dr Grant Sellar carried out cDNA-RDA comparing reciprocal OHN and 11OH2.1 gene expression and I performed sequencing and analysis of products. The method used was modified from Hubank and Schatz (Hubank and Schatz, 1994). Briefly, double stranded cDNA from tester and driver samples is generated and DpnII digested. R linkers (R12- 5'-GATCTGCGGTGA-3', R24 – 5'AGCACTCTCCAGCCTCTCACCGCA-3') are ligated onto the DpnII fragments and amplified by PCR to generate representations. These representations are further DpnII digested. The tester population has J linkers (J12 5'-GATCTGTTCATG-3', J24 5'ACCGACGTCGACTATCCATGAACA-3') ligated to it and subtractive hybridisation between the tester and driver (in 80-fold excess) is carried out in a PCR-based step. Those fragments present more abundantly or solely in the tester are enriched and amplified. Single stranded, unpaired fragments are digested using mung bean nuclease. This generates the first difference product (DP1). To generate DP2, adapters are changed from J to N (N12 5'-GATCTTCCCTCG-3', N24 5'-AGGCAACTGTGCTATCCGAGGGAA-3') in DP1 and driver added in further excess in a second subtractive hybridisation step. This process is repeated until sufficient enrichment of products has been achieved, usually progressing to DP3 or DP4 with increasing excess of driver.

Products in high abundance (MMLV and RALDH2) were amplified by PCR and added into the driver sample at a following stage of subtractive hybridisation by a method called 'spiking'. The addition of these PCR products prevents saturation of DPs with the aim of cloning out other, less abundant genes.

Difference products were run on a low melting point gel, bands excised, then purified using QIAquick gel extraction kit (Qiagen). Briefly, the gel is melted at 50^0C with three volumes buffer QG. Once dissolved, one volume of isopropanol is added and the DNA is bound to a spin column. The DNA is washed with 0.75ml buffer PE and eluted in 50μl EB (10mM Tris-Cl).

These fragments were ligated into pGEM-T Easy TA vectors (Promega) and inserts sequenced using SP6/T7 primers. Sequence files were analysed using EditView software.

2.1.3 Clontech Atlas™ array hybridisation.

Clontech provide an Atlas™ nylon array hybridisation and analysis service.

Frozen cells (>10^7) for OHN and 11OH2.1 clonal lines were sent to Clontech who carried out hybridisation of three Atlas™ arrays. The nylon arrays carry 1,176 cDNA fragments (200-600bp) each. Three arrays were hybridised; Human 1.2k I, Human 1.2k II and Human Cancer array. The method for hybridisation is available on the Clontech web site (www.clontech.com).

Briefly, RNA is extracted from the cell samples and bound to fluorochromes to generate labelled probes. This is then hybridised to the array of choice and phosphoimages of the membranes are produced. These images are scanned and analysed using Atlas Image™ software that quantitatively analyses and compares signal intensities on the arrays. The spots are normalised to nine housekeeping genes carried on the array and signal intensities a corrected for background by subtracting the average of several non-hybridised regions of on the membrane. Clontech carried out the analysis of these arrays. A report of up and down regulated genes is provided for further analysis containing gene identifiers, signal intensities and fold differences in expression for each gene between our samples. Clontech recommend results be corroborated with another method of gene expression analysis such as Northern blotting or RT-PCR.

2.1.4 Identification of transcripts

cDNA-RDA and DD-RT-PCR generate transcripts which are sequenced for identification. These sequences are BLAST searched against the non-redundant (NR) and high throughput genome sequencing (HTGS) databases using the online facility at NCBI (www.ncbi.nlm.nih.gov). Characterised sequences were then researched for cellular function using the OMIM and GeneCards databases (at NCBI and http://bioinformatics.weizmann.ac.il/cards/). Other sequences were analysed in LocusLink and UniGene for homologues in other species (NCBI).

2.2 Gene expression analysis-verification

2.2.1 RNA extraction for use in Northern blotting and RT-PCR

RNA was extracted from monolayers of cells using TRI REAGENT (Sigma-Aldrich). 1ml of TRI REAGENT was used per $10cm^2$ of tissue culture plate. Cells were pipetted into RNase free tubes. After standing at room temperature for 5 minutes, 0.2ml chloroform per ml of TRI REAGENT was added. The solution was vigorously shaken for 15s and allowed to stand for 10 minutes at room temperature. This mixture was centrifuged at 12,000g for 15 minutes at 4^0C. Centrifugation separates the mix into three phases with the upper colourless aqueous phase containing the RNA. This layer is transferred to a clean RNase-free tube and 0.5ml isopropanol per ml of TRI REAGENT was added and mixed by inverting. The sample stood at room temperature again for 10 minutes. When centrifuged at 12,000g for 10minutes at 4^0C a pellet of RNA formed on the side of the tube. This pellet was washed with 1ml 75% ethanol per ml TRI REAGENT and mixed. The RNA was re-pelleted by centrifugation at 12,000g for 5 minutes at 4^0C. The pellet was transferred to an eppendorf tube with 1ml 75% ethanol and re-compacted by centrifuging at 13,000 rpm in a microfuge for 5 minutes at 4^0C. It was then air dried and resuspended in 50μl DEPC-treated H_2O. Once quantitated the RNA was aliquotted in 20μg amounts and stored at -70^0C.

2.2.1.1 Quantitation of DNA/RNA by spectrophotometry

5μl DNA or RNA solution is added to 995μl DEPC-treated H_2O (1 in 200 dilution). A reading of absorbance was taken at 260nm and also at 280nm for RNA samples. A ratio of absorbance at 260nm divided by 280nm indicates purity of the sample. A value of 1.6 to 1.8 indicates pure RNA. A higher value of about 2.0 indicates DNA contamination whereas a low value <1.5 suggests protein contamination. The calculation below determines the concentration of DNA/RNA in the solution and relies on the fact that an OD_{260} of 1.0 indicates a DNA concentration of 50μg/ml.

$$\frac{\text{Concentration } A_{260} \text{ x dilution factor (200) x X}}{1000} = \mu g/\mu l$$

Where X is 40 for RNA, 50 for DNA, 33 for single stranded DNA.

2.2.2 Quick RNA extraction from cell lines for RT-PCR

An Absolutely RNA™ RT-PCR mini prep kit (Stratagene) was used for ease and time as per manufacturer's instructions to extract RNA from transfected clonal lines. Briefly, $25cm^3$ tissue culture flasks of cells at 70% confluence were washed twice in PBS. 350μl of lysis buffer containing 2.5μl ß-mercaptoethanol was added onto the monolayer of cells which were then transferred to a microfuge tube. This mix was transferred to a prefilter spin cup and centrifuged at 4^0C for 5 minutes at maximum speed. The filtrate was then added to an equal volume of 70% EtOH and vortexed to mix. This was transferred to a RNA binding spin cup and centrifuged at 13,000 rpm for 30-60 sec. Bound RNA was then DNase I treated after washing with 600μl 1x low salt wash buffer. A mix of 50μl DNase I digestion buffer and 5μl DNase I was added to the spin cup and incubated at 37^0C for 15 minutes. The cup was then washed in turn with 600μl high salt buffer, 600μl low salt buffer, then 300μl low salt buffer, and each time centrifuging at 4^0C. The DNase I treated RNA was then eluted in 50μl EB and quantitated by spectrophotometry. RNA was stored at -70^0C long-term. This RNA is suitable for use directly with the Roche 1st strand cDNA synthesis kit.

2.2.3 DNase I treatment of RNA

20-40μg of total RNA, made up to a total volume of 50μl in DEPC-treated water, was DNase I treated with 5.7μl 10x buffer and 10 Units DNase I for 30 minutes at 37^0C on a MJ thermocycler. Treated RNA was then extracted with 40μl phenol/ chloroform in a 3:1 ratio for 10 minutes on ice. After centrifugation at 10,000g for 5 minutes at 4^0C, the upper phase was collected into a clean tube. The RNA was then ethanol precipitated with 5μl NaOAc and 100μl 100% ethanol at -70^0C for 2 hours. A 10 minutes centrifugation pelleted the RNA and this was then washed with 70% Ethanol in DEPC-treated H_2O. The RNA was air-dried, dissolved in 15μl DEPC-treated H_2O and 1μl quantitated by spectrophotometry. DNase I treated RNA was stored in 3μl aliquots (3μg) at -70^0C.

2.2.4 cDNA generation

A Roche 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) was used to generate cDNA as per manufacturers instructions. 1μg of DNase I treated RNA was mixed with final concentrations of 1x reaction buffer, 5mM $MgCl_2$, 1mM deoxynucleotide mix, 1.6μg oligo-p$(dT)_{15}$ primer, 50 Units RNase inhibitor, 20 Units AMV reverse transcriptase, and made up to the volume of 20μl with sterile water. This mix was then incubated at 25^{0}C for 10 minutes followed by 42^{0}C for 60 minutes and the AMV-RT denatured at 95^{0}C for 5 minutes. Control reactions minus reverse transcriptase were also performed. cDNA was then aliquotted into 2μl or 3.2 μl volumes for use directly in RT-PCR and real-time RT-PCR respectively.

2.2.4.1 RT-PCR

RT-PCR was carried out using multiple primer pairs at the standard conditions of 2mM $MgCl_2$ normally using a touchdown 67-55^{0}C program on a MJ thermocycler. The RT-PCR reaction volume was 25µl and contained 2µl cDNA, 2mM $MgCl_2$, 12.5 pMol each primer, 1.25 Units PicTaq polymerase (Cancer Research UK), 200µM dNTPs and 1x buffer. Products were run on a 2% agarose gel and visualised under UV light with ethidium bromide.

Products were quantitated for use as Light Cycler standards by spectrophotometry. PCR products were diluted to standards of 1x10^{9}copies/µl, 1x10^{7}copies/µl and 1x10^{5}copies/µl, as calculated from product size and OD_{260} reading.

2.2.5 Primer Design

Primers for use in RT-PCR and Light Cycler analysis were designed using the Primer3 program at www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi/. All primers were synthesised at the Cancer Research UK oligonucleotide synthesis service, London. Sequences for the primers used in validation can be acquired from Dr Hani Gabra.

2.2.6 Light Cycler analysis.

Quantitative RT-PCR analysis used Roche and Idaho Technologies Light Cyclers. Initially PCR standards were run to check the melt temperature (Tm) of the primers.

RT-PCR reaction volume was 10μl containing 1X PCR master mix (GeneSys Ltd.) 5pMol forward and reverse primers, 4mM $MgCl_2$, 1:20,000 volume SYBR Green I fluorescent dye (BioGene Ltd.) in a 10mM Tris (pH8) – 1mM EDTA buffer and 1μl cDNA. Quantitative RT-PCR was performed in thin-walled glass capillaries by initial denaturation at 95°C for 10 sec followed by 45 cycles of 95°C for 0 sec, 55°C for 2 sec, and 72°C for 15 sec. Reading of fluorescent emission from SYBR Green I bound to double stranded DNA was taken over 2 sec after each PCR cycle at a temperature ~4°C lower than the Tm. A final extension at 72°C for 10 sec was included before a temperature ramp from 72°C to 95°C at 0.1°C/sec continuously reading fluorescence. Inspection of melt curves generated by this process demonstrated amplification of a single product. Standards were run in duplicate and RT-PCRs in triplicate. Expression levels were normalised with respect to β-Actin.

2.2.7 Northern Blotting

2.2.7.1 DEPC treatment of water and glassware

All glassware, gel tanks and consumables used in Northern blotting were sterile / DEPC-treated to avoid RNA degradation. Glassware was treated by filling with a solution of 0.1% DEPC in distilled water and left in a fume hood over night. The water was then poured into a pre-treated tub and autoclaved along with the empty glassware. RNase AWAY (Invitrogen-Life Technologies) was used on surfaces such as Pyrex dishes and gel tanks to ensure removal of residue RNases and rinsed with DEPC-treated water.

2.2.7.2 Sample electrophoresis and transfer

20μg RNA samples were run on an agarose / formaldehyde gel with 1x MOPS/EDTA running buffer. Sample buffer was made from 0.75ml Deionised formamide, 0.15ml 10% MOPS/EDTA, 0.24ml 37% formaldehyde, 0.10ml glycerol, 0.10ml DEPC-treated water, and 0.08ml 10%w/v bromophenol blue and stored in aliquots at –20°C. RNA samples and marker, with 25μl sample buffer, were denatured at 65°C for 15minutes then 1μg ethidium bromide added to each sample then loaded onto the gel. It was run overnight at 30-40V until the dye front was 4/5 down the gel.

The RNA was then transferred onto hybond-N membrane after washing in 0.05M NaOH and 20x SSC. Transfer apparatus was assembled as in Maniatis (Maniatis, 1989) and transferred overnight in 10x SSC. Once disassembled, the gel lanes were marked on to the membrane and the RNA was UV cross-linked using a stratalinker at 150MJ.

2.2.7.3 Hybridisation of Membrane

120μl sonicated salmon sperm DNA (sssDNA) was boiled for 5 minutes and quenched on ice. The membrane was then rolled in 6x SSC separated by a 0.1% SDS boiled gauze and placed into a hybridisation bottle, RNA facing inwards. Hybond mix was made from 6x SSC, 5x Denhardts (0.4g BSA, 0.4g Ficoll, 0.4g PVP, 0.1% SDS), 0.1% NaPPi, and 10% dextran sulphate made up to 500ml with water and filtered to store at room temperature. 20ml Hybond hybridisation mixture, containing 100μl of the boiled sssDNA, was then incubated with the blot overnight at 68^{0}C.

DNA probes for labelling were generated by RT-PCR and products purified using the QIAquick PCR product kit. The probes were quantified on 2% agarose gel as compared to known dilution of DNA marker.

25ng of probe in 13μl distilled H_2O was boiled for 5 minutes then incubated with 4μl High prime (Roche) and 3μl ^{32}P dCTP redivue ambient 1.11MBq (Amersham Biosciences) for 15 minutes at 37^{0}C.

Incorporation of ^{32}P dCTP was determined by 5% TCA extraction of 0.5μl probe through a filter using a vacuum manifold. All probes used were well labelled.

A NICK sephadex 50 column (Pfizer) was used to remove unincorporated nucleotides from the labelled probe. 400μl of label in 1xTE was dripped through the column into 100μl sssDNA and boiled for 10 minutes then quenched and added to the hybridisation bottle to incubate overnight at 68^{0}C.

Non-specific background labelling was removed from the membrane by washing in 2x SSC/ 0.1%SDS for 20 minutes followed by four washes with 0.2x SSC/ 0.1% SDS. Each wash was done at 68^{0}C. The membrane was then sealed in plastic to remain moist and exposed to Hyperfilm™-MP (Amersham Pharmacia Biotech Ltd) in a sealed cassette at –70^{0}C.

Exposed films were developed using an AGFA curix 60 developer. The blot was re-exposed until a suitable autoradiograph was produced. Densitometry on the scans of the autoradiographs was carried out using LabWorks software (UVP)

Blots were stripped up to four times in a solution of boiling 0.1% SDS, and residue radioactivity was detected again by exposure to film.

2.3 Protein expression analysis

2.3.1 Making protein lysates.

Lysis buffer made up of 50mM TRIS pH 7.5, 5mM EDTA pH8.5, 150nM sodium chloride, 100μg apotonin, 2mM sodium orthovandate, 50mM sodium fluoride, 1mM PMSF, 100μg leupeptin, 10mM sodium molybdate, 20μM phenylarsine oxide, 100μl 1% triton X-100 and made up to 10ml with distilled water.

A 70-80% confluent flask of cells is washed with cold PBS and lysis buffer added to the bottom of the flask for 10 minutes at 4^0C. Cells were scraped off the plastic and collected into tubes. They were then centrifuged at maximum speed for 6 minutes at 4^0C. Pellets are then quantitated by a Bradford assay.

2.3.2 Bradford assay

TUBE	PROTEIN (µL)	WATER (µL)
A	20	0
B	60	20
C	60 from B	20
D	60 from C	20
E	60 from D	20
F	60 from E	20
G	60 from F	20
H	60 from G	20
I	0	20

Table 2.2 Serial dilution of protein standard for Bradford assay.

Control protein standards were made as in Table 2.2. Dilution of lysates in a final volume of 20μl was made. To each of these and the controls was added 1ml Bio-Rad protein assay dye. 200μl of each sample then transferred to a 96-well plate and run

on a spectrophotometer at 600nm (Kruger, 2001). The Assayzap programme was used to determine to concentration of the samples as compared to protein standards.

2.3.3 Western Blotting

Cell lysates were prepared and quantified as described. 20 μg of protein was run on a mini-gel apparatus (Bio-Rad). Protein samples were aliquotted, and their volumes equalised using lysis buffer. Samples were denatured at 95°C for 5 minutes in loading buffer containing SDS, ß-mercaptoethanol and bromophenol blue. Protein was loaded onto a 10% polyacrylamide gel, along with 10μl protein marker (New England BioLabs Inc.)

Mini tanks were run at 80mA for 25 minutes, then up to 200mA for 1 to 1.5 h. After electrophoresis, proteins were transferred to a permeablised Immobilon-P membrane (Millipore) via a wet transfer method at 30 V, 4°C for 1-4 h. Proteins were detected using a chemiluminescence Western Blotting Kit (Roche). Membranes were blocked using 1% blocking agent (diluted in Tris Buffered Saline (TBS): 6.05g Trisma base, 8.76g NaCl, made up to 1L with dH_20, pH adjusted to 7.5) for 1 h at room temperature. They were then incubated overnight with CTSD primary antibody (Abcam) at a dilution of 1/1000 in 0.5% blocking solution, at 4^0C. Membranes were washed 3 times for 5 minutes in TBS-Tween (TBS-T, TBS containing 0.1% Tween 20), then twice for 5 minutes with 0.5% blocking solution, and treated with secondary anti-mouse and anti-rabbit IgG antibody (Roche) at a dilution of 1/1000 in 0.5% blocking solution for 1 h at room temperature. Membranes were then washed three times for 5 minutes with TBS-T and 3 times for 5 minutes with TBS. After a 1 minute incubation with the luminescence substrate solution, light emission was detected on Hyperfilm™ECL™ (Amersham Pharmacia Biotech Ltd).

2.3.4 Avidin-Biotin Conjugate Immunohistochemistry

Sections were cut to a width of 10μM using a microtome and mounted on glass slides.

Sections were dewaxed in xylene and then hydrated in ascending grades of ethanol. They were then incubated in 3% hydrogen peroxide for 30minutes. Antigen presentation was enhanced by boiling samples in citric acid (pH6.0) for 3x 5minutes

in a full power microwave and standing for 20 minutes in a fume hood. 0.05M TBS was used to rinse sections which were then blocked with 20% FCS in TBS. This was replaced with CTSD primary antibody at 1/200 dilution in 20% FCS in TBS and incubated for 1hour at room temperature. Sections were rinsed briefly with TBS and washed twice for 5 minutes. A StrAviGen MultiLink kit (BioGenex) was used for the labelling. Linking antibody (100μl in 20%FCS/TBS) was added onto the sections for 20 minutes and again rinsed and then washed twice with TBS. IgG anti-mouse labelling antibody (100μl made up in TBS) was incubated for another 20 minutes and again the sections were rinsed and washed with TBS. A 1mg/ml of DAB, containing 0.003% hydrogen peroxide, was dropped onto the slide, incubated for 5 minutes and then removed with water. Haematoxylin was used to counter stain the slides, which are then rinsed, in order, in water, 0.1%HCl in ethanol, water, lithium carbonate and then water again. Sections were dehydrated in ascending grades of alcohol and cleared twice, for 2 minutes each, in xylene. Slides were then mounted with a coverslip using a drop of DPX mounting medium (Fisher Scientific Chemicals) and analysed under a microscope for protein staining.

CTSD staining was scored for intensity and location by Dr Owatif Al-Nafusi, Senior Lecturer in Pathology, Edinburgh University Dept. of Pathology.

2.4 Loss of heterozygosity and revertant analysis studies

2.4.1 Rapid mini-prep extraction of DNA for microsatellite analysis

DNA was extracted using a QIAamp DNA mini kit (Qiagen). 10^5-10^6 cells were harvested, pelleted and resuspended in 200μl PBS. 20μl proteinase K was added along with 200μl buffer AL, vortexed, then incubated for 10 minutes at 56^{0}C. 200μl 100% ethanol was added and again vortexed. This mix was applied to a QIAamp spin column and centrifuged for 1 minute.

The column bound DNA was washed with 500μl buffer AW1 and spun for 1 minute. The DNA was washed with 500μl buffer AW2 and spun for 3 minutes. Carryover buffer AW2 was removed by a further 1 minute spin. DNA was eluted in a total of 100μl of warmed buffer AE (10mM Tris-Cl; 0.5mM EDTA) over two spins and stored at 4^{0}C.

DNA levels were too low to quantify by spectrophotometry so concentrations were determined by PCR (normally 2μl per reaction) using ß-actin primers.

2.4.2 Clinical data

I examined LOH in 38 fresh and 49 paraffin embedded epithelial ovarian tumours and their corresponding normal tissues (blood, normal ovary, and normal omentum). The specimens were collected between 1989 and 2001 at ICRF medical oncology unit.

A gynaecological pathologist confirmed all histopathological diagnoses and grades of the ovarian cancers. The average age at presentation was 59.8 years (range, 30 to 87 years). Patient characteristics are collated in Table 8.1.

Sample DNA was extracted by Diane Scott.

2.4.3 Identification of LOH primers

Forty-four polymorphic markers were identified on chromosome 11p15 using GeneMap '99 (www.ncbi.nlm.nih.gov/genemap/) and Genethon and Marshfield maps at NCBI (www.ncbi.nlm.nih.gov/cgi-bin/Entrez/map_search). Markers were confirmed as polymorphic using links to the UniSTS database. The position of these markers along 11p15 was determined by electronic PCR of marker sequences again non-redundant and HTGS databases using BLAST. The order of the markers was further refined using UCSC June 2002 web browser (http://genome.ucsc.edu/).

2.4.4 PCR of microsatellite markers.

Oligonucleotides were obtained from the Cancer Research UK oligonucleotide synthesis lab, London. Primers were selected on basis of location and spread over chromosome 11p15. Table 2.3 shows the 39 markers that were optimised for PCR and used in LOH and revertant analysis. Primers consist of fluorescinated primer pairs, 5' end labelled with fluorochromes TET/FAM that amplify dinucleotide repeat fragments of 90-348 bp.

Essentially two PCR programs were used for LOH and revertant analysis. Touchdown 67-55^{0}C is composed of 2 cycles of 94^{0}C for 30 sec, 67^{0}C for 30 sec and 72^{0}C for 45 sec followed by repeats of the above but at decreasing temperatures of

64^0C to 61^0C to 58^0C, for two cycles each, then 30 cycles of 94^0C for 30 sec, 55^0C for 30 sec and 72^0C for 45 sec finishing off with 72^0C for 5 minutes. All other touchdown programmes are similar but with altered annealing temperatures. Standard 55^0C program runs for 30 cycles of 94^0C for 30 sec, 55^0C for 30 sec and 72^0C for 45 sec followed by 5 minutes at 72^0C.

PCR reaction volume was 25μl and mixes consisting of 1.25-2.5mM $MgCl_2$, 12.5pM each primer, 1.25U taq polymerase, 200μM dNTPs, 1xBuffer and 1-2μl of DNA were run on an MJ thermocycler.

2.4.5 Agarose gel electrophoresis

PCR products were run on 2% agarose gels at 40-100V in 1xTAE with 0.1μg/ml of ethidium bromide (Sigma) and visualised and photographed under UV light. Products were quantitated by visually analysing digital images of PCR products.

MARKER	5' PRIMER	3'PRIMER	LABEL	SIZE (bp)	PCR programme	Mg2+ conc. (mM)	Heterozygosity	GDB number	Accession No
D11S1363	GAAAATGGTATTTAGAAACCAA	CCCAAGGGCTTACAAC	FAM	242-252	TD67-55	2	0.6	GDB:200143	Z24657
D11S4177	GACGGTGAACCCAGTCATT	TGTCAGCACAGAGGCAGAGT	TET	179-213	TD67-55	2	0.802	GDB:611871	Z53859
D11S2071	AGGGCAATGAGGACATGAAC	ATGTGGCTGGTCCACCTG	TET	168-202	TD67-55	1.25	0.85	GDB:373277	U12896
D11S4046	ACTCCAGCCTGGGAAAC	TGATAGACACACCCATTGC	FAM	183-203	TD68-56	2	0.87	GDB:592618	Z53199
DRD4	ACAGGCCCTGAGGTTTCC	GTGGGGAAGGGGTGTTTC	FAM	139-143	TD68-56	1.25	0.533	GDB:374242	-
HRAS	CTGTGGGTTTGCCCTTCAGA	CTCCTACAGGGTCTCCTGCC	TET	106-118	TD68-56	1	0.537	GDB:187026	K00654
D11S922	GGGGCATCTTTGGCTA	TCCGGTTTGGTTCAGG	FAM	88-138	TD67-55	2	0.935	GDB:188402	Z16988
D11S4088	GGGCAGAGGCAGTGGAG	GCATGTTTCGGGGGTG	FAM	204-252	TD68-56	1	0.92	GDB:603872	Z52321
D11S4146	AACACGAGGTTAAGCAGAG	GAATGAAGAATTTTCCAAACTAC	FAM	195-211	TD67-55	2	0.703	GDB:609885	Z53263
D11S1758	GTCAAGGTAGCCCAGGAAAT	CCAGTGTTCTCACAAACTGAGTA	TET	241-251	TD67-55	2	?	GDB:269766	Z50963
D11S4181	GGGCACCTGTAATCCCA	GAACTGAGACCAAGAACATTATTCC	FAM	201-217	TD67-55	1	0.803	GDB:612153	Z53945
D11S1760	GATCTCAAGTGTTTCCCCAC	AAACGATGTCTGTCCACTCA	TET	90	TD67-55	2	?	GDB:269768	Z50981
D11S4124	GAACACAATCCCATTTATATTAGC	TCAGCCTCACAAAGTGC	TET	165-177	TD67-55	1.25	0.67	GDB:608709	Z52902
D11S1338	GACGGTTTAACTGTATATCTAAGAC	TAATGCTACTTATTTGGAGTGTG	TET	255-265	TD67-55	2	0.74	GDB:199807	Z24082
D11S1331	GCTGCTTCCATGAGAGGATACTG	GCAGAGCCCTTTGCAGTCTT	TET	191-205	TD67-55	2	0.705	GDB:199705	Z23980
D11S932	TCGTATAGCACACCTTGGC	CTTATCATCTCTGGGTAGTGAAGTC	TET	150-164	TD67-55	2	0.637	GDB:188498	Z17083
D11S909	GATATAACACCAAAAGCGCG	GGTATTCTTACAGCACAAAAGTTCT	FAM	113-125	TD67-55	2	0.623	GDB:188088	Z16685
D11S4149	TGAATTATACCCCTGACCAA	CCCAGCCAATATCAGCA	FAM	214-226	TD67-55	2	0.772	GDB:610110	Z53329
D11S4188	TAACACCATCAAATAGACCAGG	TTTTATCCAGAAGGCAGCA	TET	109-123	TD69-57	2	0.633	GDB:612960	Z51398
D11S1329	TGTGGAACATCAAGCAAG	GGCACTACAGGGACAATC	TET	257-269	TD67-55	2	0.77	GDB:199647	Z23920
D11S1346	GCCAAATTAAGAGGCG	CCCAGGGTTGTTGTGA	TET	268-281	TD67-55	2	0.792	GDB:200347	Z24350
D11S1349	AGAGAGCACTTGAGTGAAGG	GGACTTTTCATCCCAAATCT	FAM	133	TD67-55	2	0.841	GDB:199969	Z24387
D11S1315	CTCTGGCCTTTGTCATACAG	GAGTAATCACCGTCAGTGTGA	FAM	148	TD67-55	2	0.555	GDB:199294	Z23620
D11S1334	TGCAGCATAGNCCTGT	AGCTTTATTGAAAGTCATTTTTG	TET	134-150	TD67-55	2	0.8	GDB:199747	Z24022
D11S4116	AACTGGTCCTTTAGACAGACAA	GAAAGCATCTCTATCTGCTGTG	TET	205-244	TD67-55	2	0.803	GDB:608187	Z52745
D11S1794	GCTCCTAAAGGGTGGAGAC	TGCACTCAAAGCTGACAA	FAM	249-273	TD67-55	2	?	GDB:270372	Z51378
D11S926	GTGTGATGTATATGCTCATTATTC	CAATTCTGGTGTACAATTTGC	TET	135-145	TD67-55	2	0.74	GDB:188432	Z17018
D11S1307	TGTCCGTTCTAGGAATTTGA	AGCGTTTTCACAGTCAGAGA	TET	147	TD67-55	2	?	GDB:4560016	Z23425
D11S4170	CACACATCATTCCTCAGAAG	AATTTAATGCAATCCCAATC	FAM	158-198	TD67-55	2	0.78	GDB:611490	Z53745
D11S861	CTGAAACCAAGTGAAAAGGAGA	AAGCTCCATTGTCTTCTGGC	FAM	154	TD67-55	2	0.7	GDB:182240	M87674
D11S4193	AAAGTAAAGGTACGGTGCTCTT	GTGAGCCAATCCCTTAAAAC	TET	158-162	TD67-55	2	0.522	GDB:613236	Z50822
D11S4121	GAACACAATCCCATTTATATTAGC	TCAGCCTCACAAAGTGC	TET	92-122	TD67-55	2	?	-	Z52872
D11S1791	ATGAAAAGTAATGACCCCA	TCTCAGATGCCTCACAGT	FAM	256-264	STD55	2	?	GDB:270369	Z51298
D11S921	TGCATTCAACAAATCAACA	CTTGGACCATTTAATCTAAAGTAAT	FAM	243-255	TD67-55	2	0.708	GDB:188380	Z16968
D11S902	CCCGGCTGTGAATATACTTAATGC	CCCAACAGCAATGGGAAGTT	FAM	145-163	TD67-55	2	0.806	GDB:187919	Z16521
D11S4138	GTGCTGACCGCTCCAAGG	CCAAAGGGGTTAATAGGGGTCCA	TET	181-211	TD70-58	2	0.822	GDB:609603	Z53178
D11S1888	CCCCAGTACCCTGTATAGGC	CACTTGTGTGTTTGTATCGAGTCA	TET	207-227	TD67-55	2	0.79	GDB:307892	Z52228
D11S1310	GGCCTGGGAAACATCC	ACTTCCAGTAGGTNAGGGG	TET	222-228	TD67-55	1.25	?	GDB:583400	Z23518
D11S4096	TTAGGATTCACGGGCA	AGCTAATACATAGTGTAGCAGGG	FAM	262-348	TD69-57	2.5	0.912	GDB:606088	Z52420

Table 2.3. Chromosome 11p15 polymorphic markers used.
Name, primer sequences, PCR conditions, size and heterozygosity rate of alleles are shown.

2.4.6 Use of ABI310.

PCR products were pooled at suitable dilutions, from 1 in 5 to 1 in 50, with distilled water to a total volume of 50μl.

3μl of diluted PCR products were mixed with 10.5μl deionised formamide and 0.5μl internal size standard (TAMRA 350). Before electrophoresis, samples were denatured for 5 minutes at 95^{0}C and then snap cooled on ice. Electrophoretic separation was conducted in an ABI Prism 310 Genetic analyser (Applied Biosystems). Samples were electrokinetically injected for 5 sec at 15kV and run on a 47cm (36cm length to detector) 50μl ID capillary filled with the denaturing polymer POP4. The separation was conducted for 24 minutes at 15kV, 9μA, 10mW and 60^{0}C. Raw data was analysed with GeneScan software Version 2.1 (Perkin Elmer). LOH was determined by calculating the ratio of peaks between normal and tumour samples.

$$\frac{\text{Normal B x Tumour A}}{\text{Normal A}} = \text{Expected Tumour B (E)} \qquad \frac{\text{Exp. E}}{\text{Actual Tumour B}} = \text{R value}$$

LOH was present when the ration of alleles in tumour was 60% or less than the ratio of alleles in normal tissue DNA ($r \leq 0.6$).

2.4.7 Statistical methods

Computer packages InStat 2.01 (GraphPad software) and SPSS for Windows (SPSS Inc) were used for all clinicopathological correlations. A two-tailed Fisher's exact test was used to determine significant associations between marker/region LOH and clinicopathological variables. Kaplan-Meier and Log-rank analyses were done using SPSS to determine any associations of LOH with survival. Protein and gene expression associations were also determined using a Fisher's exact test. P values were determined on the basis of sample numbers used in each analysis. The null hypothesis is that there will be no correlation of samples with the variables representing the normal situation in the population. The p value is a measure of discrepancy between our data and the null hypothesis. A p=0.05 shows that there is a 1 in 20 chance of this event happening in the normal population. The p value does not however take into account the size of the sample set. An arbitrary cut-off should

therefore be decided upon. For LOH analyses, the number of samples used in each correlation ranged from n=21 to n=80. A p value of 0.05 is therefore not suitable so p=0.01 (1 in 100) was chosen. Survival analyses for LOH used a lower sample number so p=0.05 was used to indicate significance. The correlations of gene expression in 18 HOV tumours also used this cut-off.

All of the analyses performed are univariate, that is each variable does not take into account any dependencies on other variables. For example, the serous histology maybe associated with a more advanced stage and therefore these variables are not independent. A multivariate analysis of data would be more precise showing independent correlations of LOH or gene expression changes with clinicopathological variables.

Correlation between expression of individual genes was done using the data analysis tools on Microsoft Excel.

2.5 Bacterial culture and Plasmid preparation

2.5.1 Media and additives

L-Broth was made with 2.46g magnesium sulphate, 10g Bacto-tryptone, 5g yeast extract, and 5g sodium chloride made up to 1L with distilled water at pH 7.0. L-agar has 15g agar added per 1L of L-Broth.

SOC media was prepared with 2.0g Bacto-tryptone, 0.5g yeast extract, 10mM sodium chloride, 2.5mM potassium chloride, 20mM Mg^{2+}, and 20mM glucose in 100ml distilled water. This was filter sterilised and adjusted to pH 7.0.

Ampicillin was added to the above L-broth and L-agar at a concentration of 100μg/ml to select for transformants carrying the *amp* resistance gene. Ampicillin (Sigma-Aldrich) stock was prepared sterile at a concentration of 50mg/ml and stored at –20^{0}C.

X-gal and IPTG were used for blue/white colour selection with the pGEM-T Easy plasmid (Promega) to select for bacteria successfully transformed by insert containing plasmids. IPTG stock was made to a concentration of 100μM and used at a concentration of 0.5μM. A 2% (20mg/ml) X-Gal stock was made up in DMF to use at a concentration of 80μg/ml. Solutions were stored at –20^{0}C.

2.5.2 Bacterial strains

JM109 competent cells (Promega) were used for all transformations. They are a highly efficient cell line and compatible with blue/white colour screening. Stock cells were stored at -20^0C and thawed prior to use.

2.5.3 Plasmids

The pGEM-T Easy (Promega) plasmid was chosen for convenience of use. The 3Kb plasmid has 3' T overhangs for ease of cloning PCR products and *lac* operon sequences for use with blue/white detection. Insertional inactivation of the alpha-peptide of the enzyme ß-galactosidase disallows X-gal to be cleaved and white colonies are formed. It also carries an amp^R gene for selection.

CMV promoter driven vector pcDNA3.1/Zeo (+) (Invitrogen-Life Technologies) carries a zeo^R gene to allow selection with the antibiotic Zeocin. This 5kb plasmid was chosen for use in transfection of both Hyg and/or Neo resistant cell lines and has a BGH polyadenylation signal for efficient termination and increased stability of the transcript. The plasmid is not suitable for blue/white colour selection, but carries an *amp* resistance gene.

2.5.4 Bacterial transformation.

Full length PCR products with 3' A overhangs were generated and ligated overnight into pGEM-T Easy vector. A Promega ligation kit was used to ligate 3μl PCR product into 50ng vector with 3 units of T4 DNA ligase and Rapid ligation buffer. After incubation overnight at 4^0C, 2μl was added to 50μl JM109 cells and left on ice for 20 minutes. This mix was then heat shocked at 42^0C for 45 sec and added to SOC media to be incubated at 37^0C for uptake of the plasmid into bacterial cells. Culture was plated onto LB/ampicillin/IPTG/X-Gal plates and left overnight at 37^0C.

For the pcDNA3.1/Zeo (+) vector, no blue/white screening was used so the culture was plated onto LB/ampicillin plates.

2.5.5 Plasmid DNA preparation

Plasmids were isolated from lysed bacterial cells using the QIAprep miniprep kit (Qiagen). Briefly, cells from an overnight culture in L-broth at 37^0C were pelleted

and resuspended in 250μl buffer P1 (RNase A). 250μl of buffer P2 then added and inverted to mix. Protein was precipitated out using 350μl buffer P3 and pelleted by centrifuging for 10 minutes at maximum speed in a microfuge.

The DNA in solution was bound to a spin column and washed with 0.5ml buffer PB, then with 0.75ml buffer PE.

Plasmid DNA was eluted off the column with 50μl buffer EB (10mM Tris-Cl, pH8.5).

Presence of inserts in the purified plasmid was checked by EcoRI digestion for 2hrs at 37^{0}C and 1μl visualised on 2% agarose gel with ethidium bromide under UV light. Directionality of inserts was determined by DNA sequencing.

2.5.6 Sequencing of inserts.

Sequencing was carried out using an ABI prism dRhodamine terminator cycle sequence ready reaction kit (Applied Biosystems). A mix was made up of 8μl ready reaction mix (containing labelled oligonucleotides, deoxynucleoside triphosphates, AmpliTaq DNA polymerase, $MgCl_2$, and TRIS HCl buffer), 4μl (~50ng) plasmid preparation, 1μl (3.2pM) primer (either SP6 or T7), and made up to a total of 20μl with water. This mix was then incubated on an MJ thermocycler for 25 cycles of 94^{0}C for 10 sec, 50^{0}C for 5 sec and 60^{0}C for 4 minutes and kept at 4^{0}C until ready to precipitate. To each reaction was added 2μl 3M NaOAc at pH4.6, 50μl 100% EtOH and 1μl pellet paint (Novagen). This precipitated out the amplified DNA by standing at room temperature for 1 hour. The DNA was then pelleted by a 20 minutes spin at 4^{0}C. The pellet was then rinsed with 250μl 70% EtOH and air dried. The samples were sequenced on an ABI 3700 at the sequencing core of the MRC Human Genetics unit, Edinburgh.

2.5.7 Digestion of plasmids

Once directionality was established, plasmids were digested with SspI restriction enzyme for 2 hours at 37^{0}C. This mix contained 6μl plasmid, 1μl SspI, 1μl 10x buffer and 2μl water. SspI cuts pcDNA3.1/ Zeo once.

Complete plasmid linearisation was checked by running 1μl digested product on a 1.5% agarose gel compared to undigested plasmid.

2.5.8 Plasmid purification

Linearised plasmids were purified using QIAquick PCR purification kit. Five volumes of buffer PB are added to the digested plasmid. This is then placed into a spin column and centrifuged for 1 minute. The DNA is washed with 0.75ml buffer PE and then the plasmid is eluted in 50μl 10mM Tris/ HCl.

Plasmid DNA was quantified by spectrophotometry.

2.6 Human cell culture techniques

Sterile conditions were used throughout. Cell lines maintained on DMEM/10% FCS/ penicillin and streptomycin. Predetermined levels of antibiotics hygromycin and G418 were also added to the media as required. They were cultured at 37^{0}C, 5% CO_2 in humidified incubators. Sterile flasks, plates, glassware, solutions and other consumables were used. Tissue culture was carried out in a laminar flow hood.

Cells were harvested by washing twice in PBS and incubating with a minimal volume of trypsin. Then resuspended in 10% FCS media and pelleted at 1,000g for 5 minutes for further manipulation.

Cells were frozen in liquid nitrogen in a freezing mix composed of 10% DMSO in FCS. They were stored initially for 24 hours at –70^{0}C prior to this.

Recovery was by rapid thawing of vials in a water bath at 37^{0}C followed by pelleting in media and plating with fresh DMEM in a suitable sized flask.

2.7 Transfection of plasmid DNA into cell lines.

2.7.1 Transfection reagents

Two different transfection solutions were used in the transfection of sense and antisense plasmids into OH1 cells. Lipofectin was initially used with the pCDNA3-Neo RALDH2 plasmids. Lipofectin (Invitrogen-Life Technologies) is a 1:1 (w/w) liposome formulation of the cationic lipid DOTMA and DOPE in membrane filtered water. It interacts spontaneously to form a lipid-DNA complex. Fusion of complex with cells results in efficient uptake and expression of DNA.

FuGene6 was the other method used in transfection of the candidate TSG plasmids into OH1 cells. This switch to using FuGene6 was because of problems with transfection efficiency and toxicity of Lipofectin. FuGene6 (Roche) is a non-

liposomal lipid based transfection reagent with low toxicity and high transfection efficiency.

2.7.2 Method of transfection

Both reagents are added to cells in a similar manner. Initially, $2x10^5$ cells are plated into each well of a six-well plate and left overnight or until ~70% confluent. Each sense or antisense plasmid transfection is done in triplicate.

Serum-free DMEM is warmed to 37^0C and the transfection reagent and plasmid DNA are warmed to room temperature. 100μl DMEM is then mixed with either 3μl or 6μl of FuGene6 and 1μg DNA. Using Lipofectin, 200μl DMEM is mixed with 2μl DNA and 10μl Lipofectin. These mixes are left to stand at room temperature for 15-30 minutes. Meanwhile, cells are washed with serum-free DMEM. The FuGene6 mixture is added directly to the cells. The Lipofectin mixture is added to 1.8ml DMEM, which is then poured over the cells. These are then placed in an incubator at 37^0C for 6hrs, after which the media is replaced with fresh DMEM containing 10% FCS.

After 48 hours each well is split 1/5 in maintenance media. Zeocin/Neomycin selection is added 24 hours later.

2.7.3 Selection conditions

Bacterial gene *zeo* confers resistance to the antibiotic zeocin in mammalian cells. Zeocin is the commercial name of a formulation containing Phleomycin D1, a copper-chelated glycopeptide antibiotic of the bleomycin family. Zeocin allows the selection of cells expressing the *Sh ble* gene, which encodes a small protein (MW 13.665 kDa). The Sh ble protein binds zeocin with a strong affinity and inhibits its DNA strand cleavage activity allowing further cell growth.

Geneticin/G418/neomycin is an aminoglycoside related to gentamycin that inhibits translation by binding to the small subunit of ribosomes. The *neo* resistance gene encodes aminoglycoside 3'-phosphtransferase whose expression allows resistance to geneticin through its phosphorylation so allows translation and cell growth.

Hygromycin is an aminoglycoside antibiotic, which inhibits protein synthesis. The bacterial gene hph^R, encodes a kinase which inactivates hygromycin by phosphorylation so protein synthesis may occur.

Kill curves for antibiotics were previously determined by Dr Hani Gabra for neo/hyg and for zeo by Dr Karen Watt. Cells with resistance are maintained on 150μl Hyg (7.5mg), 175μl Zeo (17.5mg) and 2ml G418 (400mg) per 100ml DMEM.

2.7.4 Picking of resistant clones

Cells were maintained under selection conditions which killed controls. resistant colonies arising from single cells were picked at 2-6 weeks post antibiotic selection. . When colonies reached >1mm, the flask was washed twice with PBS and colonies picked by dislodging cells into the tip of a fine tip pastette containing trypsin. They were transferred to 24 well plates containing 1ml of selection media, refed every 3-4 days and passaged up to larger flasks as required.

2.7.5 Determination of Insert expression and endogenous expression change

2.7.5.1 Determination of insert presence and correct orientation.

DNA was extracted from each of the individual clones and used in PCR to determine insert presence. Using SP6 and T7 primers, which bind to sequence within the plasmid, and the forward/reverse primer used in cloning the full-length cDNA, both the presence and direction of the insert could be seen.

2.7.5.2 Determination of insert expression.

RNA was extracted from each of the clones using the Absolutely RNA™ RT-PCR mini prep kit as previously mentioned. From this, cDNA was made and used in RT-PCR to determine insert expression. The SP6 and T7 primers lie within the region of plasmid which is transcribed. These primers were therefore used in conjunction with the forward/reverse primer used above. A control cDNA was generated for each clone which were all found to be negative.

2.7.5.3 Determination of endogenous expression.

Those clones that have insert expression were used in RT-PCR to determine if there is an alteration in the levels of endogenous cDNA as compared to normal. For example, if an antisense transcript knocks out endogenous expression, this alteration can be detected using primers designed outwith the full-length coding sequence within the plasmid.

Clones with alterations in endogenous expression can then be used in functional studies to determine whether the gene has an effect on phenotype.

2.7.6 Growth analysis of transfected clones

In order to determine cell concentration for experimental set up, trypsinised cells were resuspended in a minimum of 1:1 trypsin to media and counted on a haemocytometer, cells were then diluted down appropriately for seeding.

Cells were seeded from 70% confluent flasks into 24 well trays at 2 x 10^3 cells per well, in 1 ml normal growth media (day 0) in triplicate wells. Cells were counted every 5 days, here after for a total of 15 days.

To count cells, they were washed with 1ml PBS then 250μl trypsin added and plates incubated at 37°C. Once cells were detached, 250μl of normal growth media was added and the plates kept on ice. Cell suspension was syringed to break up any aggregations and 200μl added to 9.8ml sodium chloride (0.9%), which was then counted by a coulter counter. Each well was counted twice, generating six counts for each cell-lines that were then averaged.

3. MMCT CLONAL HYBRID ANALYSIS

3.1 Microsatellite mapping of MMCT hybrids

Prior to my arrival, MMCT of chromosome 11 was carried out. OVCAR3 is an ovarian cancer cell-line from which clonal lines OH1 and OHN were generated by hygromycin and hyg/neomycin tagging. 556.1.5 is a mouse line containing a complete copy of neo-tagged human chromosome 11 for use as a resource for MMCT.

The transfer chromosome 11 (556.1.5) into ovarian cancer cell line OVCAR3 generated a number of clonal cell lines. The origins of these can be seen in Figure 3.1.

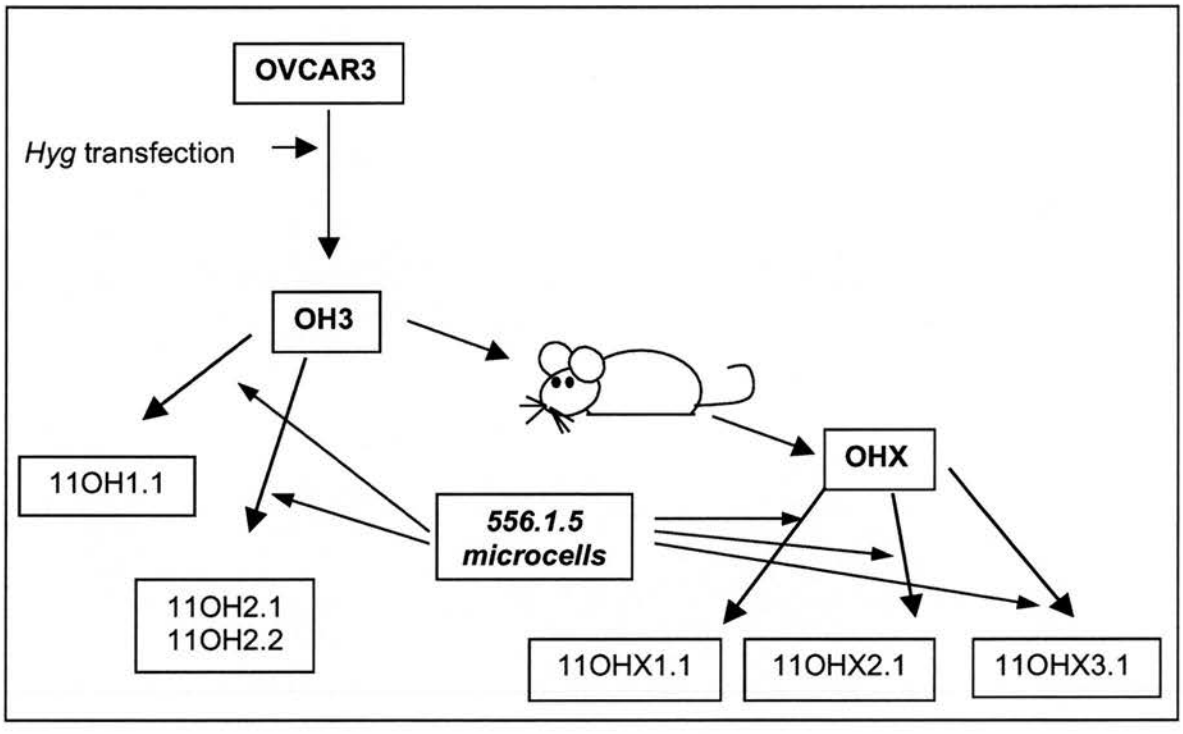


Figure 3.1. The generation of OVCAR3 derived hybrid cell lines using MMCT. OH3 and OHX are clonal derivatives of OVCAR3. OHX is generated from a xenograft of OH3. Independent MMCT were carried out to form clonal hybrids.

To determine the extent of chromosome 11p15 transferred into each of the hybrids, microsatellite maps were generated. Of 14 microsatellite markers situated along 11p15, only 8 were found to be polymorphic between the parental lines OVCAR3 and 556.1.5. Examples of chromosome transfer as detected by peak size differences are seen in Figure 3.2.

The regions of chromosome 11p15 transferred in the hybrid cell lines 11OH1.1, 11OHX1.1, 11OH2.1, 11OHX2.1, 11OH2.2 and 11OHX3.1 was determined using these 8 polymorphic markers (Figure 3.3). The original microsatellite mapping of these lines for 11p15 used only two markers so was not very comprehensive (see Figure 1.4).

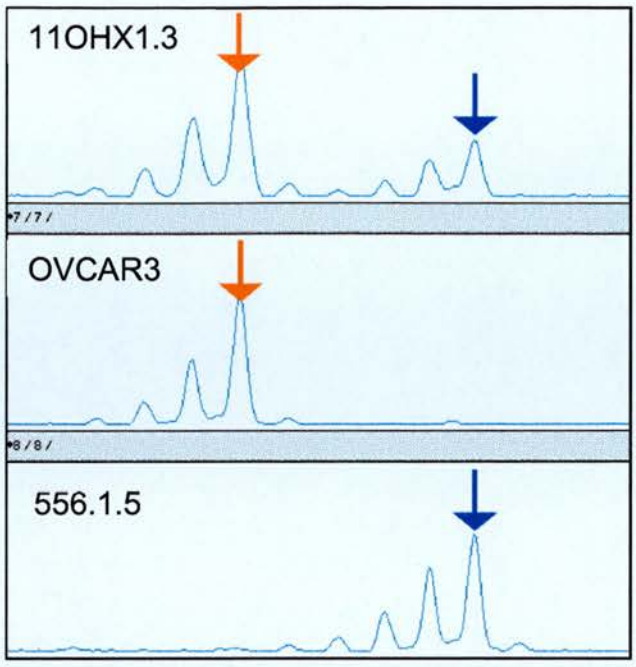
Hybrid cell-lines 11OH1.1, 11OHX1.1 and 11OHX3.1 contain the whole 11p15 region which agrees with the original two marker data. 11OH2.1, 11OH2.2 and 11OHX2.1 have only partial transfer. This fragmentation at 11p15 was not detected in the preliminary maps. 11OH2.1 and 11OH2.2 were generated from the same Chr 11 transfer and show loss of the same regions and the same growth characteristics. Further mapping has successfully minimised the areas along 11p15 which may contain putative TSGs to pTel-D11S932 and D11S569-D11S419.

3.2 Revertant hybrid mapping

Clonal hybrid cell lines 11OHX1.1, 11OHX2.1, 11OH2.2 and 11OHX3.1 were passaged ten times the aim being to generate revertant clones which will have lost the growth suppression due to loss of donated chromosome 11 regions (assisted by Eric Miller). In all, from these cell lines, we generated 48 clones. It should be noted that these clones have not yet been assessed for growth and tumorigenicity characteristics, they are therefore only putative revertants.

Again, using the eight polymorphic markers, microsatellite maps were generated showing the regions of transfer and by comparison to the parent hybrid, any regions of loss of the donor 556.1.5 chromosome (Figure 3.4).

a)



b)

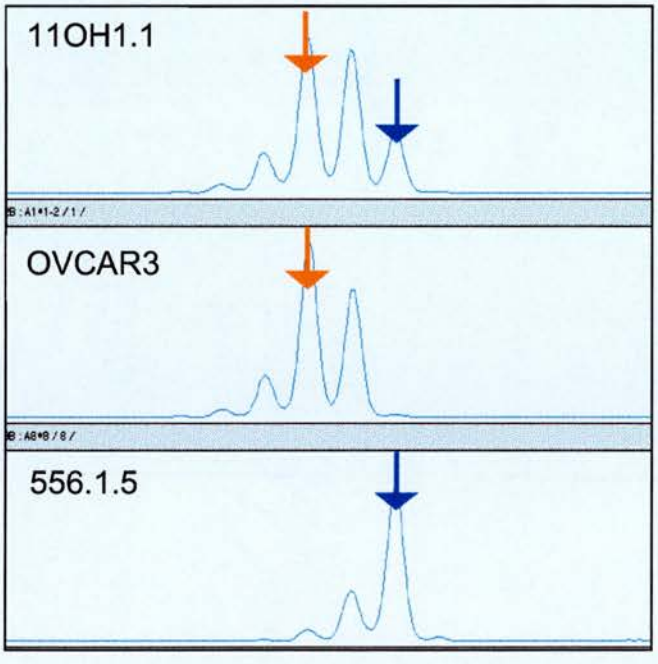


Figure 3.2
Example traces of allele peaks from parental and hybrid cell-lines. Fam-labelled primers for a)D11S922 and b)D11S921. Arrows point to polymorphic peaks between parents OVCAR3 and 556.1.5.

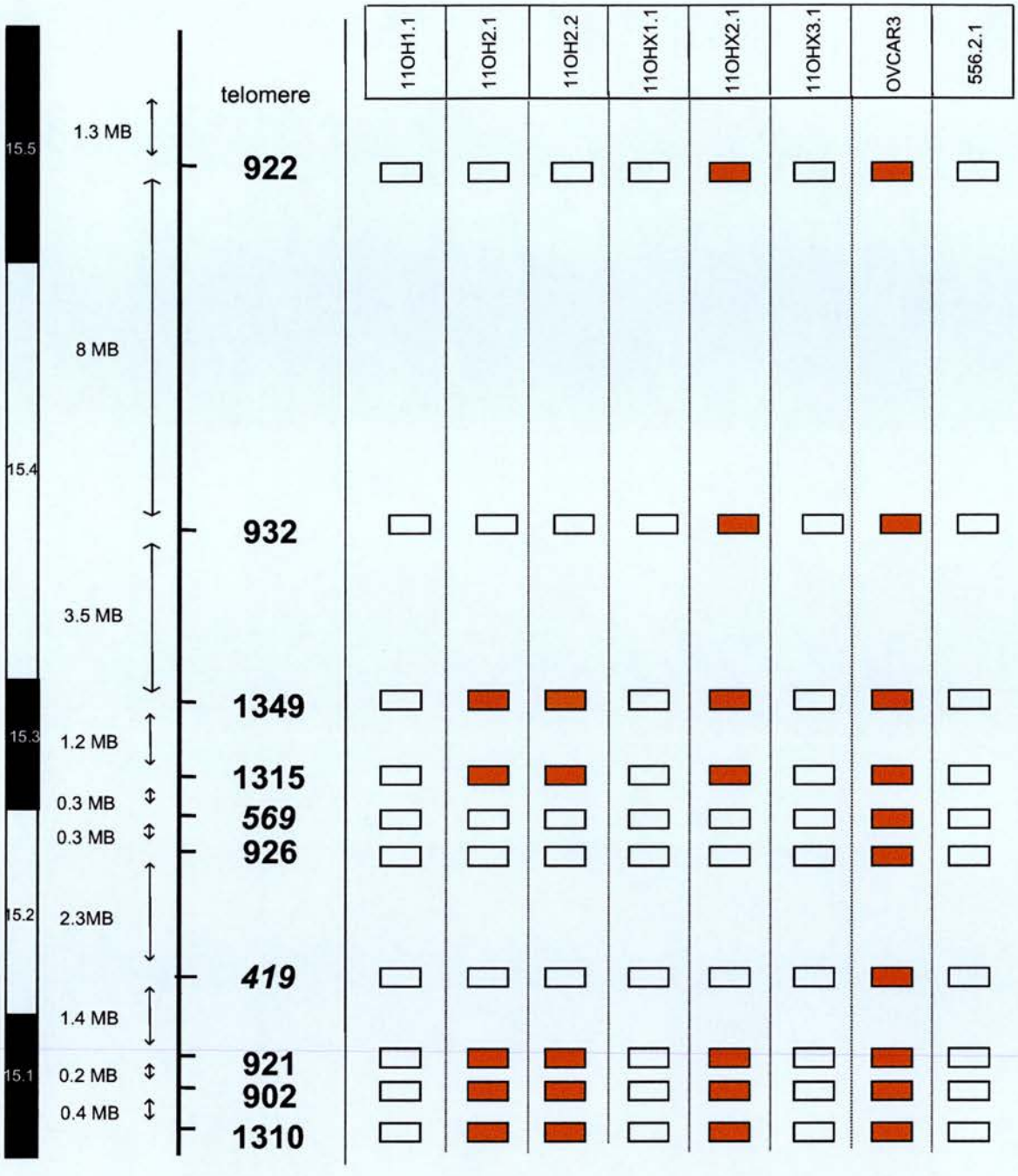


Figure 3.3
The extent of chromosome 11p15 transfer in clonal hybrid cell-lines generated by MMCT. A white box= transfer of the 556.1.5 Chr 11 marker, a red box= endogenous OVCAR3 Chr 11. Microsatellite loci and distances between these are seen on the left. Data for markers *D11S569* and *D11S419* from original mapping.

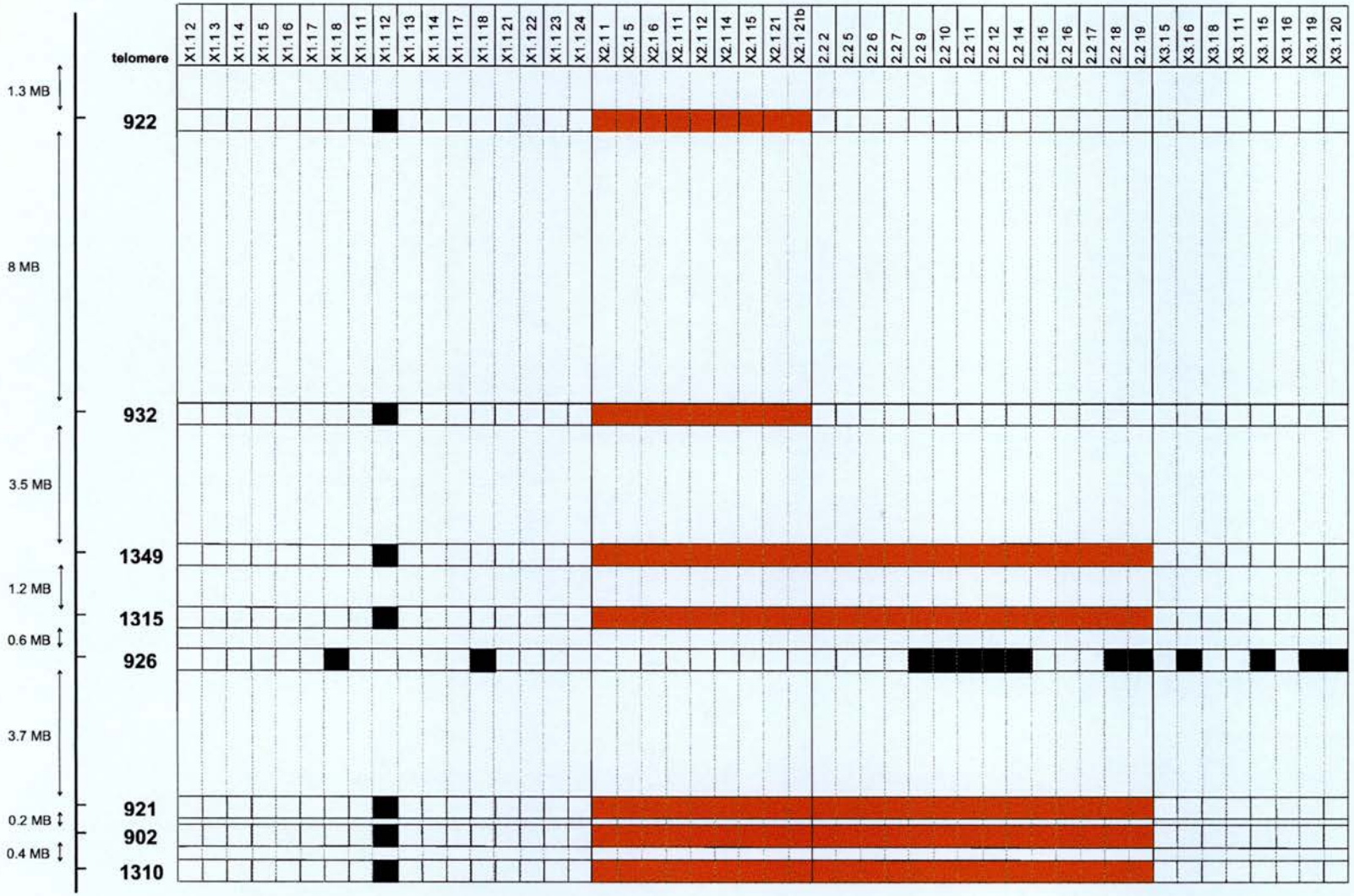


Figure 3.4 Extent of Chromosome 11p15 transferred in passaged clones.
A white box= transfer, a red box= OVCAR3 chr 11 only (as parents), a black box= loss of marker.

Revertant clones were generated from cell-lines 11OHX1.1, 11OHX2.1, 11OH2.2 and 11OHX3.1.

Mapping of the 11p15 region in the passaged clones reveals few further losses of the donated chromosome. 11OHX2.1 parental line contains only the region spanning D11S569-D11S419 of 11p15. Passaged clones from this line did not show further losses at 11p15. This line interestingly exhibits faster growth than the other two 11OHX lines, suggesting that a growth suppressor may lie in those regions not contained in this clone. D11S926 marker appears to be the only marker that is lost in any of the passaged clonal derivative lines, with the exception of clone 11OHX1.1 12. Seven 11OH2.2 clones have lost D11S926 along with 4 11OHX3.1 and 2 11OHX1.1 clones.

The frequent loss of D11S926 in these clones may correlate with growth suppression and these functional studies are still to be undertaken.

The microsatellite map of clone 11OHX1.1 12 closely resembles a 11OHX2.1 clone and it is likely that this clone has been mislabelled.

3.3 Further mapping of 11OH2.1

MMCT hybrid 11OH2.1 was chosen as the cell-line to be used in difference analysis techniques with the aim of identifying genes associated with growth suppression. When previously mapped with the two markers at 11p15.2, the hybrid showed retention of this region. When I mapped further with the eight polymorphic markers used in the revertant analysis, regions of loss between D11S932 and D11S926 and also D11S926 to 11p15.1 were detected. In order to refine this region a further 14 microsatellite markers were used. Again some of the markers were found not to be polymorphic between OVCAR3 and the donor cell-line 556.1.5 so only 9 markers were added to the map (Figure 3.5). Nineteen markers were mapped in all.

The regions of transfer in 11OH2.1 span 9.9 MB from D11S1363 to D11S4188 and 4 MB from D11S569 to D11S419. It is hypothesised that these regions may therefore carry the genes causing growth and tumorigenic suppression.

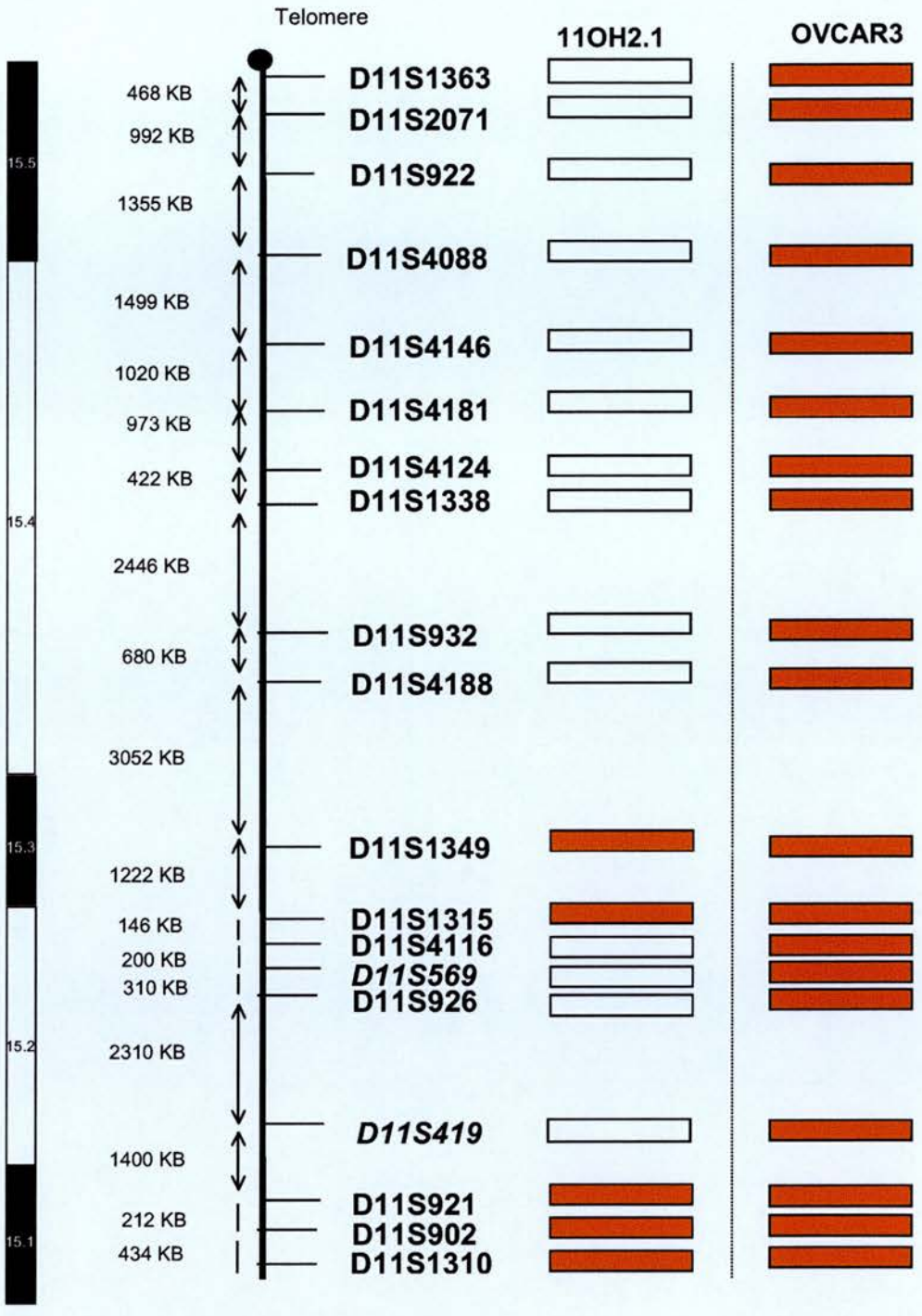


Figure 3.5 Extent of chromosome 11p15 transfer in MMCT hybrid 11OH2.1.
Distance between markers is seen on left. Markers run from telomere to centromere, 11p15.5 to 11p15.1. A white box= transfer, a red box= endogenous OVCAR3 Chr 11. D11S569 and D11S419 determined previously.

4. GENE EXPRESSION DIFFERENCE ANALYSIS: UNVALIDATED PRODUCTS

4.1 Identification of differentially expressed genes

With the aim of identifying genes involved in the growth suppressed phenotype, gene expression in microcell hybrid 11OH2.1 was compared to parental clone OHN using three difference analysis techniques. Transfer of chromosome 11 into hybrid 11OH2.1 caused growth suppression both in vivo and in vitro. The products identified as differentially expressed between this clone and OHN will therefore be associated with the growth suppressed phenotype. The methods of DD-RT-PCR, cDNA-RDA and Clontech HDFA were used to identify up and down regulated genes.

Dr Grant Sellar and Genevieve Rabiasz carried out DD-RT-PCR and the subsequent cloning and sequencing of differentially expressed products.

Dr Grant Sellar also performed the cDNA-RDA experiments which compare cDNA samples prepared from two different sources (11OH2.1 and OHN). The method identifies those genes present in the tester population as opposed to the driver so reciprocal experiments are needed to identify genes both up and down regulated. In all, five experiments were done, three identifying candidate oncogenes and two finding candidate TSGs that are down and up regulated in 11OH2.1 respectively.

The final difference analysis method used in the comparison of 11OH2.1 and OHN was doing Clontech High Density Filter Array (HDFA) hybridisations. Three arrays, each containing 1,176 oligonucleotide spots, were hybridised with RNA extracted from OHN and 11OH2.1.

4.2 DD-RT-PCR results

DD-RT-PCR products were subcloned and sequenced. In silico analysis of these sequences was performed to determine identity, location and function of products. Sequence matches were identified using online BLAST searches of the NR and HTGS databases. As the Human genome sequencing project progressed, more of these transcripts could be identified. The introduction of the NCBI Mapviewer also allowed pinpointing of transcripts within the human genome. Using these resources, the majority of transcripts were identified and all were subsequently positioned on chromosomes. Cellular functions and alternate gene nomenclature were found using OMIM at NCBI and the GeneCards database and subsequent links from this site. A

summary of these in silico analyses is shown in Table 4.1. Overall, 47 transcripts, representing 18 genes were identified as either up or down regulated in 11OH2.1 as compared to the parent line.

Of the 10 genes upregulated in the hybrid clone, one was located on chromosome 11 (KIAA0409).

a)

ACCESSION NO	GENE	TIMES SEEN	CHR	FUNCTION
AL158802	NPAS-3/MOP6	1	14q12-13	Signalling
AA283103	KIAA0409	1	11p15	Unknown
X67247	RPS8	10	1p34	Ribosomal protein
AA181431	Cytochrome c oxidase	3	8q	Mt protein
AL519840	ZDHHC4	2	7p	Unknown
various	mouse	7	-	-
AI381647	CGI-72	1	8q	Unknown
BF590583	MRPL41	3	9q34	Ribosomal protein
BE898409	PSMD1	1	2q37	Protein degradation
AW591192	ZAK	1	2q31	Protein modification

b)

ACCESSION NO	GENE	TIMES SEEN	CHR	FUNCTION
AF014807	CDIPT/PIS	3	16	Signalling
BE898762/V00662	MTCYT B	1	MT	MT protein
AB015228	RALDH2	5	15q	Protein modification
AC005823	?	1	17q	Unknown
BE671874	SKP2	1	5p	Cell growth/cell cycle
AC083805	?	1	12q	Unknown
AX061637	HSPC194	3	6p22	Membrane bound
AJ243666	NICE-5	2	1q	Protein degradation

Table 4.1 Genes a) up and b) downregulated in hybrid clone 11OH2.1 as compared to OHN as identified by DD-RT-PCR. GenBank accession number, gene name, chromosomal location, number of times transcript was detected and cellular function shown.

4.3 cDNA-RDA results

Gel extracted and purified difference products were shotgun cloned into plasmid pGEMt-easy. Having transformed competent cells, colonies were picked and inserts sequenced using dRhodamine. In all, 572 were successful. I took this sequence data and analysed the transcripts in silico, as described for the DD-RT-PCR products. A large number of up and down regulated genes were identified from the 5 cDNA-RDA experiments (Tables 4.2/4.3). Having collated the data, the 572 sequenced products were found to represent 231 different genes that are either up or down regulated in 11OH2.1 (Chr 11 hybrid) with respect to OHN.

One hundred and four genes were identified as upregulated in 11OH2.1. Of these, 9 were ribosomal proteins, 3 mitochondrial proteins, 22 of unknown function, 4 identified as mouse contaminants (due to the murine cotransfer with chromosome 11 from mouse donor line 556.1.5) and 66 characterised genes. Nine of the genes are

located on chromosome 11. Twenty-eight percent of genes are seen twice or more within the sequenced difference product transcripts.

One hundred and twenty seven genes were identified as upregulated in the oncogenic parental line OHN. These consist of 23 ribosomal proteins, 8 mitochondrial proteins, 8 of unknown function and 88 characterised transcripts.

In all, more candidate oncogenes than TSGs were identified. However, three experiments were done to identify genes upregulated in OHN resulting in 391 sequenced transcripts as compared to the 181 sequenced from the reciprocal two experiments. It is therefore not surprising that we identified more genes that are downregulated than upregulated in 11OH2.1. The majority of genes are only detected once within the sequenced transcripts with only 27% of genes detected twice or more. Fifteen percent of all genes were identified by two or more of the repeated RDA experiments. Curiously, SLPI was identified by all five RDA experiments, regardless of direction of expression.

Those genes identified as upregulated in OHN consisted of 18% ribosomal proteins and 6% mitochondrial products. Fewer ribosomal and mitochondrial products were found to be upregulated in 11OH2.1 (9% and 3%) suggesting that in general these proteins are associated with proliferation. Novel and uncharacterised genes were more commonly associated with growth suppression (21% vs 6%), probably because proliferation associated genes are well annotated and have been more widely studied.

cDNA-RDA is known to produce false positives. In our experiments, thirteen genes were found in both directions of subtraction. These are BCAT2, RPS16, RPL10, RPLPO, RPS14, RPL27A, GNAS1, ENO1, SLPI, SPINT2, PSMA7, DLC1 and beta-actin. These genes may not represent actual differentially expressed genes and would need to be validated further.

ACCESSION NO	PRODUCT	FUNCTION	CHR	Times seen	RDA exp.
X03559	F1ATPASEß/ ATP5B	MT protein	12p	1	9A-SPIK
U68418	BCAT2	MT protein	19q	1	9A-SPIK
U78678	TXN2/Thioredoxin 2	MT protein	22q	1	9A-SPIK
NM_001020	RPS16	Ribosomal protein	19	1	9A
NM_000999	RPL38	Ribosomal protein	17q	1	9A
NM_000994	RPL32	Ribosomal protein	3p	1	9A
M73791	RPL10/QM	Ribosomal protein	Xq	1	9A-SPIK
M17885	RPLPO	Ribosomal protein	12q	1	9A-SPIK
AF116710	PRO2640/RPS14	Ribosomal protein	5q	1	9A-SPIK
X69150	RPS18	Ribosomal protein	6p	1	9A-SPIK
U14968	RPL27A	Ribosomal protein	11p15	2	9A-SPIK
X73460	RPL3	Ribosomal protein	22q	1	9A-SPIK
NM_004494	HDGF/HMG1L2	Signalling	Xq	2	9A
NM_000214	JAGGED1/HAG1/AGS	Signalling	20p	1	9A
NM_016592	NESP55/GNAS1/POH	Signalling	20q	2	9A
NM_002087	Granulin/GRN/PCDGF	Signalling	17q	1	9A
NM_005682	TM7XN1/GPR56	Signalling	16	23	9A, 9A-SPIK
AF147747	BOG25/SH3BP4	Cell cycle/Signalling	2q	1	9A-SPIK
AF025304	EPHB2/DRT	Signalling	1p	1	9A-SPIK
L10612	MIF/GLIF	Signalling	22q	1	9A-SPIK
D16227	BDP-1/Recoverin/HPCAL	Signalling	2p	1	9A-SPIK
X76678	MAL/MTKL1	Signalling	2	1	9A-SPIK
X00588	EGFR	Signalling	7p	1	9A-SPIK
L11285	MEK2/MAP2K2	Signalling	19p	1	9A-SPIK
AF297709	NTKL/P105	Signalling	11q12	1	9A-SPIK
U21909	COFILIN/CFL1	Cytoskeleton/Signalling	11q13	1	9A-SPIK
AK022227	GBL	Signalling	c16p	1	9A-SPIK
AK026462	PGPL	Signalling	Xp	1	9A-SPIK
NM_001064	Transketolase/TKT	Metabolism/Protein modification	3p	3	9A
NM_003334	UBE1/GXP1/A1S9T	Protein Modification	Xp	3	9A, 9A-SPIK
NM_001084	PLOD3/LH3	Protein Modification	7q	1	9A
M98252	PLOD	Protein Modification	1p	2	9A-SPIK
D29643	KIAA0112/DDOST	Protein Modification	1p	1	9A-SPIK
E06719	P4HB/DSI/PDI	Protein Modification	17q	1	9A-SPIK
NM_001961	EEF2/EF2	Translational elongation	19p	5	9A, 9A-SPIK
U77700	HSGCN1/KIAA0219	Translation regulation	12	3	9A
NM_017827	FLJ20450	?Translation	19	1	9A
AF193054	PP3241/DDX27	Translation/Splicing	20q	2	9A-SPIK
NM_013282	ICBP90/UHRF1/TCF19	Transcription factor	6p	1	9A
U57316	GCN5L2/GCN5	Transcriptional activation	17q	1	9A
NM_001428	ENO1	Transcription factor	c1	2	9A
NM_006892	DNMT3B	Methylation/Gene expression	20q	1	9A
NM_003072	SMARCA4/BRG1/BAF190	Transcriptional activation	19	3	9A, 9A-SPIK
NM_001312	CRIP2	Transcriptional regulation	14q	1	9A
AF108459	UBN1	Transcription factor	16p	1	9A-SPIK
NM_003064	SLPI	Protease inhibitor	20q12	2	9A, 9A-SPIK
U78095	Bikunin/SPINT2	Protease	19	2	9A, 9A-SPIK
U44385	TIMP-2	Protease inhibitor	17q	1	9A
X64190	CAPNS1	Protein degradation	19q	1	9A-SPIK
AF054185	PSMA7	Protein degradation	20q	1	9A-SPIK
AB009398	PSMD13	Protein degradation	11p15	1	9A-SPIK
AF007162	CRYAB	Protein folding/ heat shock	11q22	1	9A-SPIK
U27515	MIP224/PSMC4	Protein degradation	19q	1	9A-SPIK

Table 4.2 Genes upregulated in 11OH2.1- candidate TSGs. As identified by cDNA-RDA.

ACCESSION NO	PRODUCT	FUNCTION	CHR	Times seen	RDA exp.
AB023154	KIAA0937	Unknown	11	8	9A, 9A-SPIK
AB002323	KIAA0325	Unknown	14	4	9A, 9A-SPIK
NM_006360	GA17	Unknown	11p13	1	9A
AB028991	KIAA1068	Unknown	7p13	1	9A
AK022702	Timeless/TIM/HTIM	Unknown	12q	2	9A-SPIK
BF115985	FLJ14775	Unknown	17	1	9A-SPIK
AB028965	KIAA1042	Unknown	3p	1	9A-SPIK
AF119848	PRO1580	Unknown	5q	1	9A-SPIK
AB040897	KIAA1464	Unknown	16q	1	9A-SPIK
AB033011	KIAA1185	Unknown	1	1	9A-SPIK
AB038651	LOC57106/KLP1	Unknown	19q	1	9A-SPIK
AF289485	MYG1/ C12ORF10	Unknown	12q	1	9A-SPIK
D38549	KIAA0068/CYF1P1	Unknown	15	1	9A-SPIK
AF175966	AMOTL2/LCCP	Unknown	3q	1	9A-SPIK
AB002308	KIAA0310	Unknown	9	1	9A-SPIK
AB040924	KIAA1491	Unknown	9	1	9A-SPIK
AK025994	FLJ22341	Unknown	17q	1	9A-SPIK
AF151912	CGI-54/PRO0989/BR-84	Unknown	20q	1	9A-SPIK
E27354	HM13/H13	Unknown	20q	1	9A-SPIK
AJ001258	NIPSNAP1	Unknown	22q	1	9A-SPIK
AB002313	KIAA0315/PLXNB2	Unknown	22q	1	9A-SPIK
AK025875	FLJ22222	Unknown	?	1	9A-SPIK
NM_001660	ARF4	Protein trafficking	3	1	9A
U32944	DLC1/PIN	Protein trafficking	12q	1	9A-SPIK
AF002020	NPC1	Protein trafficking	18q	1	9A-SPIK
T10807	SLC1A5	Transporter	19q	1	9A-SPIK
AF051976	DFNB3/MYO15	Cytoskeleton	17p	8	9A, 9A-SPIK
NM_001101	ßACTIN	Cytoskeleton	7p15	2	9A, 9A-SPIK
NM_005572	LAMIN A/C /LMNA	Cell structure	1q	3	9A, 9A-SPIK
AK000860	CORO1B	Cell structure	11q	1	9A
X51521	Ezrin/Villin2/VIL2	Cytoskeleton	6q	2	9A-SPIK
M34482	CK8/KRT8/CARD2	Cytoskeleton	12q	4	9A-SPIK
AF216941	CLIC5	Cytoskeleton	6p	1	9A-SPIK
NM_000424	KRT5/EBS2	Cytoskeleton	12q	2	9A-SPIK
M11315	COL4A1	Structural protein	13q	1	9A-SPIK
M95178	ACTN1/Actinin	Cytoskeleton	14q	1	9A-SPIK
AF202321	KRT19	Cytoskeleton	17q	1	9A-SPIK
M80899	AHNAK/Desmoyokin	Cell differentiation	11q12	1	9A
NM_006445	PRP8/DBP2	RNA splicing	6	1	9A
AB018331	KIAA0788	RNA splicing	7	2	9A
NM_001752	CAT/Catalase	Cell protection/ cell stress	11p13	1	9A
NM_001305	Claudin4/CLDN4/CPE-R	Membrane protein	7	4	9A, 9A-SPIK
NM_016561	BFAR/LOC51283	Apoptosis regulator	16p	1	9A
M57638	ASL	Urea cycle	7q	1	9A-SPIK
AF261085	GAPDH	Metabolism	12p	1	9A-SPIK
M81735	POLD1	DNA replication	19q	1	9A-SPIK
NC_001501	MMLV	Mouse contaminant	-	8	9A
Z93724	MURINE Shuttle Vector	Mouse contaminant	-	3	9A
AF090190	MURINE KINESIN	Mouse contaminant	-	3	9A
NM_007472	MURINE Peroxiredoxin	Mouse contaminant	-	6	9A

Table 4.2 cont. Genes upregulated in 11OH2.1- candidate TSGs. As identified by cDNA-RDA. GenBank accession number, gene name, function, chromosome location, number of times identified by RDA and experiment number shown.

ACCESSION NO	PRODUCT	FUNCTION	Times seen	RDA exp.
AF172368/U09500	Mt cytochrome b/MTCYTB	Mt Protein	4	5A,9B,9B-SPI
NM_005918	malate dehydrogenase 2/MDH2	Mt Protein	2	5A, 9B
U12694	cytochrome oxidase subunits I/II/COII	Mt Protein	5	5A,9B,9B-SPI
NM_001098	aconitase 2 /ACO2	Mt Protein	1	5A
L06328/L08666	VDAC2	Mt Protein	1	9B-SPI
X83218	atp5O/OSCP/TATPO	Mt Protein	1	9B-SPI
J04469	CREATINE KINASE/CKMT	Mt Protein	1	9B-SPI
NM_001190	BCAT2	Mt Protein	1	9B-SPI
NM_002952	LLREP3/RPS2/RPS4	Ribosomal protein	13	5A,9B,9B-SPI
NM_006013	QM/RPL10/NOV	Ribosomal protein	1	5A
NM_001025	RPS23	Ribosomal protein	2	5A, 9B-SPIK
NM_001013	RPS9	Ribosomal protein	1	5A
NM_001032	RPS16	Ribosomal protein	3	5A, 9B-SPIK
NM_001009	RPS5	Ribosomal protein	2	5A, 9B-SPIK
NM_001002	RPLPO	Ribosomal protein	5	5A,9B,9B-SPI
NM_000969	RPL5	Ribosomal protein	1	5A
NM_001006	RPS3A/MTFL	Ribosomal protein	1	9B
AF161494	HSPC145/MRPL15	Ribosomal protein	1	9B-SPI
AF116710/M13934	RPS14/PRO2640/EMTB	Ribosomal protein	1	9B-SPI
U12465	RPL35	Ribosomal protein	1	9B-SPI
X06617	RPS11	Ribosomal protein	2	9B-SPI
U14968	RPL27A	Ribosomal protein	1	9B-SPI
D87735	RPL14/CAG-ISL7	Ribosomal protein	3	9B-SPI
M36072	SURF 3/RPL7A	Ribosomal protein	1	9B-SPI
X67247	RPS8	Ribosomal protein	1	9B-SPI
AF325707	RPML2	Ribosomal protein	1	9B-SPI
U43701	RPL23A	Ribosomal protein	1	9B-SPI
NM_000987	RPL26	Ribosomal protein	2	9B-SPI
NM_000976	RPL12	Ribosomal protein	1	9B-SPI
NM_000981	RPL19	Ribosomal protein	1	9B-SPI
NM_021104	RPL41	Ribosomal protein	2	9B-SPI
NM_002574	PRDX1/peroxiredoxin/NKEFA/PAGA	Signalling	2	5A, 9B-SPIK
NM_006098	GNB2L1/RACK1	Signalling	7	5A,9B,9B-SPI
NM_000877	IL1R1	Signalling	1	9B
NM_000516	GNAS1/NESP55/CARD3/POH	Signalling	4	9B,9B-SPI
NM_004039	annexin a2/ANXA2/LIP2/CAL1H	Signalling	2	9B,9B-SPI
U61538	calcineurin b homol/CHP/P22	Signalling/ membrane traffic	1	9B-SPI
S80794/Z82248	YWHAH/14-3-3 ETA	Signalling	1	9B-SPI
M84349	CD59/ PROTECTIN/MIC11	Signalling	1	9B-SPI
NM_012302	latrophilin/LPHH1/LEC1	Signalling	1	9B-SPI
NM_007273	REA/GEFSP2/SCN1A	Signalling	1	9B-SPI
AB015226/NM_009022	RALDH2/ALDH1A2	Protein modification	50	5A, 9B
AB028981/XM_090586	KIAA1058	Protein modification	1	5A
NM_003333	UBA52/RPL40	Protein modification	6	9B,9B-SPI
XM_084111	RIBOPHORIN1/RPN1	Protein modification	1	9B-SPI
NM_001418	DAP5/NAT1/EIF4G2	Translation initiation	4	5A, 9B-SPIK
AF267861	EF1A/EEF1A1	Translation	1	9B-SPI
X69150	RPS18/KE3	Translation initiation	4	9B-SPI
NM_003754	EIF3S5	Translation	1	9B-SPI
NM_001959	EEF1B2	Translation	1	9B-SPI
U63131/NM_007065	CDC37	Cell cycle/ protein chaperone	1	5A
NM_006999/AF089896	TRF4-1/ POLS	Cell cycle	1	5A
NM_005196/NM_016343	CENPF	Cell cycle	2	5A, 9B-SPIK
J02763/M18981	calcyclin/S100A6/2A9/CACY	Cell cycle	1	9B-SPI
D84296/D84294	TCC3/TPRDIII/DCRR1	Cell cycle	1	9B-SPI

Table 4.3 Genes upregulated in OHN – candidate oncogenes. As identified by cDNA-RDA.

ACCESSION NO	PRODUCT	FUNCTION	Times seen	RDA exp.
X68060/NM_001068	topoisomerase IIß/ TOP2B	Transcription	1	5A
NM_006800	MSL3L1	Transcriptional activation	2	5A, 9B
NM_005762	KAP1/TIF1ß/TRIM28	Transcriptional repression	1	5A
AB020692	KIAA0885/D1S155E/UNR	Transcription	2	5A, 9B-SPIK
NM_003663	CGGBP1	Transcription	1	5A
U69127	FBP3/FUBP3	Transcriptional activation	1	5A
X77588/NM_003491	TE2/ARD1/DXS707	Nuclear organisation	1	5A
AF074723	MED6	Transcription	1	9B
NM_000970	TAXREB 107/RPL6	Transcription	5	9B,9B-SPI
AF0077044/NM_015972	RNA pol1 16K subunit/LOC51082	Transcription	1	9B
NM_006711	RNPS1	Splicing/ transcription	1	9B
X74070	BTF3	Transcription factor	1	9B-SPI
M37197	CBF2	Transcription factor	1	9B-SPI
M11353/NM_002107	H3F3A/HISTONE H3	Nuclear organisation	1	9B-SPI
NM_014263	YME1L1/MEA	Transcription	2	9B-SPI
NM_022731	NUCKS	Transcription	1	9B-SPI
NM_005644	TAF2J/TAF12	Transcription	1	9B-SPI
NM_002512	NME2/NME23B	Transcription	1	9B-SPI
NM_005517	HMG17	Transcription	1	9B-SPI
NM_005967	NAB1/NGF1A-BP	Transcriptional repression	1	9B-SPI
NM_003286	topoisomerase I/TOP1	DNA structure/ transcription	1	9B-SPI
NM_014071	AIB3/RAPL50/NCOA6	Transcriptional activation	1	9B-SPI
NM_003064	SLPI/HUSI-1	Protease inhibitor	10	5A,9B,9B-SPI
NM_003470	usp7/HAUSP	Protein degradation	1	5A
AF022815/NM_002792	xapc7/PSMA7	Protein degradation	2	9B-SPI
E12605/E12459	POLY UBIQUITIN	Protein degradation	1	9B-SPI
X12548/NM_001610	acp2	Protein degradation	1	9B-SPI
AF097362/J03909	ip-30/IFI30/GILT	Protein degradation	1	9B-SPI
U78095	bikunin/SPINT2/HAI-2/KOP	Protein degradation	1	9B-SPI
NM_016237	APC5/ANAPC5	protein degradation	2	9B-SPI
AF161425	HSPC307/DC2	Unknown	3	5A
AF077051/AB019392	PTD001/M9/HSPC029	Unknown	2	5A, 9B-SPIK
NM_012111	HSPC322/C14ORF3/CEP52	Unknown	1	9B
NM_006070/AF143407	trk fused gene/TFG	Unknown	1	9B
AF161519/NM_014187	HSPC171	Unknown	1	9B-SPI
AC006499	CB1	Unknown	93	5A, 9B
AC087369	CB2	Unknown	37	5A
AK001985	CB3	Unknown	1	5A
NM_018840/AF112213	RIP-5	Membrane traffic	1	5A
NM_004911	erp72/DCK	Protein secretion	1	5A
NM_022449/AL136938	RAB17	Transcellular transport	1	9B-SPI
AJ251830/AF317550	PIGPC1/ THW	Transmembrane protein	1	9B-SPI
NM_006831	HEAB	Protein transport	1	9B-SPI
NM_03746	dynein light polypeptide/PIN/DLC1	Protein transport	2	9B-SPI
NM_006280	trap delta/SSR4	Protein transport	1	9B-SPI
X00351	ß-actin	Cytoskeleton	1	5A
V00599	tubulin ß5	Cytoskeleton	3	9B-SPI
NM_021103/XM_002498	thymosinß10/TMSB10	Cytoskeleton	1	9B-SPI
L54057	COACTOSIN-LIKE PROTEIN/LMO4	Cytoskeleton	1	9B-SPI
NM_003234	TFRC/p90/CD71/TFR	Iron transport	1	5A
NM_003610	MRNP41/RAE1	Nuclear metabolism	1	5A
Y00339/NM_000067	carbonic anhydrase II/ CA2	metabolism	5	5A,9B,9B-SPI
AF024631	ANG2	Angiogenesis	1	5A
NM_004499	ABBP1/HNRNP A/B	RNA Binding	2	5A, 9B-SPIK
AF043338/NM_006273	Mt MCP3/ SCYA7	Invasion	1	5A

Table 4.3 cont.1 Genes upregulated in OHN – candidate oncogenes. As identified by cDNA-RDA.

ACCESSION NO	PRODUCT	FUNCTION	Times seen	RDA exp.
NM_001428	alpha enolase/ENO1	metabolism	4	5A,9B,9B-SPI
AL049733	tNOX/APK1 ANTIGEN/COVA1	Cancer antigen	1	5A
NM_006802	SF3A3/PRP9/SAP61	Splicing factor	1	5A
NM_000071	cystathionine ß synthase/CBS/HIP4	Homocysteine metabolism	1	5A
NM_003217	TEGT	Apoptosis	1	5A
AF028832/X15183	HSP90/HSPCA/LAP2	Stress response	2	9B
NM_005566	LDHA/LDH1	metabolism	2	9B
AF216292	GRP78/HSPA5/BIP	Stress response	1	9B
U45955	M6B1/GPM6B	Development	1	9B-SPI
AF107405	SFRS3/SPP20/X16	Splicing	1	9B-SPI
U79278/NM_005742	P5/D6S2650E	Protein folding	1	9B-SPI
XM_007502	HNRPC	Splicing	1	9B-SPI
AL035456	LOC200247	Stress response	1	9B-SPI
NM_020983	ADCY6/KIAA0442	cAMP biosynthesis	1	9B-SPI

Table 4.3 cont. 2. Genes upregulated in OHN – candidate oncogenes. As identified by cDNA-RDA. GenBank accession number, gene name, function, number of times identified by RDA and experiment number shown.

4.4 HDFA results

I cultured and counted the 11OH2.1 and OHN cells, and sent them to Clontech for hybridisation onto the cDNA arrays. The three arrays used were the Human 1.2k-I and –II arrays and the Human Cancer array.

From the arrays, 230 genes were found to be upregulated in 11OH2.1 and 384 were downregulated. In order to minimise this data set into a reliable number of candidates cut-off values were applied. An expression difference of >3-fold on the array and a difference of 50 or more units of signal intensity were used. Hybridisation of the two human 1.2k arrays however was poor so few genes met these criteria. For those genes upregulated in 11OH2.1 on these arrays, a cut-off of >2-fold was applied.

These cut-off values minimised the data set into a workable number. In all, 45 genes were upregulated in 11OH2.1 and 28 clones were downregulated, with two representing the same gene but on different arrays (Tables 4.4/4.5).

Of the 45 genes upregulated in hybrid 11OH2.1, four are found on chromosome 11. The fold differences range from 9.8 to 2.1 fold with the vast majority of genes being upregulated by <5-fold. All genes are characterised and none are ribosomal or mitochondrial protein encoding. This is most likely due to the selection of genes contained on the chips.

ACCESSION NO	PRODUCT	ROLE	CHR	RATIO	ARRAY
J05593	TIMP2	Protein degradation	17q25	8.9X	Cancer 1.2k
M62403	IGFBP4	Cell growth/signalling	17q12	7.3X	Cancer 1.2k
M99061	KRT2E	Cytoskeleton	12q11	7.0X	Cancer 1.2k
S85655	PHB	Cell growth	17q21	6.3X	Cancer 1.2k
X00588	EGFR	Signalling	7p12	6.3X	Cancer 1.2k
M63959	LRPAP1	Cell growth	4p16	5.5X	Cancer 1.2k
X16707	FRA1	Transcription factor	11q13	5.3X	Cancer 1.2k
U40434	CAK1/MSLN	Cellular adhesion	16p13	5.3X	Cancer 1.2k
M98776	KRT1	Cytoskeleton	12q12	5.0X	Cancer 1.2k
X53587	ITGB4	Signalling/ cell adhesion	17q25	4.9X	Cancer 1.2k
X94991	ZYXIN	Signalling	7q34	4.7X	Cancer 1.2k
M11233	CTSD	Protein degradation	11p15	4.7X	Cancer 1.2k
X67683	KRT4	Cytoskeleton	12q13	4.4X	Cancer 1.2k
M74387	L1CAM	Cell adhesion	Xq28	4.3X	Cancer 1.2k
D13889	ID-1	Transcription	20q11	4.3X	Cancer 1.2k
L11285	MEK2	Signalling	7q32	4.2X	Cancer 1.2k
U17077	BENE	Membrane protein	2q13	4.1X	Cancer 1.2k
D84064	HGS	Signalling	17q25	3.7X	Cancer 1.2k
X61587	RHOG	Cell cycle/ signalling	11p15	3.6X	Cancer 1.2k
U19796	P15/MAAT1	Melanoma antigen	16p13	3.5X	Cancer 1.2k
X13293	B-MYB	Transcription/ cell cycle	20q13	3.5X	Cancer 1.2k
U58048	PCOLN3	Protein degradation	16q24	3.4X	Cancer 1.2k
M59371	ECK	Signalling	1p36	3.4X	Cancer 1.2k
U33053	PRK1	Signalling	19p12	3.4X	Cancer 1.2k
U18018	ETV4	Transcription factor	17q21	3.1X	Cancer 1.2k
Y07604	NME4	Nucleoside metabolism	16p13	3.0X	Cancer 1.2k
U93236	MENIN	Transcription	11q13	3.0X	Cancer 1.2k
X56681	JUN-D	Transcription factor	19p13	3.0X	Cancer 1.2k
L08096	TNFSF7	Cell growth/ signalling	19p13	9.8X	Human 1.2k-I
M80397	POLD1	DNA synthesis	19q13	4.3X	Human 1.2k-I
M81934	CDC25B	Cell cycle	20p13	3.8X	Human 1.2k-I
M11886	HLAC	Immune response	6p21	3.8X	Human 1.2k-I
X58079	S100A1	Signalling /cell structure	1q21	3.4X	Human 1.2k-I
M96577	RBBP3/E2F1	Transcription factor	20q11	3.3X	Human 1.2k-I
AF060515	CYCLIN K	Transcription	14q32	3.0X	Human 1.2k-I
M86400	YWHAZ	Signalling	2p25	2.9X	Human 1.2k-I
L27211	CDKN2A	Cell growth	9p21	2.7X	Human 1.2k-I
M92381	TMSB10	Cytoskeleton	2p11	2.4X	Human 1.2k-I
U05340	CDC20	Cell cycle	1p34	2.4X	Human 1.2k-I
M73482	NMBR	Signalling	6q21	2.1X	Human 1.2k-I
M29874	CYP2B6	Electron transport	19q13	4.7X	Human 1.2k-II
U66199	FGF-11	Signalling	17q21	2.3X	Human 1.2k-II
U53476	WNT7A	Signalling	3p25	2.2X	Human 1.2k-II
U03865	ADRA1B	Signalling	5q33	2.1X	Human 1.2k-II
L19711	DAG1	Cytoskeleton	3p21	2.1X	Human 1.2k-II

Table 4.4. Genes upregulated in 11OH2.1 – candidate TSGs. As identified by HDFA. GenBank accession number, gene name, cellular function, chromosomal location, fold upregulation and array title all shown.

Twenty-seven different genes were identified by HDFA as being upregulated in OHN. Of these, the expression differences range between 17.0 and 3.0 fold. There are fewer genes upregulated in OHN than down regulated but that may be due to the differences in cut-offs used to identify expression changes (3-fold Vs 2-fold).

ACCESSION NO	PRODUCT	ROLE	RATIO	ARRAY
U35048	TCS22	Transcriptional repressor	17.0X	Cancer 1.2k
M11810	OAS1	Translation inhibition	7.4X	Cancer 1.2k
X05323	OX2	Membrane glycoprotein	7.4X	Cancer 1.2k
Z17227	IL10RB	Signalling	5.1X	Cancer 1.2k
M65062	IGFBP5	Cell growth/ signalling	4.9X	Cancer 1.2k
U69161	CC3/TIP30	Apoptosis	4.7X	Cancer 1.2k
U51004	PKCI1/HINT	Signalling	4.6X	Cancer 1.2k
M3410	IGFBP2	Cell growth/ signalling	4.2X 3.7X	Cancer 1.2k Human 1.2k-I
X17644	GSPT1	Cell cycle	4.1X	Cancer 1.2k
M12623	HMG17	DNA structure/ transcription	4.0X	Cancer 1.2k
U18321	DAP3	Transcription / apoptosis	4.0X	Cancer 1.2k
U83117	SUMO1/UBL1	Protein degradation	3.8X	Cancer 1.2k
M27364	EEF1A1	Translation	3.8X	Cancer 1.2k
L22253	9G8/SFRS7	Splicing	3.6X	Cancer 1.2k
M60974	GADD45A	Stress response	3.5X	Cancer 1.2k
J03824	UROS	Haem production	3.5X	Cancer 1.2k
AF015592	Cdc7	Cell cycle	3.3X	Cancer 1.2k
U22431	HIF1A	Transcription factor	3.3X	Cancer 1.2k
D14878	D123	Unknown	3.2X	Cancer 1.2k
X52946	PTN	Signalling	3.1X	Cancer 1.2k
S74678	HNRNPK	Cell cycle/ DNA structure	3.1X	Cancer 1.2k
D38551	RAD 21	Cell cycle	3.0X	Cancer 1.2k
M74816	CLU	Apoptosis	3.7X	Human 1.2k-I
X66945	N-Sam	Signalling	3.6X	Human 1.2k-I
U71207	EYA2	Development	4.5X	Human 1.2k-II
U10492	MEOX-1	Transcription	3.1X	Human 1.2k-II
X78669	ERC-55/RCN2	Signalling	3.0X	Human 1.2k-II

Table 4.5. Genes upregulated in OHN – candidate oncogenes. As identified by HDFA. GenBank accession number, gene name, cellular function, fold upregulation and array title all shown.

4.5 Comparison of genes identified by difference analysis.

Comparison of the products from each of the difference analysis techniques shows some genes have been identified as differentially expressed by two independent methods. None of the genes were identified by all three techniques.

Four products were identified as differentially expressed by both DD-RT-PCR and cDNA-RDA; MTCYTB, Cytochrome C oxidase (COII), RPS8 and RALDH2. Confusingly, there is a discrepancy between techniques in the direction of expression difference for RSP8 and COII. However, the fact that two independent methods have identified MTCYTB and RALDH2 as upregulated in OHN gives support to them as true candidate oncogenes.

There was no overlap of products between DD-RT-PCR and HDFA but this may be because so few genes were identified by DD-RT-PCR. The arrays represent a limited set of genes so this may further limit overlap of the techniques.

Of the 45 genes identified as associated with growth suppression by HDFA, four were also detected as upregulated using cDNA-RDA. These are TIMP2, EGFR,

MEK2 and POLD1. Only one gene was identified as upregulated in OHN by both cDNA-RDA and HDFA and that is the translation elongation factor EEF1A1. Thymosin beta 10 transcripts were identified by cDNA-RDA as more abundant in OHN. A 2.4-fold decrease in expression, however, was seen using HDFA. This discrepancy may be due to differences between the techniques.

To summarise, three difference analysis techniques have been used to identify, in total, 153 up and 157 down regulated genes in the growth suppressed microcell hybrid 11OH2.1. Due to the false positive rates of the methods, these products need to be validated for expression differences between OHN and 11OH2.1.

5. VALIDATION OF DIFFERENTIALLY EXPRESSED GENES

All three difference analysis methods are liable to identify false positive genes. In order to calculate the false positive rates and determine those genes that are truly differentially expressed, further validation of expression is required. Of the 321 transcripts identified as differentially regulated from the three difference analyses, 178 were further validated. Both Northern blotting and quantitative RT-PCR were used for validation. Genes were chosen for validation in different ways depending upon the method by which they were identified. Those genes which were identified as 'interesting', that is any which were previously associated with cancers, any positioned on chromosome 11 and others with interesting cellular functions such as roles in apoptosis and cellular proliferation were taken forward from DD-RT-PCR and cDNA-RDA. Genes from the HDFA hybridisations were chosen by strict cut-off values of >3-fold difference (>2-fold for genes up in 11OH2.1 on Human 1.2K arrays) and a difference of 50 or more units of signal intensity. Some products were identified by more than one method but only validated once. A number of other products were not validated due to failure of the PCR primers on the Light Cycler.

5.1 Expression validation: Northern Blotting

Initially, products identified by cDNA-RDA were validated using Northern blotting. Only those which were upregulated in OHN were tested. Examples of gene expression on Northern blots are seen in Figure 5.1. Products were chosen for validation by Northern Blotting based upon preliminary semi-quantitative RT-PCR analyses. Ten probes were generated from the sequenced transcripts and hybridised to membrane strips carrying OHN and 11OH2.1 total RNA. Of these 10 transcripts, 3 were validated as having a >2-fold difference in expression, as a suitable arbitrary cut-off, in using densitometry (Table 5.1). KIAA1058, HSPC307 and RALDH2 had upregulation of 2.17-fold, 3.88-fold and 10.53-fold respectively.

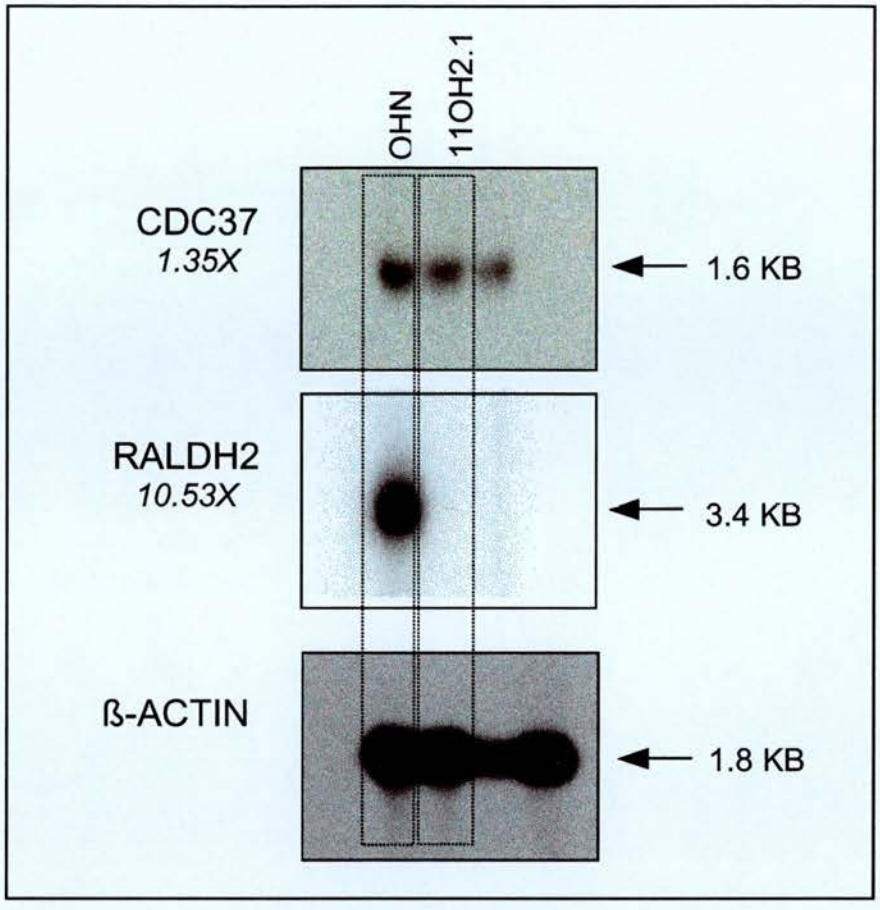


Figure 5.1 Example Northern Blots. Expression of CDC37 and RALDH2 in OHN and 11OH2.1 cell-lines.

Accession No	Product	Function	Fold difference
NM_006013	QM	Ribosomal Protein	1.70
AB028981/XM_090586	KIAA1058	Protein Modification	2.17
NM_001418	DAP5	Translation initiation	1.06
NM_003610	RAE1	Nuclear Metabolism	1.38
-	RDA43	Unknown	1.79
NM_004499	ABBP1	RNA binding	1.25
AF161425	HSPC307	Unknown	3.88
AK001985	CB3	Unknown	1.22
U63131/NM_007065	CDC37	Cell cycle/ protein chaperone	1.35
AB015226/NM_009022	RALDH2	Protein modification	10.53

Table 5.1. Transcripts examined for expression in OHN and 11OH2.1 by Northern blotting. Accession number, product, role and fold-difference is shown. RDA43 is a cDNA-RDA product with no matches to the genome databases. Differences determined by densitometry.

5.2 Expression validation: Quantitative RT-PCR

Subsequently, the method of quantitative RT-PCR using a Light Cycler was established in our Lab. This method was used in preference to Northern blotting, as it is easier and quicker to perform and is more sensitive in determining expression differences.

Seventy-eight genes down regulated with respect to growth suppression and 100 up regulated candidate TSGs were validated using quantitative RT-PCR. This represents 48% of down and 63% of all upregulated genes originally identified by the three difference analysis methods. I validated 72 of these candidate genes by Light Cycler, with the remainder being assayed by Dr Euan Stronach and Charlie Massie.

An example Light Cycler output showing expression of PSMD13 and beta actin in OHN and 11OH2.1 is seen in Figure 5.2.

For each of the difference analysis methods, the rate of false positives was high. DD-RT-PCR identified only one validated gene, RALDH2. cDNA-RDA detected the most candidates and 10% of those tested were validated by Light Cycler as differentially expressed. Just over 9% of genes found by HDFA hybridisation were verified. This data shows the importance of further validation of products identified by all difference analysis techniques. Table 5.2 summarises the number of genes identified and validated from all three methods.

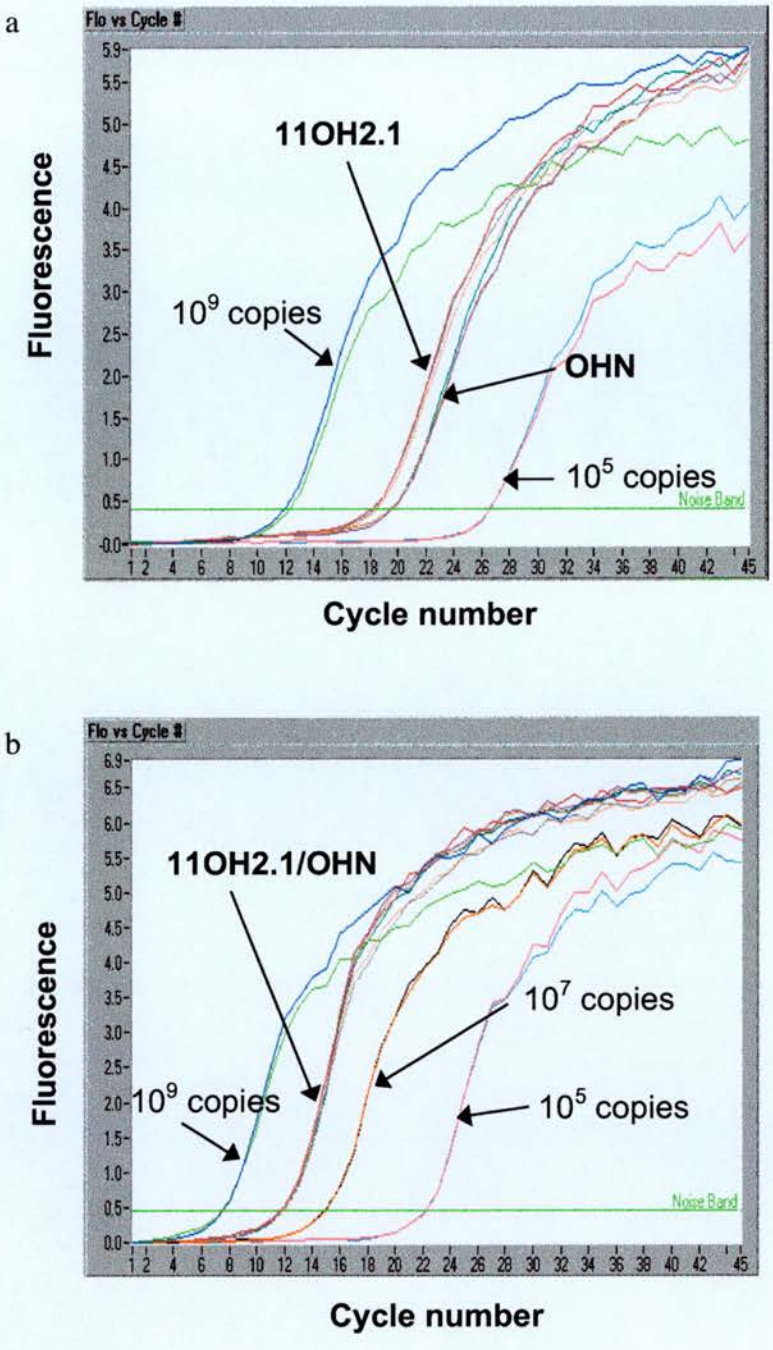


Figure 5.2 Light Cycler output graphs for expression of beta-actin and PSMD13 in OHN and 11OH2.1. a) Graph of expression of PSMD13 in triplicate samples of OHN and 11OH2.1 as compared to known standards of 10^9, 10^7 and 10^5 copies of the starting template. A clear difference between the cell-lines is seen with higher expression in 11OH2.1. b) Graph of expression of beta actin in OHN and 11OH2.1 as compared to standards. Expression is the same in the cell-lines.

Method	Number identified	Number tested on Light Cycler	Number validated as differentially expressed
Products identified as upregulated in hybrid 11OH2.1 (candidate TSGs)			
DD-RT-PCR	10	8	0
cDNA-RDA	104	63	8
HDFA	45	29	4
Products identified as downregulated in 11OH2.1 (candidate oncogenes)			
DD-RT-PCR	8	6	1
cDNA-RDA	127	48	3
HDFA	27	24	1

Table 5.2 Summary of products identified, tested and validated from all three difference analysis techniques as differentially expressed between OHN and 11OH2.1.

Twelve transcripts were validated as upregulated in 11OH2.1, and so were associated with growth suppression, representing only 12% of all genes tested by Light Cycler. These are all different genes, of which 11 have been characterised. One of these 11 represents a mouse gene that is present as contamination from the MMCT process (MMLV). Three transcripts are structural keratins and four are localised to chromosome 11. Two of the genes have been identified by two independent experiments as being upregulated in 11OH2.1 (TIMP2 and EGFR).

Five transcripts, representing four different genes were validated as potentially oncogenic. Two of these sequenced products, identified from both cDNA-RDA and DD-RT-PCR, are transcribed from the chromosome 15 gene RALDH2. Two more, both discovered by cDNA-RDA, are novel transcripts that I have named CB1 and CB2. The final product is a characterised gene IGFBP2. These five transcripts represent only 6% of the genes tested by Light Cycler.

The sixteen genes validated by quantitative RT-PCR as differentially expressed between OHN and 11OH2.1 are shown in Table 5.3.

Accession number	Gene	Function	Expression difference	Chromosome location	Method
Products Validated as upregulated in 11OH2.1 (candidate TSGs)					
U17077	BENE	Integral membrane protein	2.2	2q13	HDFA
X00588	EGFR	Signalling	2	7p12	HDFA/ cDNA-RDA
AB009398	PSMD13	Proteasome subunit	2.3	11p15.5	cDNA-RDA
M11233	CTSD	Lysosomal aspartyl protease	2.4	11p15.5	HDFA
U14968	RPL27A	Ribosomal protein	2.3	11p15.4	cDNA-RDA
AF007162	CRYAB	Heat shock protein	5.2	11q23	cDNA-RDA
NM_000424	Keratin 5	Structural protein	3.1	12q13	cDNA-RDA
M34482	Keratin8	Structural protein	2.5	12q13	cDNA-RDA
AF202321	Keratin19	Structural protein	2	17q21	cDNA-RDA
E27354	HM13	unknown	2.1	20	cDNA-RDA
JO5593	TIMP2	Matrix metalloprotease inhibitor	2.2	17q25	HDFA/ cDNA-RDA
-	MMLV	Artefact of MMCT	∞	-	cDNA-RDA
Products validated as down regulated in 11OH2.1 (candidate oncogenes)					
NM_009022	RALDH2	Aldehyde dehydrogenase	200-300	15q11.2	cDNA-RDA/ DD-RT-PCR
NM_000597	IGFBP2	Insulin-like growth factor binding protein	2.4	2q33-q34	HDFA
AC006499	CB1	Unknown	~200	4p16	cDNA-RDA
NT_023736	CB2	Unknown	1290	8p23	cDNA-RDA

Table 5.3 Validated differentially expressed genes between OHN and 11OH2.1. Accession number, cellular function, chromosomal location, fold-difference and method by which each gene was identified are listed.

5.3 Summary of gene validation

Sixteen genes have been validated, from 178 tested, as differentially expressed between the hybrid clone 11OH2.1 and parental line OHN.

Two methods of quantitative validation were used. There were discrepancies between Light Cycler and Northern data for two genes, KIAA1058 and HSPC307. These transcripts were found to show less than 2-fold expression difference by Light Cycler suggesting that the Northern data is only reliable for larger differences.

Of the fifteen validated human genes, four were down regulated upon introduction of chromosome 11. Three of the four candidate oncogenes are overexpressed by over 200-fold. These changes are markedly different to the small expression differences seen for candidate TSGs. Generally, the magnitude of decrease in expression of TSGs in tumours is however invariably less than the magnitude of induction seen for oncogenes.

All of the genes associated with growth suppression, that is the eleven human candidate TSGs, have small fold-differences between 11OH2.1 and OHN. This suggests that slight alterations in gene expression of a larger number of genes are enough to bring about a phenotypic change. With the introduction of chromosome 11 into OVCAR3 we would expect genes located on this chromosome to be induced rather than suppressed. There are however seven non-chromosome 11 positioned genes which are increased in the suppressed hybrid 11OH2.1 supporting the theory that genes are induced rather than repressed upon instigation of growth suppression.

6. VALIDATED CANDIDATE TUMOUR SUPPRESSOR GENES

6.1 Gene expression of candidate TSGs

6.1.1 Expression of candidates in ovarian cell-lines and primary tumours

Of 145 genes identified as upregulated in the MMCT hybrid 11OH2.1, and therefore associated with growth suppression, only 12 were validated as true alterations by quantitative RT-PCR. This represents only 8.3% of all candidates. Of these validated 12 genes, four (33%) were positioned on chromosome 11 (Figure 6.1). The validation enriched for genes that reside on chromosome 11, in particular at 11p15. Of the other eight genes, one was an apparent artefact of the MMCT process (MMLV).

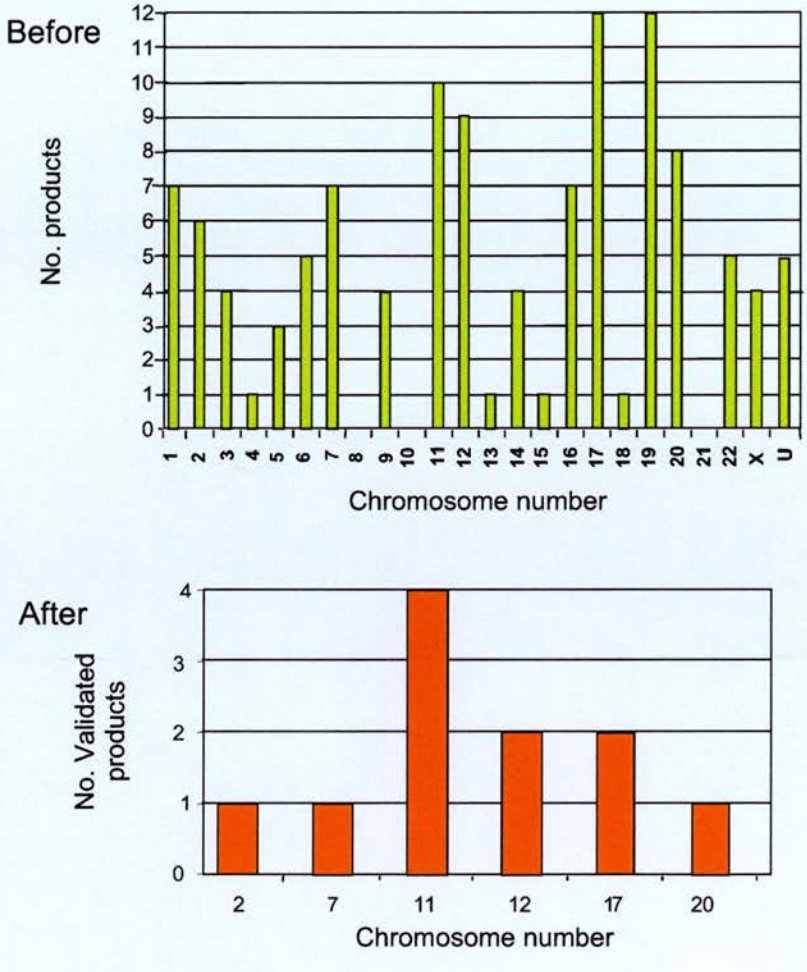


Figure 6.1 Chromosome positions of upregulated genes identified by difference analysis techniques before and after Light Cycler validation.

Our interest is in the identification of TSGs on chromosome 11 so the four differentially regulated genes located on this chromosome were further studied.

Evidence for a wider role in ovarian cancer for these genes required confirmation of their downregulation in a panel of cancer cell-lines and primary human ovarian tumours (HOV). A panel of 15 ovarian cancer cell-lines, 5 other cancer cell-lines and 18 HOVs were analysed for expression of each of the four chromosome 11 genes by quantitative RT-PCR (done by Dr Euan Stronach). A normal OSE sample Li1 was also used to determine normal epithelial expression of the genes. Light Cycler analysis was performed with OHN and 11OH2.1 in each run so results could be standardised between them. All cDNA samples were also standardised for actin ratios to ensure quantitative analysis.

Beginning with the most telomeric gene, PSMD13, expression in normal OSE sample Li1 was higher than any of the ovarian cancer cell-lines (Figure 6.2). Breast cancer cell-lines MDA and ZR75 both showed low expression but could not be corrected to its normal tissue as no such sample was available. Colon cancer cell-lines had high expression, up to >8-fold higher than the ovarian cancer lines. Only 4 HOV tumours showed a lower expression than Li1. Stromal expression may account for the high expression in the other 14 tumours.

Moving down the chromosome, CTSD shows much lower expression in all samples than in normal OSE, including all of the HOVs (Figure 6.3). The ovarian cancer cell-lines with highest expression are 59M and SKOV3. This is the only gene that shows a lower expression in all HOV samples and cell-lines than OSE.

Ribosomal protein RPL27a at 11p15.4 is expressed in a similar manner to PSMD13 (Figure 6.4). All but one of the ovarian cell-lines (PEO4) are expressed at a lower level than the normal OSE. Again the HOV samples have varied expression, possibly due to stromal expression. Expression of RPL27a is very high in the colon cancer cell-lines.

Finally at 11q22-23, CRYAB is expressed at low levels in all ovarian cancer cell-lines and expression is almost non-existent in the other cancer lines (Figure 6.5). HOVs samples vary widely in expression of the gene from 10-fold less to 5-fold more than normal OSE expression.

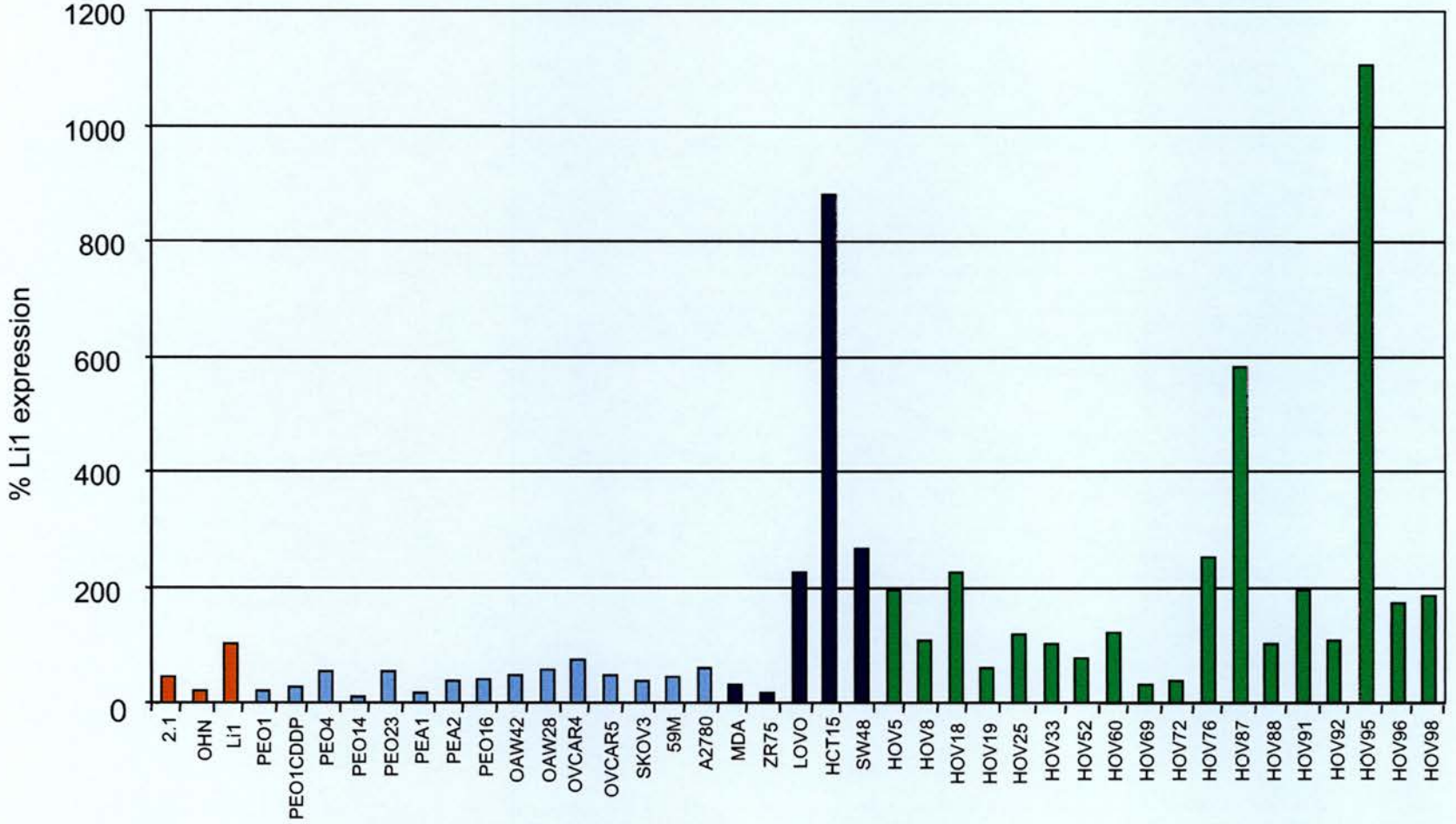


Figure 6.2 Expression of PSMD13 in a panel of cancer cell-lines and HOVs.
Green bars= HOV tumours, blue bars= non-ovarian cancer cell-lines, grey bars= ovarian cell-lines, red bars= normal OSE and original OHN and 11OH2.1 lines. Expression relative to normal OSE (100%).

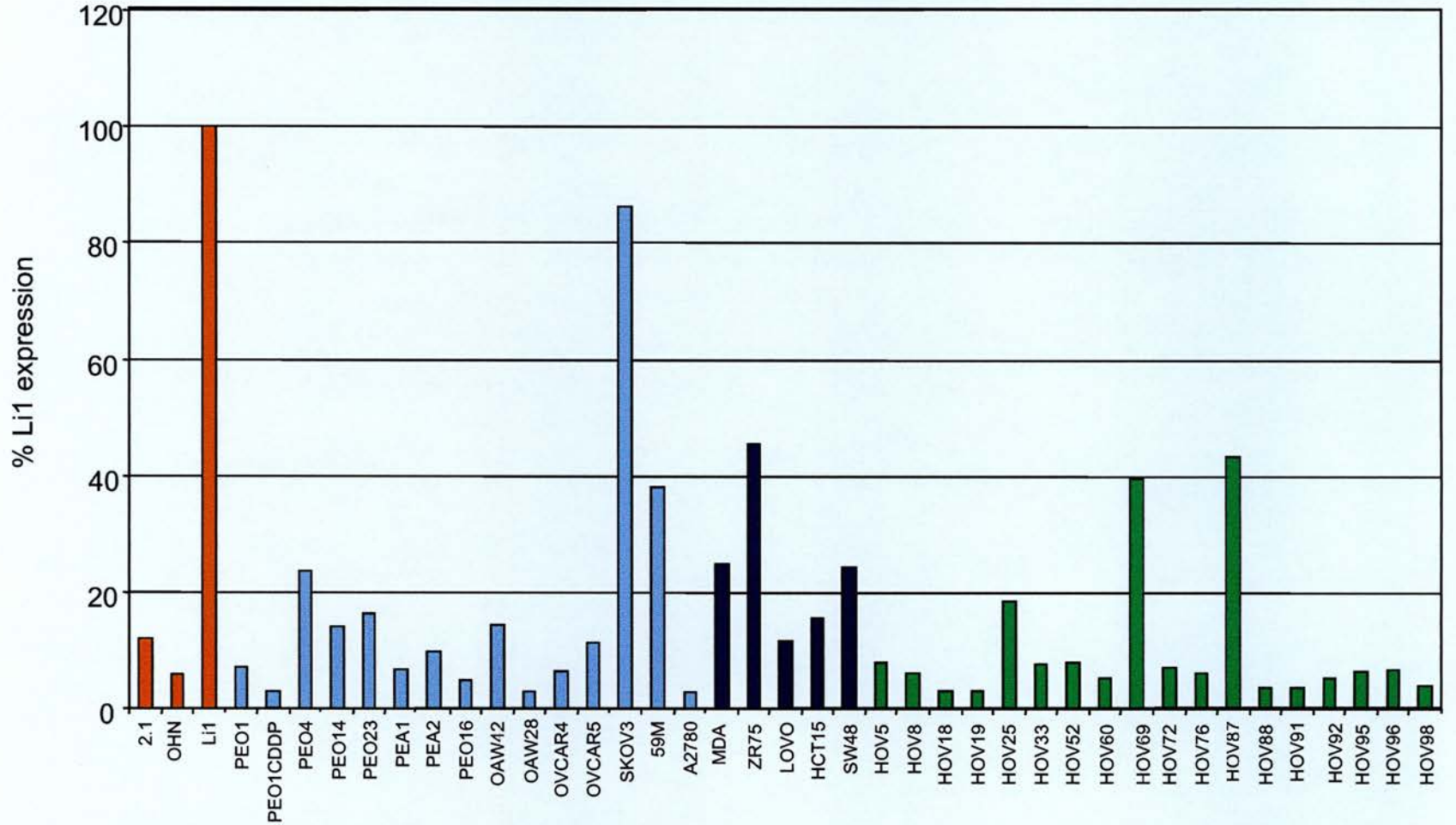


Figure 6.3 Expression of CTSD in a panel of cancer cell-lines and HOVs.
Green bars= HOV tumours, blue bars= non-ovarian cancer cell-lines, grey bars= ovarian cell-lines, red bars= normal OSE and original OHN and 11OH2.1 lines. Expression relative to normal OSE (100%).

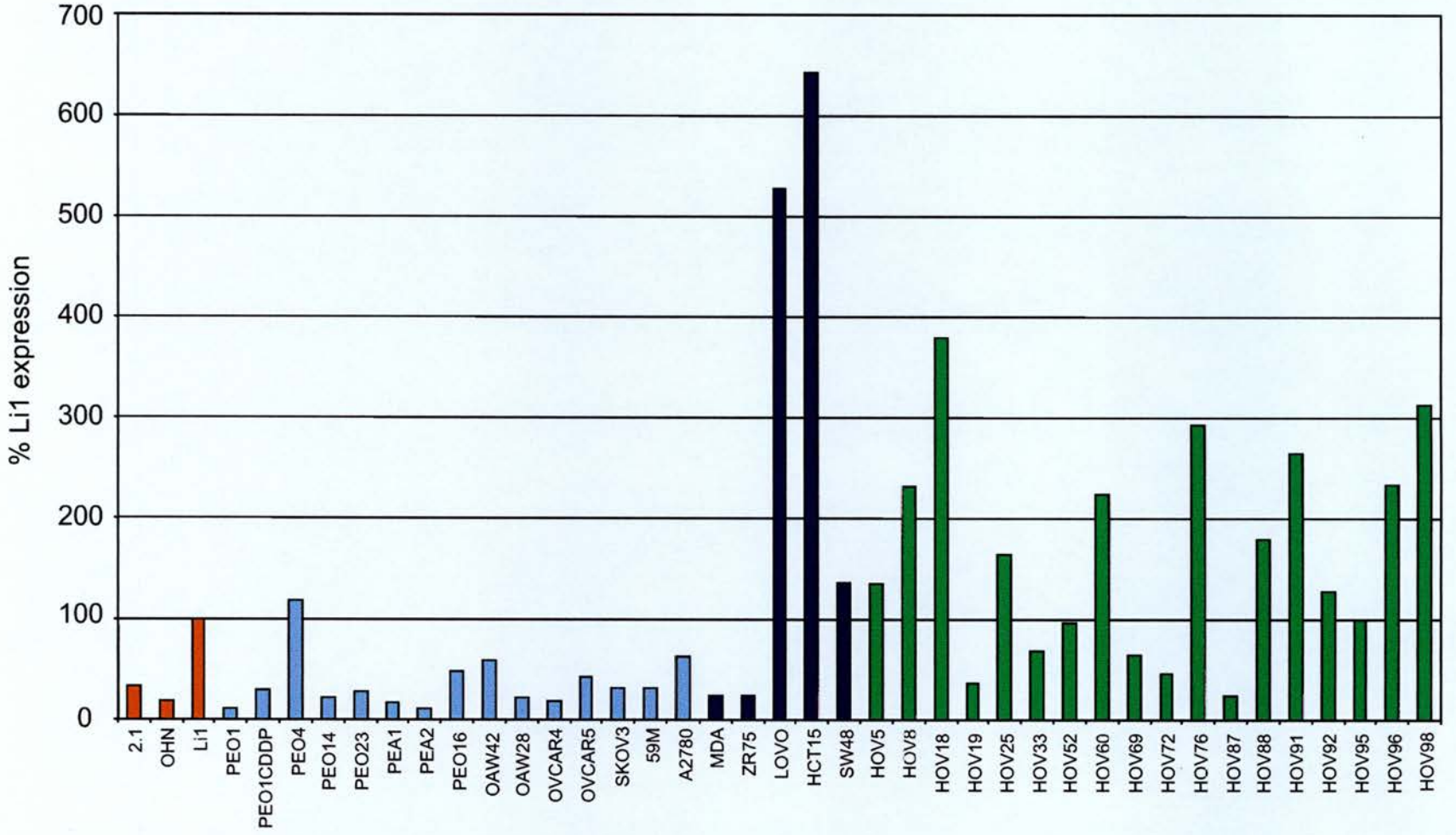


Figure 6.4 Expression of RPL27A in a panel of cancer cell-lines and HOVs.
Green bars= HOV tumours, blue bars= non-ovarian cancer cell-lines, grey bars= ovarian cell-lines, red bars= normal OSE and original OHN and 11OH2.1 lines. Expression relative to normal OSE (100%).

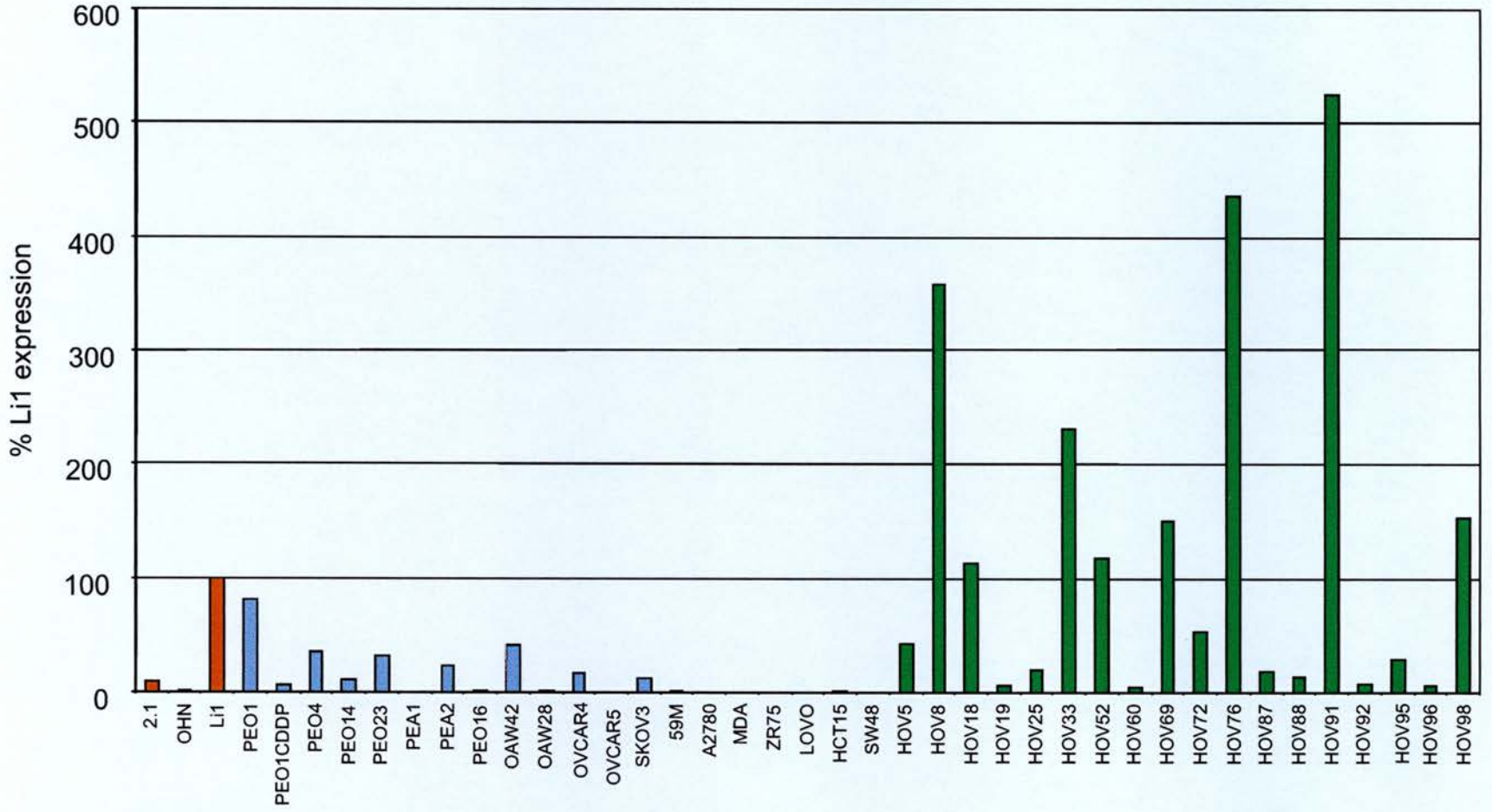


Figure 6.5 Expression of CRYAB in a panel of cancer cell-lines and HOVs.
Green bars= HOV tumours, blue bars= non-ovarian cancer cell-lines, grey bars= ovarian cell-lines, red bars= normal OSE and original OHN and 11OH2.1 lines. Expression relative to normal OSE (100%).

Northern blotting of the three genes located on chromosome 11p15 on total RNA extracted from a panel of ovarian and other carcinoma cell-lines, as performed by myself, showed none of these genes have alternative isoforms, so none under go alternative splicing. Expression of the genes on the Northern blots correlated reasonably well with data from the quantitative RT-PCR method (Figure 6.6). For all of the cell-lines, Light Cycler quantification appeared to be more sensitive in detecting levels of expression. Overall, Light Cycler RT-PCR is more accurate and sensitive than Northern Blotting.

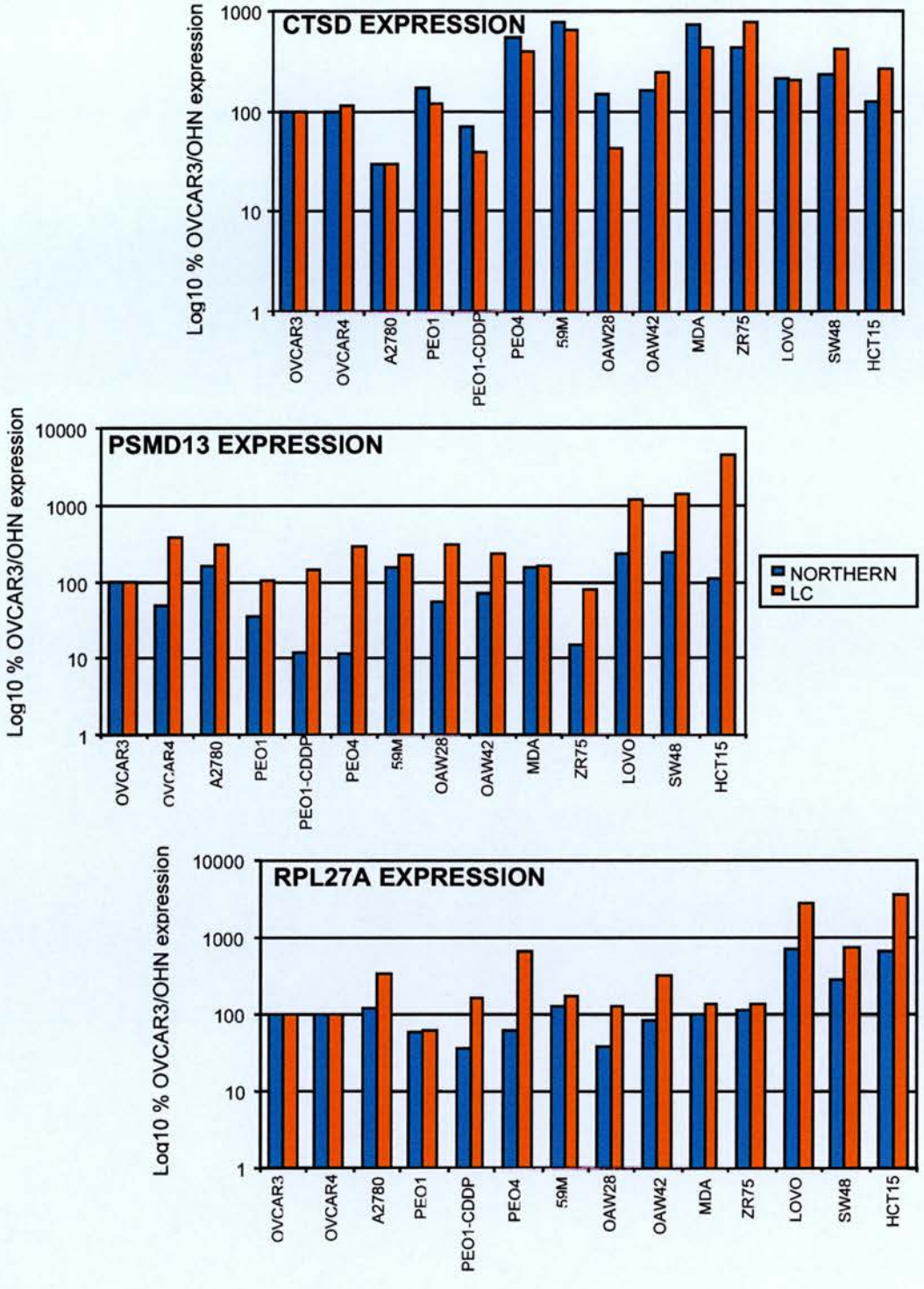


Figure 6.6.
Expression of candidate TSGs on a panel of ovarian cell-lines using two quantitative techniques. Red bars = expression determined by Light Cycler RT-PCR, Blue bars = Northern blotting. Expression shown as Log10 of percentage relative to OVCAR3/OHN expression.

6.1.2 Clinicopathological associations with candidate TSG expression

The relationship between expression of the four chromosome 11 genes in the 18 HOV samples and various clinicopathological variables was examined.

Arbitrary cut-offs of gene expression were determined for each clinicopathological variable. This therefore weakens the significance of the statistics derived from this data.

CTSD expression below 6% of normal OSE was found to correlated significantly with adverse survival (Figure 6.7). Median survival was 28 and 16 months respectively for patients with CTSD expression above or below 6% of normal OSE (p=0.0107). An expression level below 20% normal OSE for CRYAB also correlated with adverse survival with median survival at 25 months for high expression and 9 months for low expression (p=0.0126)(Figure 6.8). Fishers exact test showed that decreased expression of CTSD to below 20% of normal OSE level was associated with a high tumour grade (p=0.0179). CRYAB expression below 50% of normal OSE level was associated with non-serous tumour histology (p=0.0023). No other significant associations between expression level and tumour stage, grade or histology were found.

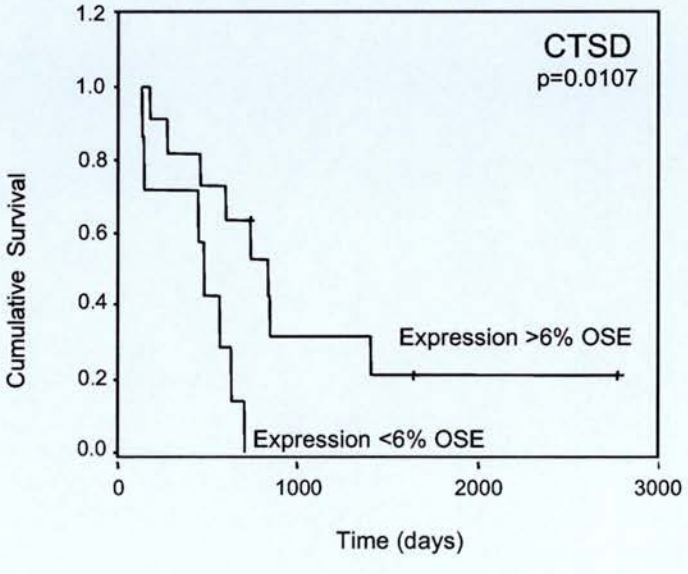


Figure 6.7 Kaplan-Meier survival curve for CTSD expression. Decreased expression, relative to normal OSE, of CTSD is associated with adverse survival (Log Rank p=0.0128).

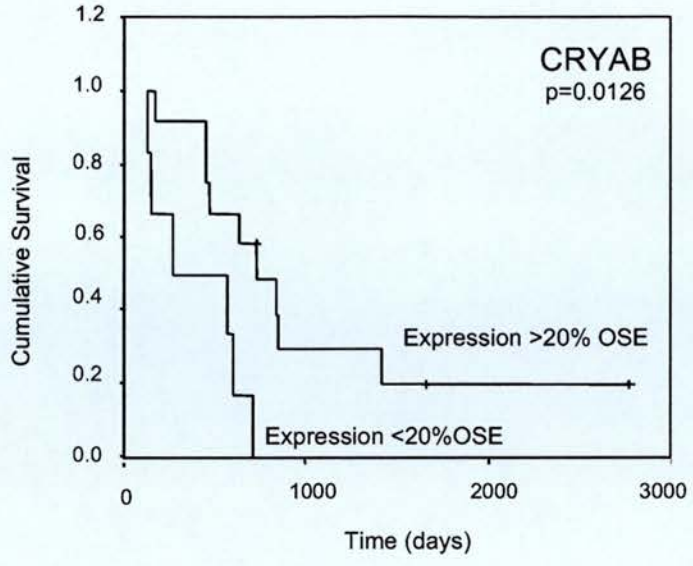


Figure 6.8 Kaplan-Meier survival curve for CRYAB expression. Decreased expression, relative to normal OSE, of CRYAB is associated with adverse survival (Log Rank p=0.0107).

Within the panel of cell-lines and HOV tumours, expression of PSMD13 and RPL27A was seen to be correlated very significantly ($p<0.001$) (Figure 6.9). This correlation is also detected on the Northern cell-line panel. Neither of the genes correlates with the expression patterns of CRYAB or CTSD.

The correlation between PSMD13 and RPL27A extended to colon and breast cancer cell-lines also suggesting that this co-regulation of expression is not ovarian tissue specific.

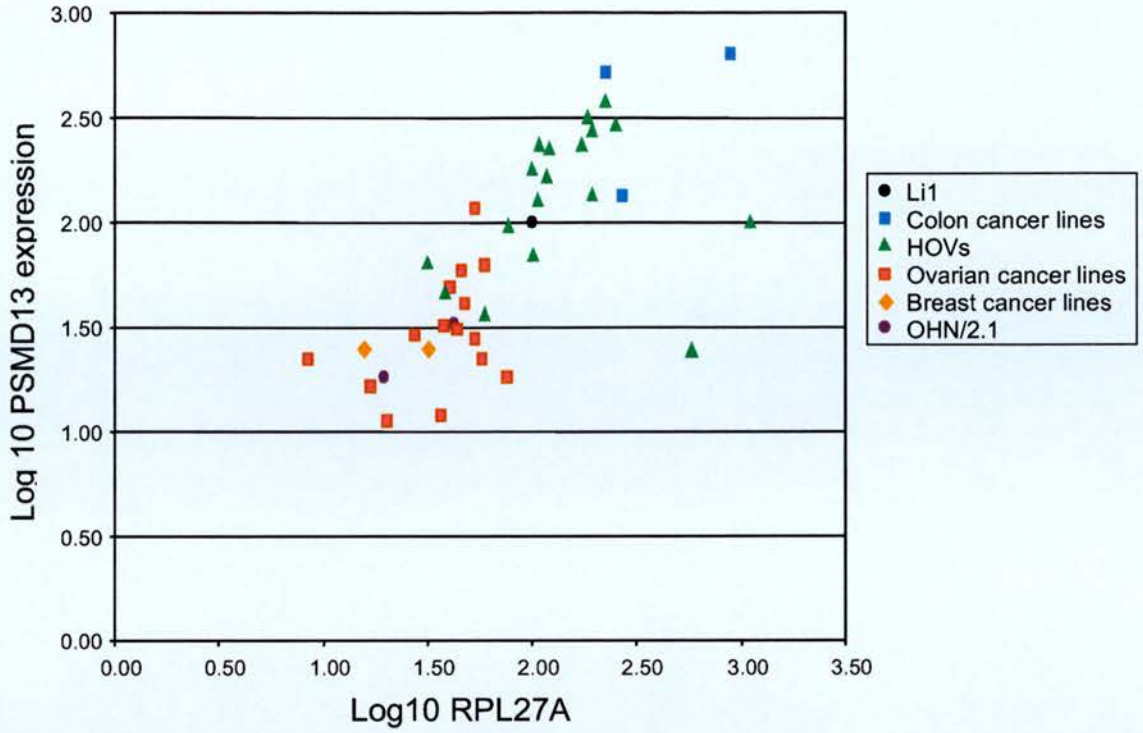


Figure 6.9 Log10 RPL27a Vs Log10 PSMD13 expression in a panel of HOVs and cell-lines. Correlation is significant ($p<0.001$).

6.2 Protein expression for candidate TSGs

Of the four chromosome 11 genes, we acquired antibodies for three. The antibody against PSMD13 was very poor and designed to identify the protein from a purified proteasome fraction and I found it very troublesome on whole cell-line lysates. At all dilutions, the background was very strong. Those bands that were seen appeared to be non-specific and a correct sized protein could not be detected.

Commercial antibodies were available for both CTSD and CRYAB. The antibody for CRYAB was unsuitable for IHC and has currently not been optimised for Western Blotting. The antibody for CTSD, which detects both the 52 kDa pro CTSD protein and the mature cleaved 34 kDa protein, was successfully optimised for western blotting and IHC.

I assayed CTSD protein expression in a panel of 16 ovarian cancer cell-lines, 3 other cancer lines and 3 breast cancer cell-lines that have been treated by estrogen (acquired from Dr Amanda O'Donnell). Western blots for CTSD are seen in Figure 6.10. Three distinct bands are seen on the blots at 52 kDa, 34 kDa and approx. 30 kDa. The larger two bands are the pro CTSD protein and its mature cleaved product. The smaller band is an unknown protein.

At a longer exposure all samples showed some expression of the mature CTSD protein, although this was very weak for PEO16 and K562. 52kDa bands are eventually detectable for all of the estrogen induced cell-lines. OVCAR5, PEO4 and SKOV3 also show expression of the pro CTSD protein. The expression difference between OHN and 11OH2.1 varied per exposure due to saturation of the bands. The average fold difference from the three gels between OHN and 11OH2.1 is 3.26-fold. This number may be skewed from the 2.5-fold ratio found at the mRNA level due to the first blot that showed a difference of about 8-fold. The other two blots demonstrated a correlation for CTSD protein and RNA differences between OHN and 11OH2.1 as a 2.5-2.6 fold increase.

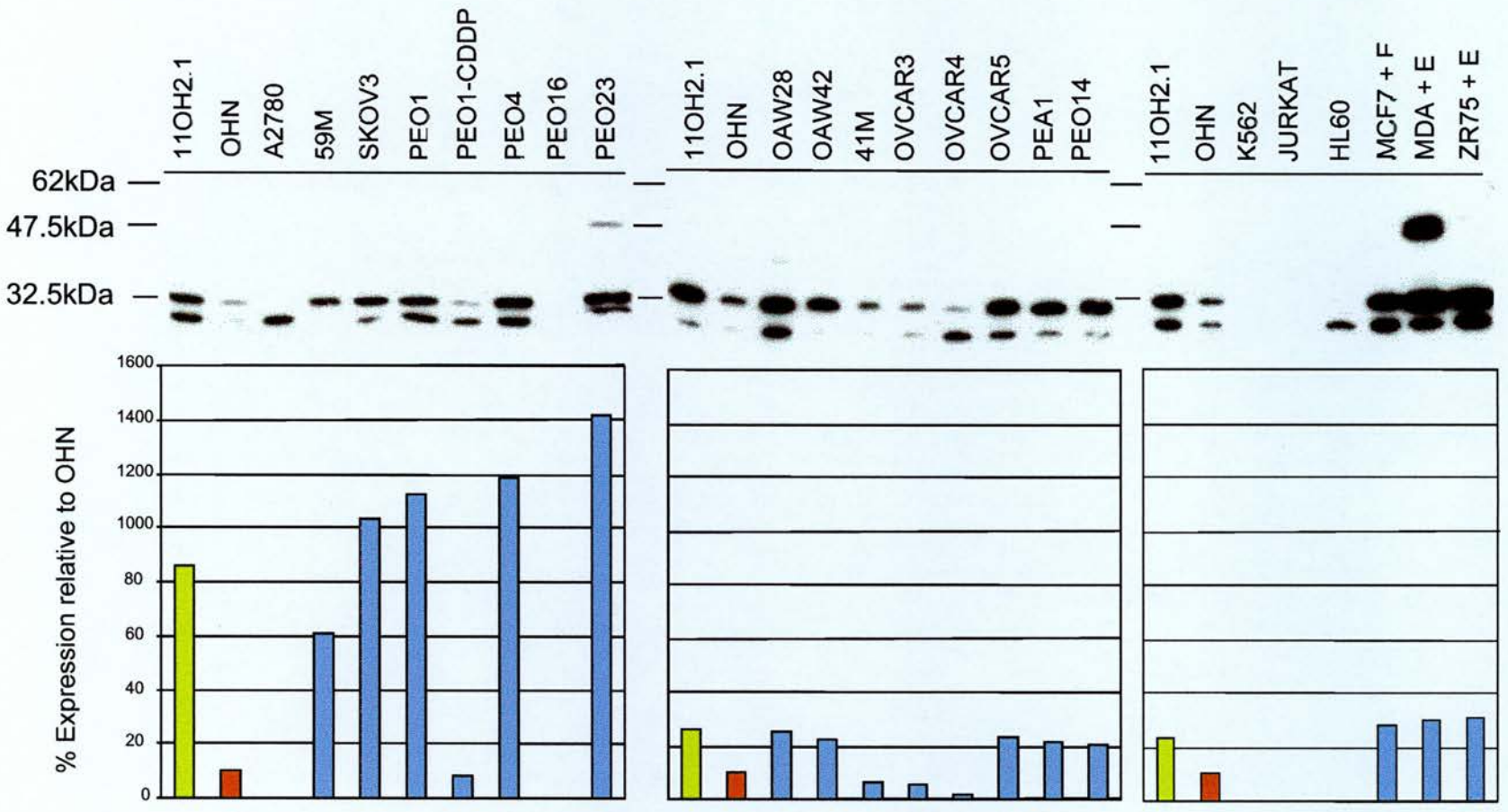


Figure 6.10 Protein expression of CTSD in 22 cancer cell-lines.
Western blots shown at top probed with CTSD antibody. Graphs at bottom show densitometric readings of the 34kDa protein band, relative to OHN levels. Yellow bar= 11OH2.1, red bar= OHN, lilac bars= cancer cell-lines. +E= cells stimulated with oestrogen.

Protein expression of CTSD correlated well with mRNA expression for most of the ovarian cell-lines (Figure 6.11). Only two samples vary widely between RNA to protein, PEO16 and A2780. This is probably due to the very low levels of RNA and protein detected. The difference between the expression levels is small but relative to the actual expression it appears to be large. The strong correlation between CTSD protein and RNA expression shows that data derived by Light Cycler analysis is representative of the actual protein levels within the cells and vice versa.

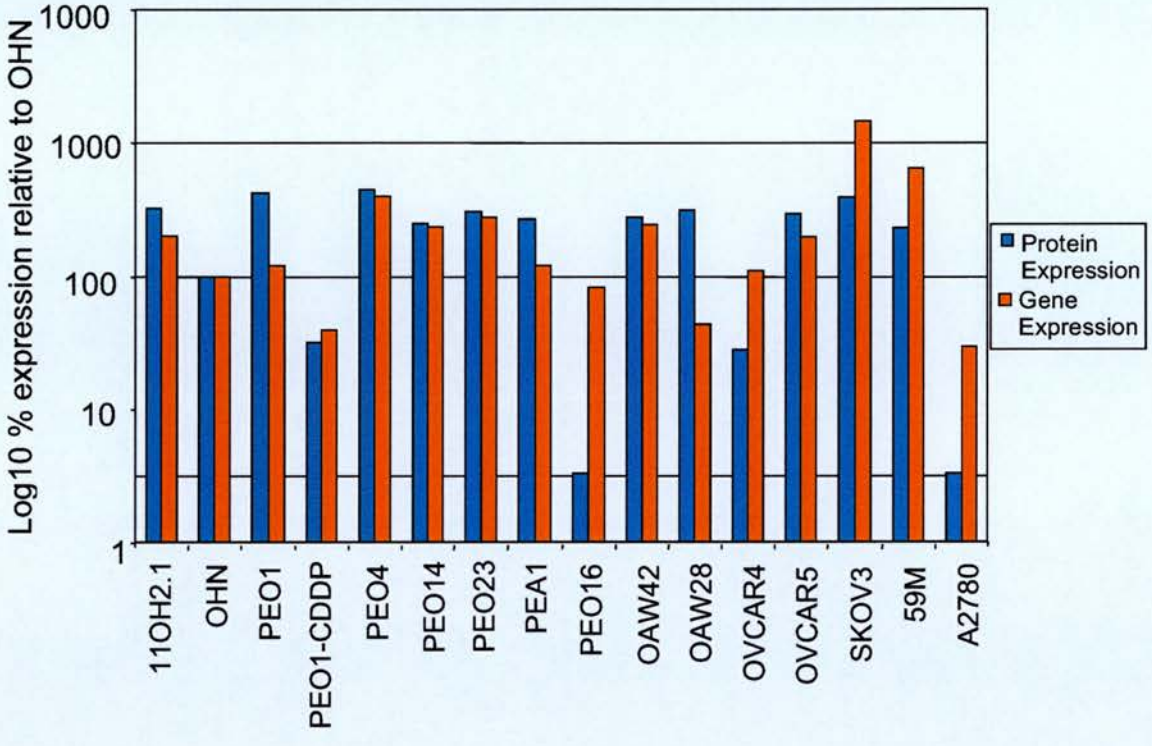


Figure 6.11 Comparison of CTSD protein and mRNA expression in 14 ovarian carcinoma cell-lines. Red bars = mRNA expression as determined by quantitative RT-PCR, blue bars = Protein expression on Western blot.

6.3 Immunohistochemical analysis of CTSD protein expression

The CTSD antibody was also optimised for use in immunohistochemistry, on fixed paraffin embedded sections. A series of 48 ovarian tumours and 5 normal Ovarian samples were treated with the CTSD antibody to detect localisation and intensity of protein staining. Clinicopathological characteristics of the 48 tumour samples are seen in Table 6.1. Mean survival is 1746 days and ranges from 38-4043 days.

After staining, expression of CTSD protein was examined for each section by Dr Owatif Al-Nafusi and scored according to intensity. The range of score was from 0-3 for the tumour samples with a low score representing low protein expression. All 5 normal ovary samples showed expression of 2/3 within the OSE with a granular cytoplasmic distribution of the protein. Cellular compartment location for the tumours was also noted. Nine tumours showed a more diffuse cytoplasmic staining pattern suggesting release of the protein from within these granular spots. The precise organelle responsible for these granules is unknown.

Number of Patients	**48**
Histology	
Serous	17
Endometrioid	14
Mucinous	8
Clear cell	7
Other	2
FIGO Stage	
I/II	30
III/IV	18
Unknown	0
Differentiation status/ Grade	
I/II	23
III	20
Unknown	5

Table 6.1 Clinicopathological characteristics of Patient samples used in CTSD IHC study.

Thirty-three tumours had decreased protein expression (score ≤1) as compared to normal OSE. Decreased expression of CTSD in these 33 tumours does not associate with any clinicopathological parameters. Very low expression of CTSD (score ≤0.5) was associated with a serous histology (p=0.0169). There were no associations of CTSD protein expression with Grade, FIGO stage or with debulk status.

Survival analysis showed a trend towards the association of very low expression (score ≤ 0.5) with adverse survival (Log rank p=0.0847) (Figure 6.12). A larger set of samples are being tested to improve numbers so true associations may become more significant.

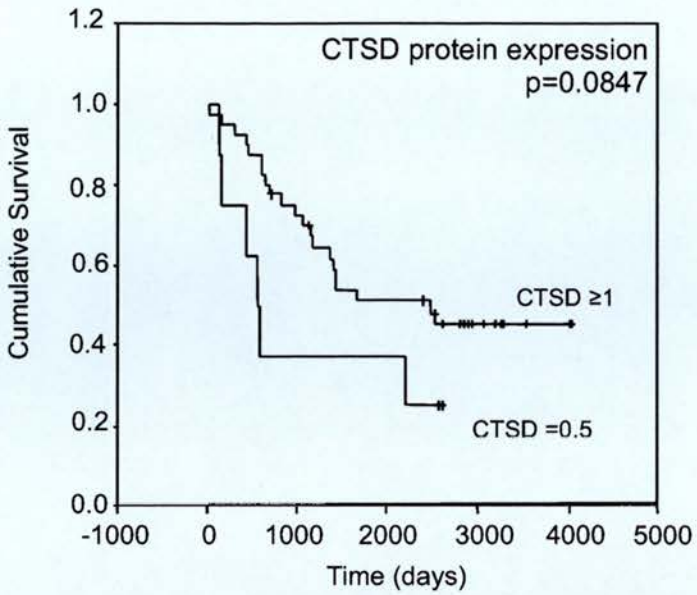


Figure 6.12. Kaplan-Meier survival curves for protein expression of CTSD in patient samples. Survival comparison between protein expression of score 0.5 and ≥1. Log rank test p=0.0847.

6.4 Functional studies using sense and antisense candidate TSG transfection.

Primers were designed which encompassed the complete cDNA coding regions for the four chromosome 11p TSG candidates. These were ligated into the plasmid pcDNA3-Zeo that carries a Zeo resistance gene. These plasmids, in the sense and antisense orientations, were transfected into OVCAR3 derived clonal line OH1. Zeo resistant clones were derived from three independent transfections. To date, clones have been generated to contain, independently, the full-length coding regions for PSMD13, CTSD and RPL27A.

Once generated, clones were assayed for insert presence and direction. I produced 4 RPL27A sense clones and 8 RPL27A antisense clones. Transfections of PSMD13 were less successful and only 1 sense and 2 antisense clones were generated. CTSD clones are yet to be assayed for inserts.

The next step was to determine if these inserts are expressed. This was done using primers within the sequence transcribed from the plasmid. SP6/T7 primers, which bind only to the transcript from the plasmid, were used to distinguish between plasmid and endogenous expression (Figure 6.13).

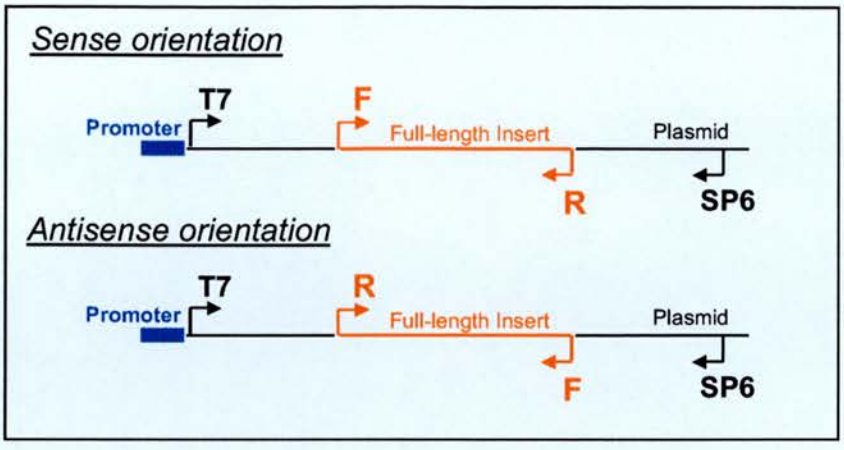


Figure 6.13 Primers used to determine insert orientation. Combinations of Insert and plasmid specific primers are used to show insert expression.

cDNA generated from the RPL27A and PSMD13 sense and antisense transfected clones were assayed for insert expression. Only one PSMD13 antisense clone and no sense clones, expressed from the plasmid. I did however, successfully generate RPL27A 4 sense and 7 antisense expressing clones. Expression levels varied between these clones suggesting some plasmids were more efficient at transcribing than others (Figure 6.14).

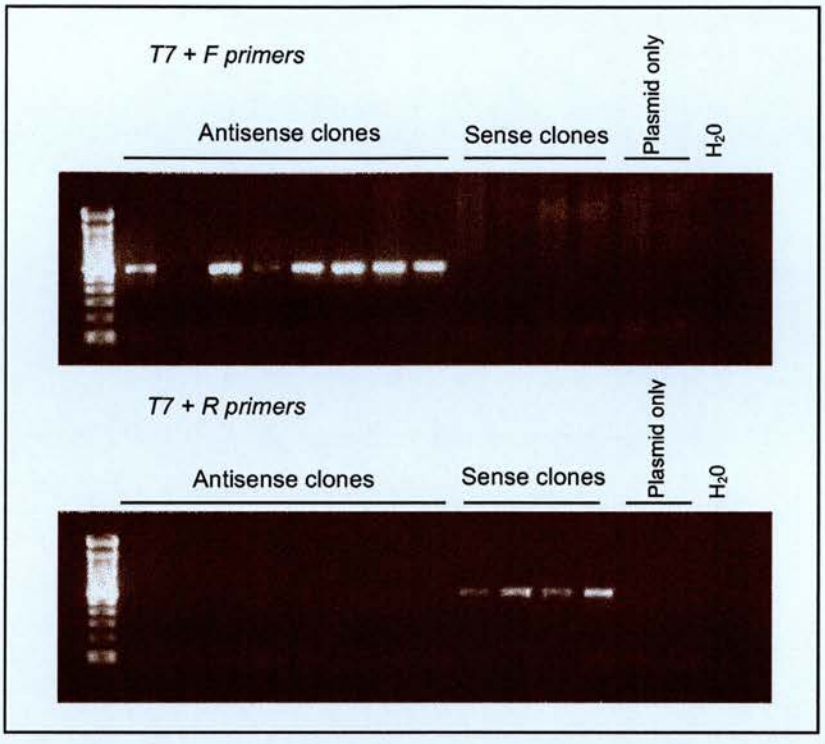


Figure 6.14 Insert expression in RPL27A sense and antisense transfected clones. Plasmid and insert specific primers used in combinations to determine orientation and expression of inserts in clones. Plasmid only clones show no expression as expected.

The most important question is whether there are any changes in endogenous expression due to the addition of these plasmids. In theory, if antisense transcripts are knocking out endogenous expression then this should be detected as a decrease in expression as compared to the sense transfected and parental cells. Endogenous expression was determined by RT-PCR using reverse primers designed to those 5' sequences not included in the plasmid.

The sense and antisense clones did not however show any differences in expression of RPL27A. A more quantitative approach may however be required to determine small differences in expression, alternatively further transfections or a different approach utilising RNAi could be adopted.

7. VALIDATED CANDIDATE ONCOGENES

7.1 Candidates

Four products were validated by Light Cycler quantitative RT-PCR as downregulated upon introduction of chromosome 11 into OVCAR3. Only two of these, RALDH2 and IGFBP2 have been previously characterised as transcribed genes. Novel transcripts CB1 and CB2 have been positioned on the NCBI map viewer on chromosomes 4p and 8p respectively (Figures 7.1 and 7.2).

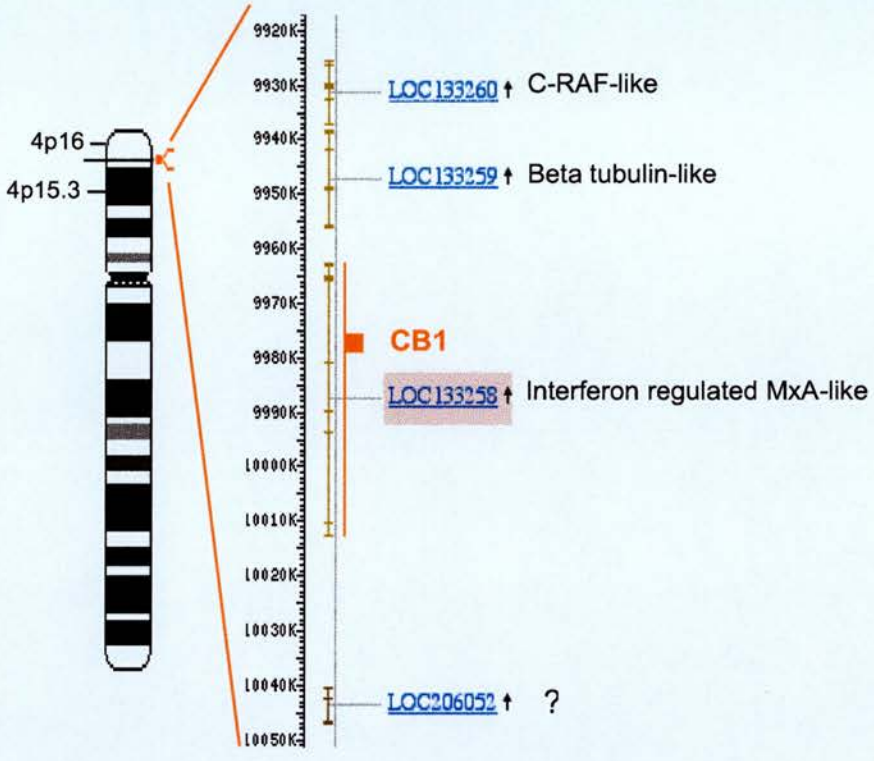


Figure 7.1 Chromosomal positioning of CB1.
Product CB1 maps to hypothetical protein LOC133258. The potential roles of genes are seen on the right. The red box = position of CB1 sequence. Distance along the Chr 4 seen right of the ideogram.

CB1 and CB2 do not match to known gene exons however suggesting that although they are high abundance transcripts they may not be translated. This is further supported when the sequences are translated using the expasy translate tool (www.ca.expasy.org). The outputs show stop codons dotted through out the postulated protein sequence, in all three codon frames and both 5'→3' and its reverse. Quantitative RT-PCR showed these products to be substantially downregulated, by 200-1300 fold in the growth suppressed clone 11OH2.1.

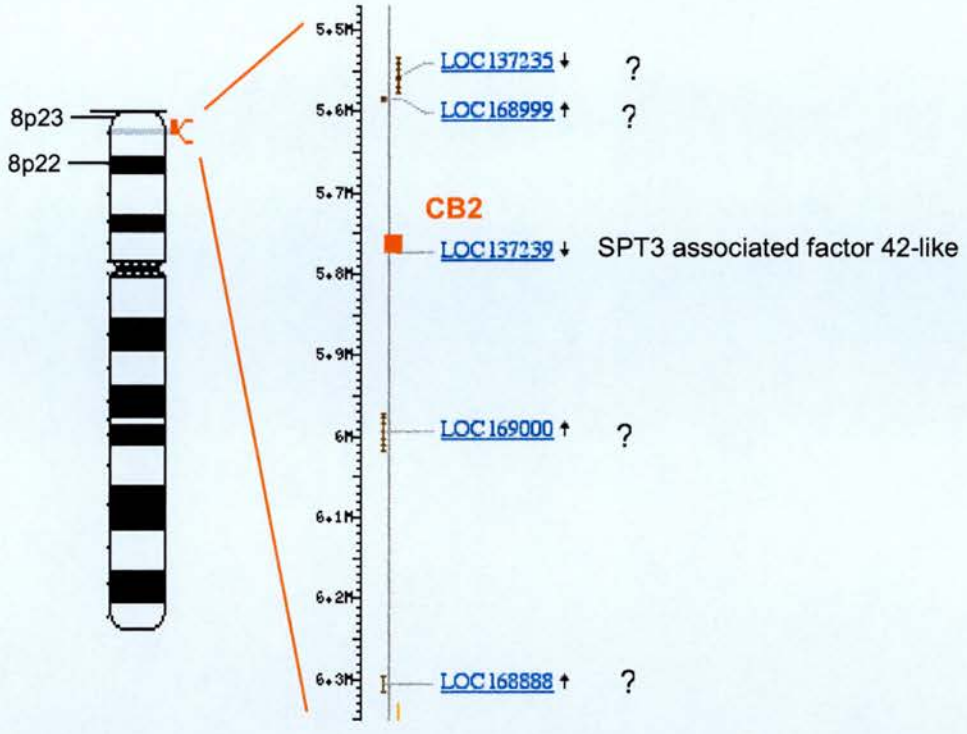


Figure 7.2 Chromosomal positioning of CB2.
Product CB2 maps between hypothetical genes LOC137239 and LOC169000. The potential roles of genes are seen on the right. The red box = position of CB2 sequence. Distance along the Chr 8 seen right of the ideogram.

7.2 Expression of candidates in cell-lines and tumours

Semi-quantitative RT-PCR on a panel of ovarian cell-lines showed that CB1 is only expressed in the line OVCAR3. This product if therefore non-representative of ovarian carcinoma cell-lines and unlikely to be involved in ovarian cancer progression. Also, Northern blotting with a CB1 probe on mRNA and total RNA from OHN and 11OH2.1 showed no bands. Northern blotting does not appear to be sensitive enough to detect this transcript. Northern blotting with a probe for CB2, again on mRNA and total RNA, showed a very feint product on total RNA alone (not shown). These results suggest that neither CB1 or CB2 are present in the mRNA population and unlikely to be transcribed.

Of the four candidate oncogenes identified and validated, three are altered in expression by >100-fold. These products, CB1, CB2 and RALDH2, were identified by the so-called open systems of difference analysis; DD-RT-PCR and cDNA-RDA.

As for the TSG candidate analysis, RALDH2 and IGFBP2 were assayed for expression across a panel of ovarian cancer cell-lines and HOV samples for further validation of actual association with ovarian cancer progression. CB1 and CB2 were not analysed as they are not likely to be translated into functional proteins.

RALDH2 was validated to be overexpressed 200-300 fold in the OVCAR3 derivative OHN. It was expected that this gene would be highly overexpressed in the majority of other ovarian lines and HOV tumours. Expression of RALDH2 was however in all but one cell-line seen to be lower than in the normal OSE (Figure 7.3). OHN (OVCAR3) expression is over 6000% relative to Li1 (normal OSE) so is not represented on the graph. The breast cell-lines MDA and ZR75 had little expression and the colon lines had similar levels to those seen in the normal OSE sample Li1. Only six HOV samples showed a higher level of expression than the OSE. Expression of RALDH2 in OHN is over 100 fold higher than in any other sample analysed quantitatively.

IGFBP2, identified by HDFA hybridisation, has a 2.4-fold change in expression between OHN and 11OH2.1. Of the panel of cell-lines, none showed higher expression than the normal OSE (Figure 7.4). Only 4 of the 17 HOV tumour samples showed elevated expression over the normal OSE. One of these samples had an increase in expression of IGFBP2 of 6-fold. The level of expression in OHN is only 22% of Li1. If this gene were truly oncogenic, I would expect expression in OHN to be higher than in normal OSE.

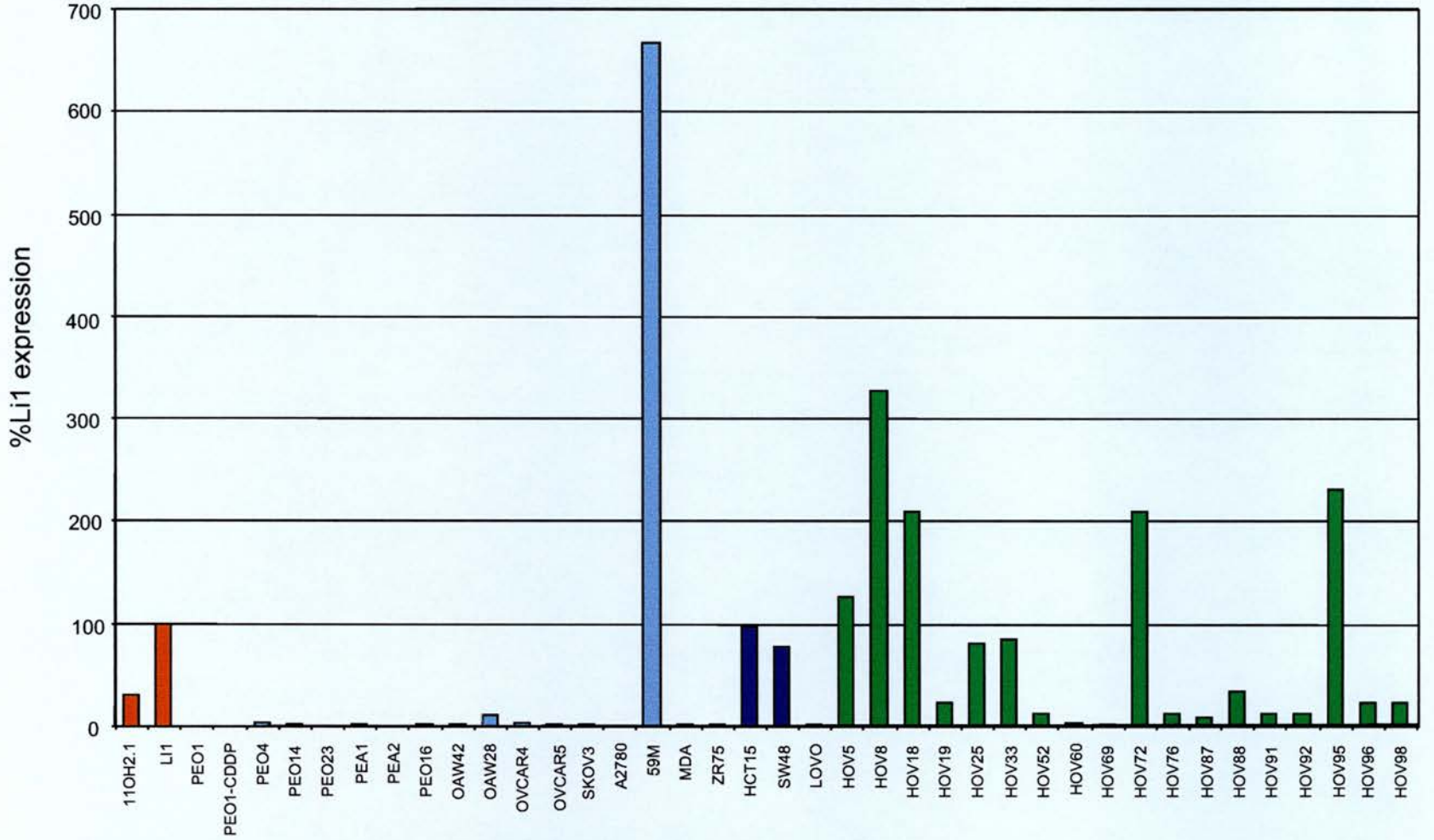


Figure 7.3 RALDH2 expression in a panel of cell-lines and HOV tumours.
Green bars= HOV tumours, blue bars= non-ovarian cancer cell-lines, grey bars= ovarian cell-lines, red bars= normal OSE and original 11OH2.1 line. Expression relative to normal OSE (100%).

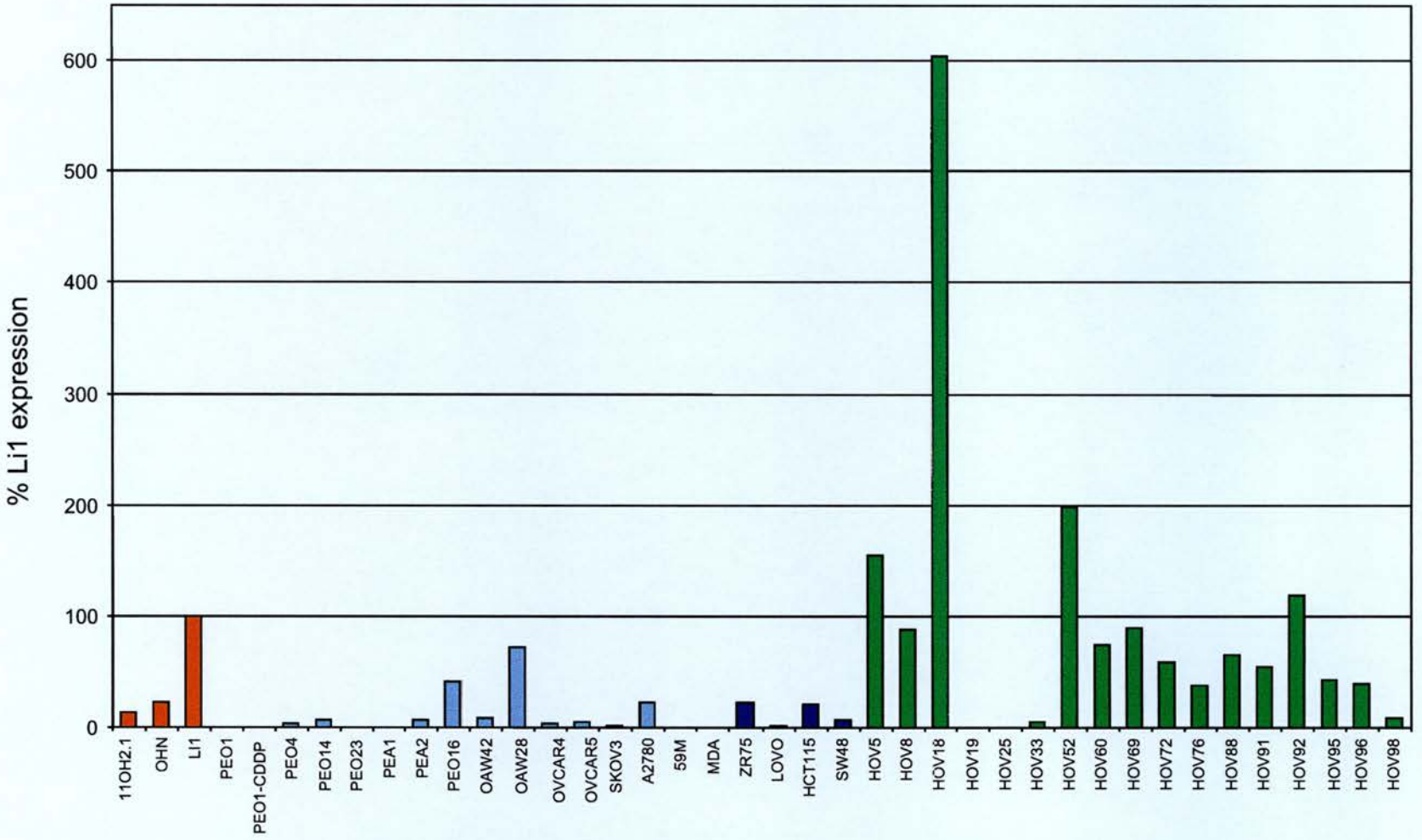


Figure 7.4 IGFBP2 expression in a panel of cell-lines and HOV tumours.
Green bars= HOV tumours, blue bars= non-ovarian cancer cell-lines, grey bars= ovarian cell-lines, red bars= normal OSE and original OHN and 11OH2.1 lines. Expression relative to normal OSE (100%).

7.2.1 Clinicopathological associations with candidate oncogene expression

No clinicopathological associations were found between RALDH2 and IGFBP2 expression and stage, grade, and histology. The only trend towards an association with adverse survival is for RALDH2 expression >80% normal OSE level (p=0.1303) (Figure 7.5).

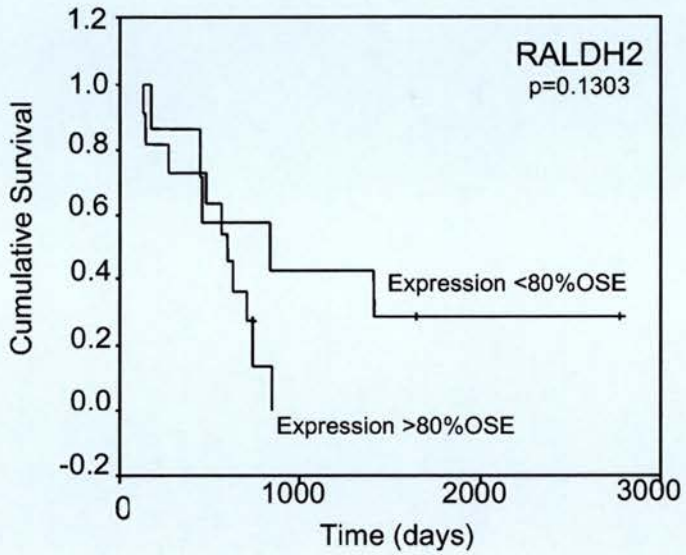


Figure 7.5 Kaplan-Meier survival curve for expression of RALDH2 above and below 80% normal OSE levels. Log rank p=0.1303.

7.3 Protein expression of candidate oncogenes

Of the two characterised genes, RALDH2 and IGFBP2, only IGFBP2 has commercially available antibodies. Two antibodies were tested on IHC and neither could be optimised. The Upstate Biotechnology UK antibody was also tested on cell-line lysates by western blotting. This antibody however produced multiple non-specific banding on the gels. Intense optimisation of this antibody would be needed for it to be used quantitatively.

7.4 In vitro antisense knockout of RALDH2 and IGFBP2

If these genes are functionally active in the carcinoma cell-lines then removal of these RNAs by antisense knockout might slow growth and tumorigenicity characteristics of the cells.

Sense and antisense constructs were generated by ligating partial cDNAs into pcDNA3.

A 499bp fragment of RALDH2 cDNA was ligated into plasmid pcDNA3-neo and sense and antisense orientation constructs were transfected into OVCAR3 clonal line OH1. After selection with G418, clones resistant to the antibiotic were selected and grown-up with DNA and RNA made from each. Two primer sets were used to determine expression of the insert and endogenous expression of the gene, with the aim being to identify clones having partial knockout of RALDH2 expression.

I was successful in generating clones with insert expression and subsequent partial endogenous knockout, showing that this method of transfecting in antisense clones does work. Initial in vitro growth studies showed mixed results. Sense and antisense transfected clones grew with unexpected variability. There were varied growth rates for both the sense and antisense clones. Those antisense clones with knockout did not necessarily grow slower than the sense controls.

A example graph of RALDH sense and antisense clone growth rates is seen in Figure 7.6. As seen, growth rates for the antisense clones are not slower than for the sense clones suggesting that knockout of RALDH2 caused no functional effect in these cell-lines. Parental line OH1 appears to grow slower than any of the transfected clones. The OH1 cells used however had been passaged on fairly rigorously so may no longer be representative of the OVCAR3 parent.

Antisense transfection experiment appears to suggest that knockout of RALDH2 has no functional consequences on clone phenotype. This data supports the cell-line expression data, leading us to believe that RALDH2 overexpression may represent merely a contextual artefact of OVCAR3.

In light of the results from RALDH2 functional experiments, IGFBP2 sense and antisense knockouts were not performed on that basis of similar cell-line panel expression data, in that IGFBP2 is not overexpressed in the ovarian cell-lines.

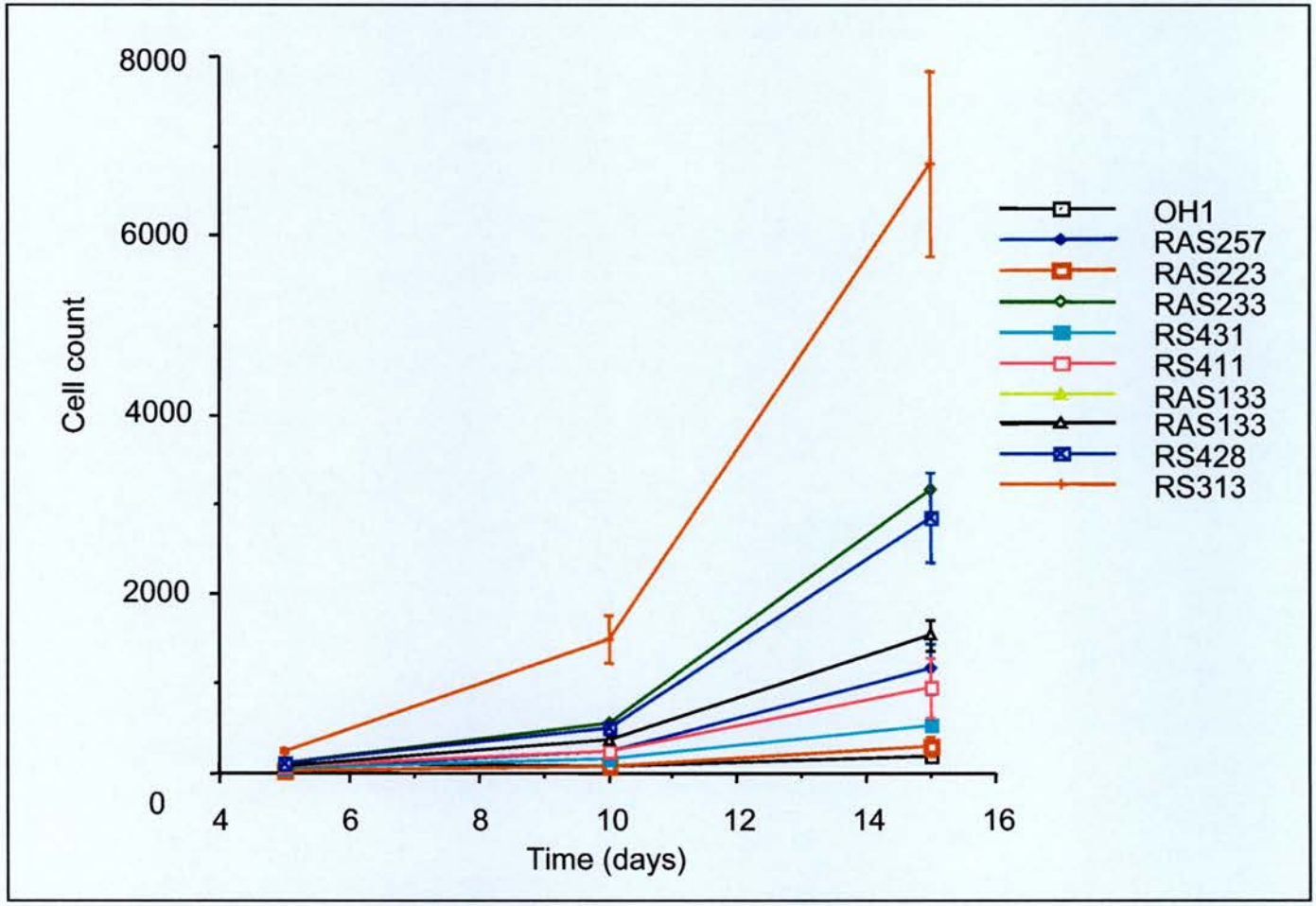


Figure 7.6 Growth curves for RALDH2 sense and antisense transfected clones.
Cell number over 15 days, counted every 5 days. RS= sense clone RAS= antisense clone, OH1= Parental line. Error bars represent standard deviation.

8. LOH ANALYSIS OF CHROMOSOME 11p15

Loss of heterozygosity has been detected at chromosome 11p15 in multiple tumour types including ovarian carcinoma (as reviewed in introduction). In order to minimise the regions of deletion on 11p15, identify potential tumour suppressor genes and place these in relation to genes identified by our functional approach, further LOH mapping was carried out.

Forty-four polymorphic markers along 11p15 were identified and optimised for PCR conditions. Of these, two markers could not be successfully optimised (D11S1348 and D11S4189). A further two markers were found to be non-polymorphic on our panel of blood-tumour pairs (D11S1318 and IGF2). D11S4194 was removed from the study as it was subsequently positioned outwith 11p15. Therefore the study used 39 polymorphic markers.

The aim was to identify as many markers along the 11p15 region as possible to create a precise map of LOH, therefore markers were chosen anywhere along this region so they are not necessarily well spaced. The largest distance between any two markers is 2Mb and the smallest 10Kb. Markers were identified from the UCSC Genome Map and NCBI Mapviewer with links to the STS and GDB databases (See Table 2.2).

8.1 Individual Marker Analysis.

A total of 87 matched tumour-normal pairs were used in the study, extracted from a combination of fresh and paraffin embedded samples. Normal samples were extracted from blood samples, normal omentum, normal uterus or paired normal ovary. All tumour samples were from epithelial ovarian cancers.

Details of the clinicopathological features of the patients used in the study are found in Table 8.1. The retrospective set is over represented in terms of the less commonly occurring early stage (FIGO I/II) carcinomas. Patients with follow-up of >2 years were used in survival analyses.

All pathology was routinely reviewed through the South-East Scotland multidiscipline gynaecological oncology meetings.

NUMBER OF PATIENTS	87
HISTOLOGY	
SEROUS	32
MUCINOUS	12
ENDOMETRIOID	24
CLEAR CELL	13
OTHER	6
DIFFERENTIATION	
WELL	10
MODERATE	24
POOR	46
UNKNOWN	6
FIGO STAGE	
I/II	42
III/IV	44
UNKNOWN	1
SURGERY	
COMPLETE DEBULK	62
PARTIAL DEBULK	9
NONE	12
UNKNOWN	4

Table 8.1 Clinicopathological information of the 87 patient samples of EOC used in the microsatellite LOH mapping of 11p15.5.

LOH analysis was carried out for all 39 markers with as many of the 87 samples as possible. It was found that two of the markers (D11S1310 and D11S1791) could not be optimised for PCR on the paraffin embedded DNA. There were also 'difficult' samples that for a number of markers could not be amplified for analysis, probably due to DNA degradation. These samples are labelled as "not determinable".

LOH results for blood tumour pairs were analysed and the R-value, a ratio of the two peaks, was generated for each pair. Example peak traces can be seen in Figure 8.1. A cut-off of 0.6, or 40% loss, was chosen based on recent discussion in the literature (Tomlinson *et al.*, 2002). This cut-off value allowed detection of markers with variable LOH rates which for 12 markers was above the LOH threshold of 35% (Shelling *et al.*, 1995).

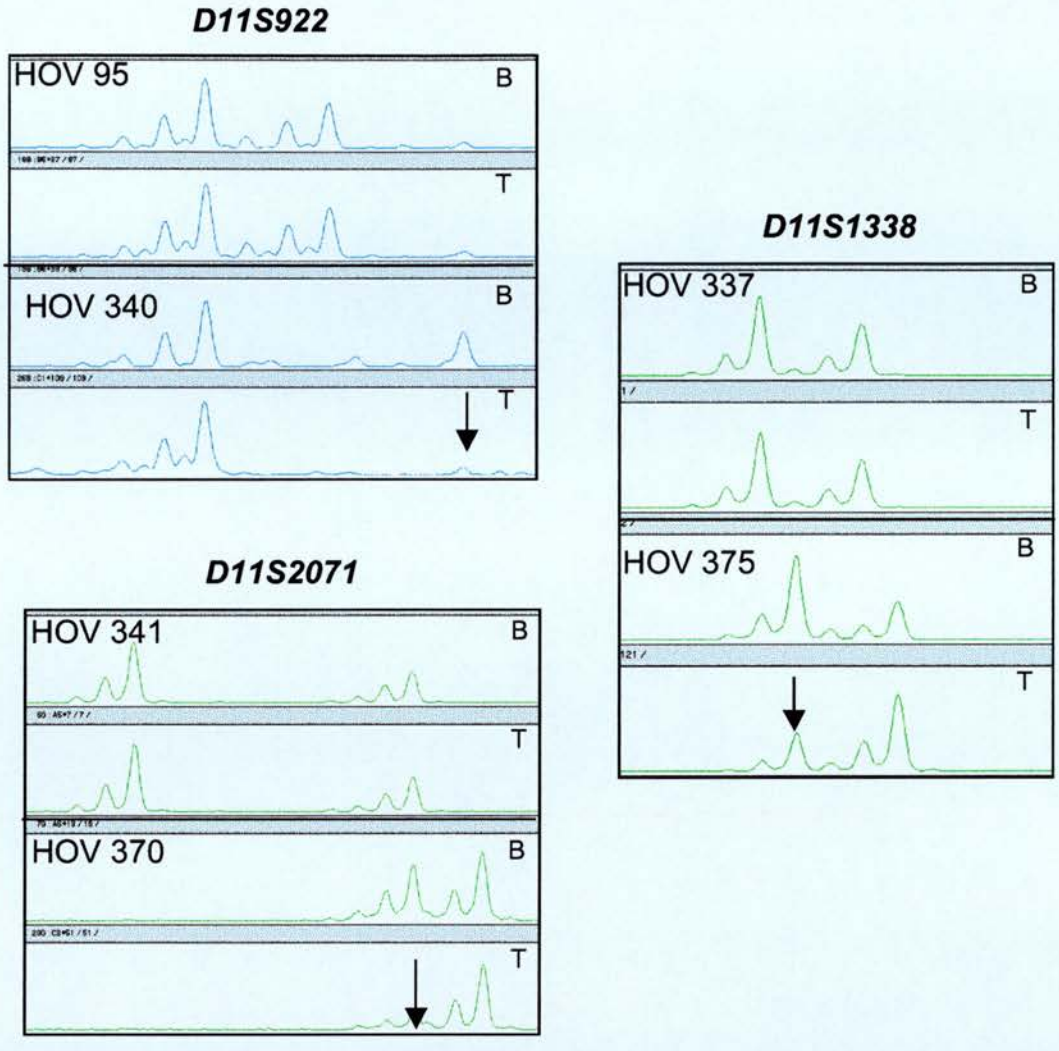


Figure 8.1 Example Gene Scan traces of allele patterns for markers D11S922, D11S2071 and D11S1338. The top pairs show blood and tumour samples with no loss. The bottom pair show allele loss in the tumour sample as indicated by an arrow.

To summarise the LOH data, 91% (79/87) of all samples had evidence of loss of 1 or more markers along 11p15. Only 9% (8/87) had no loss at all. Twenty-one samples appear to have LOH of the majority of the 11p15 region (at least 80% loss). This high rate of whole arm allele imbalance (24%) suggests that loss of the whole region is a common mechanism for the LOH observed.

There are 12 markers which show an overall LOH rate of >35%. A graph of the LOH rates pinpoints these markers (Figure 8.2).

The overall LOH for each of the clinicopathological subgroups, per marker, is represented in Figure 8.3. There appears to be a trend along the chromosome arm for increased LOH in late stage tumours and high grade. There is also an apparent association with a non-mucinous histology.

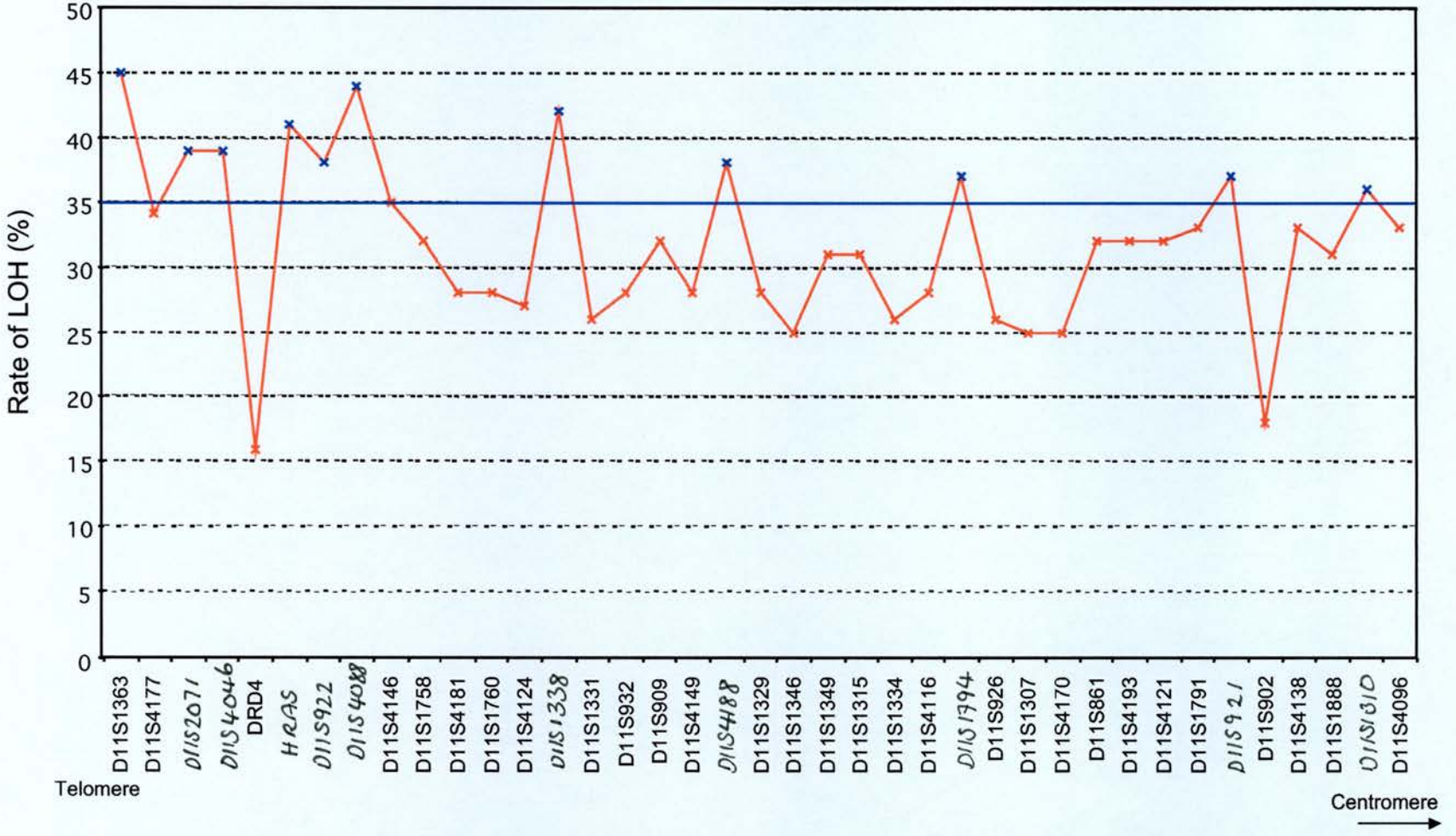


Figure 8.2. LOH rates along 11p15 showing a cut-off of 35% loss. Microsatellite markers from left to right are telomeric to centromeric. Markers in italics have an LOH rate >35%.

MARKER	ALL TUMOURS	SEROUS	ENDOMETRIOID	MUCINOUS	CLEAR CELL	EARLY STAGE	LATE STAGE	MOD/WELL DIFFERENTIATED	POORLY DIFFERENTIATED	NO DEBULK	DEBULK	ALIVE	DEAD
D11S1363	18/40 45%	8/12 67%	5/11 44%	3/3 50%	0/6 0%	9/23 39%	9/17 53%	8/18 44%	10/20 50%	1/4 25%	16/34 47%	3/12 25%	7/16 44%
D11S4177	23/67 34%	8/25 32%	10/20 50%	2/8 25%	0/8 0%	6/26 23%	15/38 39%	0/6 0%	8/24 33%	1/11 9%	20/53 38%	7/19 37%	9/24 38%
D11S2071	30/77 39%	12/28 43%	12/22 55%	1/8 13%	2/13 15%	10/34 29%	20/41 49%	8/30 27%	21/42 50%	3/11 27%	25/62 40%	9/22 41%	12/29 41%
D11S4046	28/72 39%	13/30 43%	9/18 50%	1/9 11%	2/11 18%	10/35 29%	18/36 50%	8/28 29%	18/38 47%	4/9 44%	22/59 37%	8/22 36%	12/25 48%
DRD4	8/51 16%	2/19 11%	4/15 27%	0/5 0%	1/9 11%	1/22 5%	7/28 25%	2/21 9%	6/27 22%	0/8 0%	7/41 17%	4/15 27%	3/21 14%
HRAS	18/44 41%	6/13 46%	7/15 47%	1/6 17%	2/7 29%	6/19 32%	12/25 48%	7/17 41%	10/22 45%	2/7 29%	15/37 41%	7/16 44%	6/17 35%
D11S922	28/73 38%	10/26 38%	9/18 50%	0/8 0%	3/13 23%	9/36 25%	19/36 53%	8/25 32%	20/42 48%	3/10 30%	23/60 38%	7/24 29%	12/23 52%
D11S4088	35/80 44%	12/30 40%	10/21 48%	5/12 42%	4/12 33%	15/40 38%	20/40 50%	13/30 43%	21/43 49%	5/12 42%	28/64 44%	9/20 45%	17/33 51%
D11S4146	21/60 35%	5/20 25%	11/19 58%	0/7 0%	2/10 20%	2/27 19%	16/32 50%	6/22 27%	14/33 42%	1/8 13%	18/48 38%	6/18 33%	9/24 38%
D11S1758	17/53 32%	8/22 36%	6/16 38%	1/5 20%	1/6 17%	6/24 25%	11/30 37%	4/17 24%	12/33 36%	3/9 33%	12/41 29%	4/16 25%	7/17 41%
D11S4181	19/68 28%	6/26 23%	9/19 47%	0/10 0%	2/10 20%	5/32 16%	14/35 40%	6/25 24%	13/39 33%	0/10 0%	17/54 31%	6/21 29%	7/24 29%
D11S1760	19/67 28%	6/23 26%	5/19 26%	2/11 18%	3/11 27%	8/34 24%	13/34 38%	6/27 22%	13/35 37%	1/8 13%	17/55 31%	5/21 24%	10/25 40%
D11S4124	16/60 27%	6/23 26%	7/18 39%	1/7 14%	2/10 20%	5/31 16%	11/28 39%	4/24 17%	11/31 35%	3/11 27%	12/47 26%	3/14 21%	7/28 25%
D11S1338	28/66 42%	8/25 32%	9/18 50%	3/10 30%	4/9 44%	14/33 42%	14/33 42%	12/29 41%	16/34 47%	0/8 0%	26/54 48%	9/18 50%	11/24 46%
D11S1331	18/70 26%	4/23 17%	8/21 38%	0/11 0%	3/11 27%	6/38 16%	12/31 38%	3/28 11%	13/36 36%	1/7 14%	15/59 25%	6/25 24%	6/22 27%
D11S932	19/67 28%	7/25 28%	9/20 45%	0/9 0%	0/8 0%	2/32 6%*	17/35 49%*	4/25 16%	14/35 40%	2/8 25%	17/57 29%	5/21 24%	10/25 40%
D11S909	15/47 32%	5/16 31%	4/14 29%	1/6 17%	1/7 14%	5/22 23%	10/24 42%	6/18 33%	9/25 36%	0/7 0%	13/36 36%	4/15 27%	6/17 35%
D11S4149	18/65 28%	5/23 22%	6/17 35%	1/11 9%	3/9 33%	6/34 18%	12/30 40%	7/25 28%	10/33 30%	1/7 14%	13/51 24%	6/19 32%	7/25 28%
D11S4188	25/66 38%	10/21 18%	9/19 47%	0/11 0%*	2/9 22%	7/34 21%*	18/32 56%*	4/22 18%*	20/35 57%*	4/9 44%	19/54 35%	5/21 24%	11/21 52%
D11S1329	18/63 29%	8/27 30%	6/16 38%	1/10 10%	0/6 0%	7/29 24%	11/33 33%	7/24 29%	10/34 29%	1/12 8%	17/49 35%	5/14 36%	6/26 23%
D11S1346	15/60 25%	6/24 25%	6/16 38%	0/7 0%	1/9 11%	4/23 17%	11/36 31%	4/21 19%	11/35 31%	2/11 18%	11/45 24%	2/11 18%	9/26 35%
D11S1349	24/78 31%	12/29 41%	9/22 41%	0/11 0%	0/11 0%	5/37 14%*	19/40 48%*	7/30 23%	16/42 38%	3/11 27%	19/63 30%	6/23 26%	12/30 40%
D11S1315	18/58 31%	10/25 40%	5/15 33%	0/7 0%	1/7 14%	4/25 16%*	14/37 52%*	6/23 26%	12/31 39%	2/9 22%	14/46 30%	4/13 31%	9/25 36%
D11S1334	17/66 26%	7/24 29%	6/19 32%	1/9 11%	0/8 0%	4/31 13%	13/34 38%	5/30 17%	12/33 36%	3/9 33%	12/53 23%	4/19 21%	6/23 26%
D11S4116	18/68 26%	7/26 27%	6/20 30%	0/7 0%	3/11 27%	4/32 13%*	15/35 43%*	6/24 25%	12/39 31%	4/11 36%	14/53 26%	5/18 28%	8/27 30%
D11S1794	22/59 37%	8/22 36%	7/17 41%	1/8 13%	3/9 33%	12/30 40%	10/29 34%	8/22 36%	13/34 38%	3/11 27%	17/44 39%	9/18 50%	8/19 42%
D11S926	19/72 26%	8/29 28%	4/19 21%	1/9 11%	2/8 25%	5/33 15%	14/38 37%	6/27 22%	13/39 33%	2/11 18%	15/57 26%	6/22 29%	6/25 24%
D11S1307	11/44 25%	3/19 16%	4/11 36%	1/8 13%	0/2 0%	5/28 18%	9/28 32%	5/25 20%	9/26 35%	1/9 11%	10/34 29%	5/15 33%	3/14 21%
D11S4170	17/67 25%	7/25 28%	7/21 33%	1/8 13%	0/9 0%	2/25 8%*	15/36 42%*	5/23 22%	12/40 30%	1/12 8%	14/41 34%	3/15 20%	9/29 31%
D11S861	24/76 32%	9/29 31%	8/22 36%	1/9 11%	3/9 33%	9/35 26%	15/40 38%	6/28 21%	17/43 40%	4/12 25%	19/61 31%	7/22 32%	11/32 34%
D11S4193	18/57 32%	8/20 40%	6/17 35%	2/8 25%	0/9 0%	5/30 17%	13/26 50%	7/21 33%	11/30 37%	2/7 29%	14/46 30%	3/18 17%	8/22 36%
D11S4121	22/68 32%	8/25 32%	8/20 40%	1/7 14%	2/11 18%	6/32 19%	16/35 46%	8/25 32%	14/36 39%	1/10 10%	20/54 37%	5/24 21%	11/22 50%
D11S1791	7/21 33%	1/8 13%	3/6 50%	1/2 50%	0/1 0%	3/8 38%	2/12 17%	3/7 43%	4/13 31%	0/4 0%	5/14 36%	2/5 40%	0/1 0%
D11S921	22/60 37%	7/21 33%	8/16 50%	2/8 25%	2/11 18%	10/19 53%	12/31 39%	9/23 39%	10/33 30%	2/11 18%	18/45 40%	7/16 44%	9/23 39%
D11S902	14/77 18%	11/33 33%	4/22 18%	0/10 0%	1/12 8%	5/39 13%	9/37 24%	6/32 19%	8/39 21%	1/10 10%	12/61 17%	1/21 5%	10/31 32%
D11S4138	21/63 33%	8/25 32%	8/15 53%	0/9 0%	2/9 22%	5/29 17%	16/33 48%	3/21 14%	17/36 47%	2/10 20%	17/49 35%	8/19 42%	9/24 38%
D11S1888	20/64 31%	9/24 38%	7/16 44%	0/9 0%	1/10 10%	6/31 19%	14/31 45%	4/23 17%	16/35 46%	3/10 30%	15/50 30%	6/18 33%	7/24 29%
D11S1310	9/25 36%	2/9 22%	3/5 60%	1/2 50%	1/5 20%	4/10 40%	5/14 36%	3/7 43%	6/15 40%	1/3 33%	6/17 35%	3/6 50%	3/7 43%
D11S4096	16/49 33%	5/16 31%	4/13 31%	1/7 14%	2/7 28%	5/22 23%	11/26 42%	6/18 33%	9/25 36%	2/11 18%	12/34 35%	3/13 23%	7/16 44%

Figure 8.3 LOH rates for all clinicopathological subgroups. Values are the number of cases of LOH/total number of informative cases in that group (% LOH in brackets). Early stage = FIGO I/II, late stage = FIGO III/IV. Debulk = debulking operation performed, No Debulk = no operation. *Yellow bar indicates significant difference (p=0.01) Survival for patients with >2 years follow-up.

8.1.1 Clinicopathological correlations.

All 39 markers were assessed using a two-tailed fishers exact test for correlations with stage, grade, histology and debulk status. At a significance cut-off of p=0.05, 18 markers showed an association with one or more of these variables. A more stringent cut-off of p=0.01 associated only 6 markers with variables, the most common being advanced stage.

8.1.2 Allele loss and histology

There are few markers that show biased LOH in histological subtypes (Figure 8.4). At p<0.01, only one marker associated with a histopathological subtype. Marker D11S4188 was associated with a non-mucinous histology (p=0.0046). There were trends towards significance for a number of other markers (Table 8.2).

Marker	Association with histology (p values)
D11S4188	Non-Mucinous p=0.0046*
D11S1349	Non-Mucinous p=0.0149
D11S4146	Endometrioid p=0.0191
D11S922	Non-mucinous p=0.0202
D11S4138	Non-Mucinous p=0.0234
D11S1363	Non-Clear cell p=0.0243
D11S4184	Endometrioid p=0.0366
D11S4177	Non-clear cell p=0.0435
D11S4193	Non-clear cell p=0.0452
D11S4188	Non-mucinous p=0.0473

Table 8.2 Microsatellite markers associated with Histology. Fisher's exact test used to calculate significance. * indicates p<0.01.

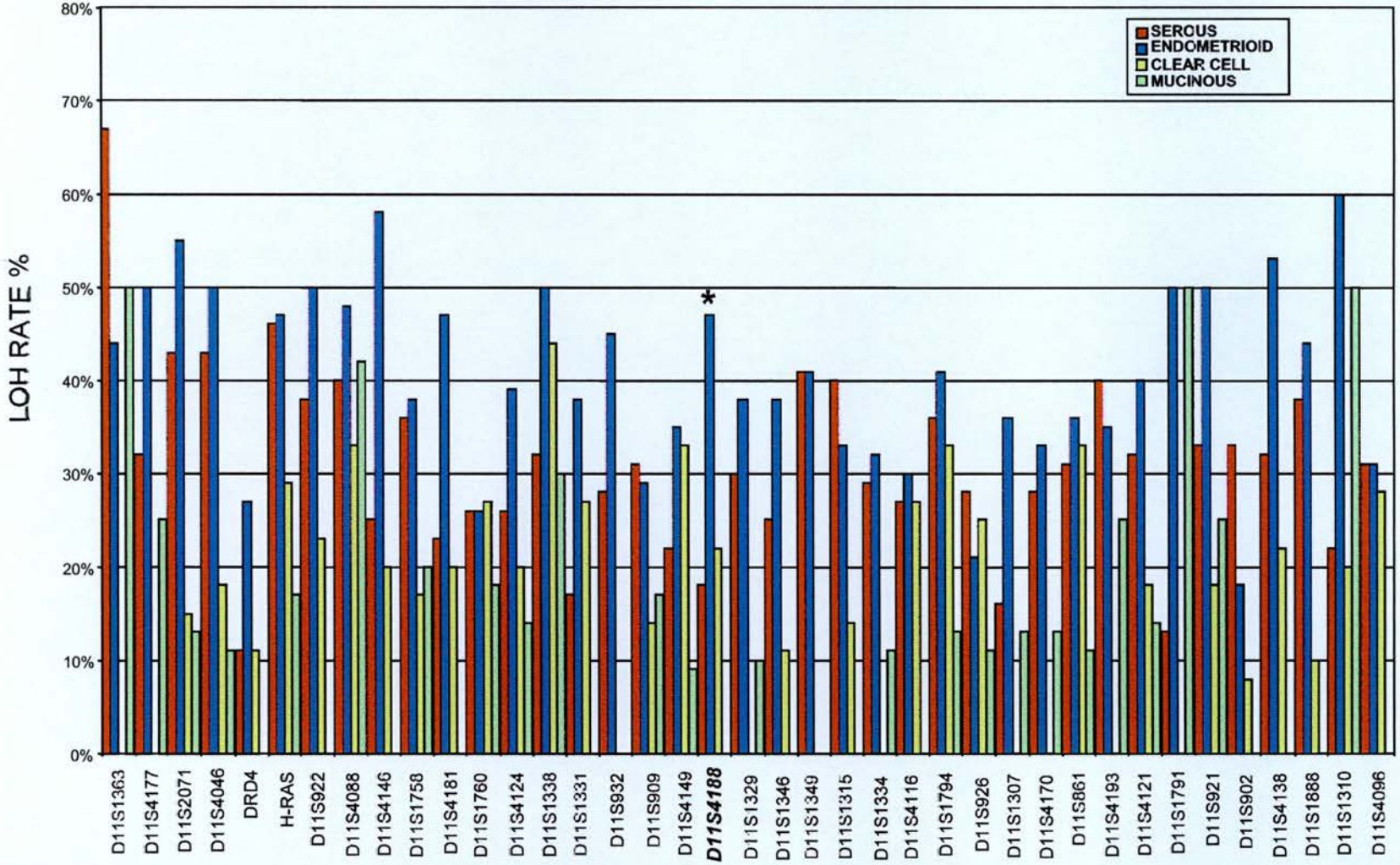


Figure 8.4 LOH rates of markers as associated with histological subtype. Key shows labelling of bars. An * and bolded italics indicate significance <p=0.01.

8.1.3 Allele loss and FIGO stage

LOH at many individual loci associate with late stage suggesting loss of the whole region may occur in late stage tumours (Figure 8.5).

Using a more stringent cut-off of p=0.01, 6 markers remain associated with late stage supporting the above theory. These markers cluster from D11S932-D11S4170 at 11p15.4-p15.2 (Table 8.3). Three markers that do not show this bias towards increased loss in late stage tumours are D11S1338, D11S1794 and D11S921.

Marker	Association with adverse stage (FIGO III/IV)
D11S932	P=0.0001*
D11S4170	P=0.0009*
D11S1349	P=0.0015*
D11S4188	P=0.0048*
D11S4116	P=0.0072*
D11S1315	P=0.0091*
D11S4138	P=0.0149
D11S4146	P=0.0152

Table 8.3 Microsatellite markers associated with adverse stage (FIGO III/IV). Fisher's exact test used to calculate significance. * indicates p<0.01.

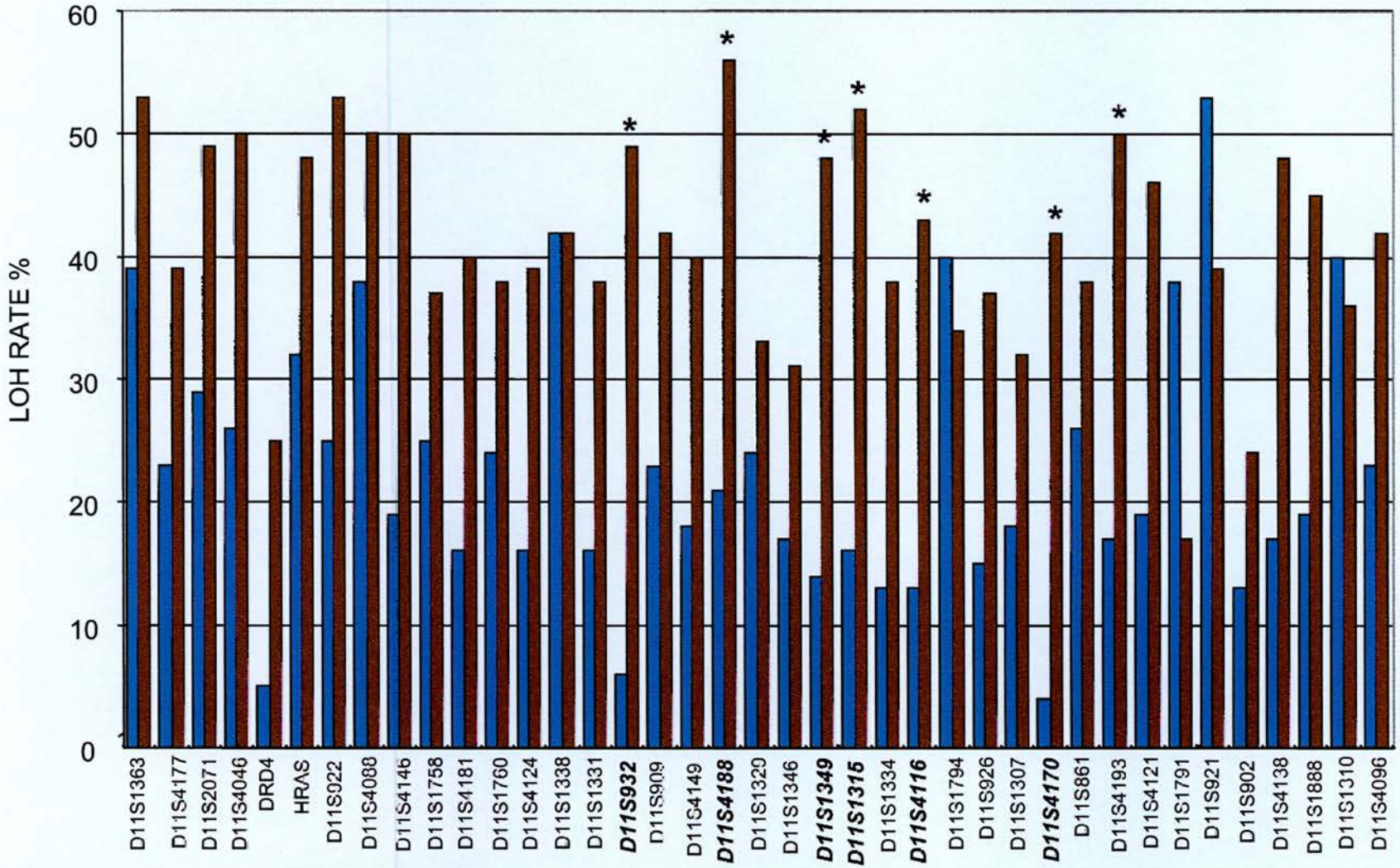


Figure 8.5. LOH rates of markers for stage. A red box shows late stage tumours (III/IV). A blue box shows early stage tumours (I/II). An * and bolded italics indicate significance at <p=0.01.

8.1.4 Allele loss and differentiation status

Only one marker associates with a poor differentiation status D11S4188 (p=0.0055) (Table 8.4). D11S1331 (p=0.0230), D11S4138 (p=0.0201) and D11S1888 (p=0.0467) all show a trend towards high grade. These few associations suggest that loss of the complete region is not associated with a poor differentiation status (Figure 8.6).

Marker	Association with Poor Grade (p value)
D11S4188	P=0.0055*
D11S4138	P=0.0201
D11S1331	P=0.0230
D11S1888	P=0.0467

Table 8.4 Microsatellite markers associated with differentiation status. Fisher's exact test used to calculate significance. * indicates p<0.01.

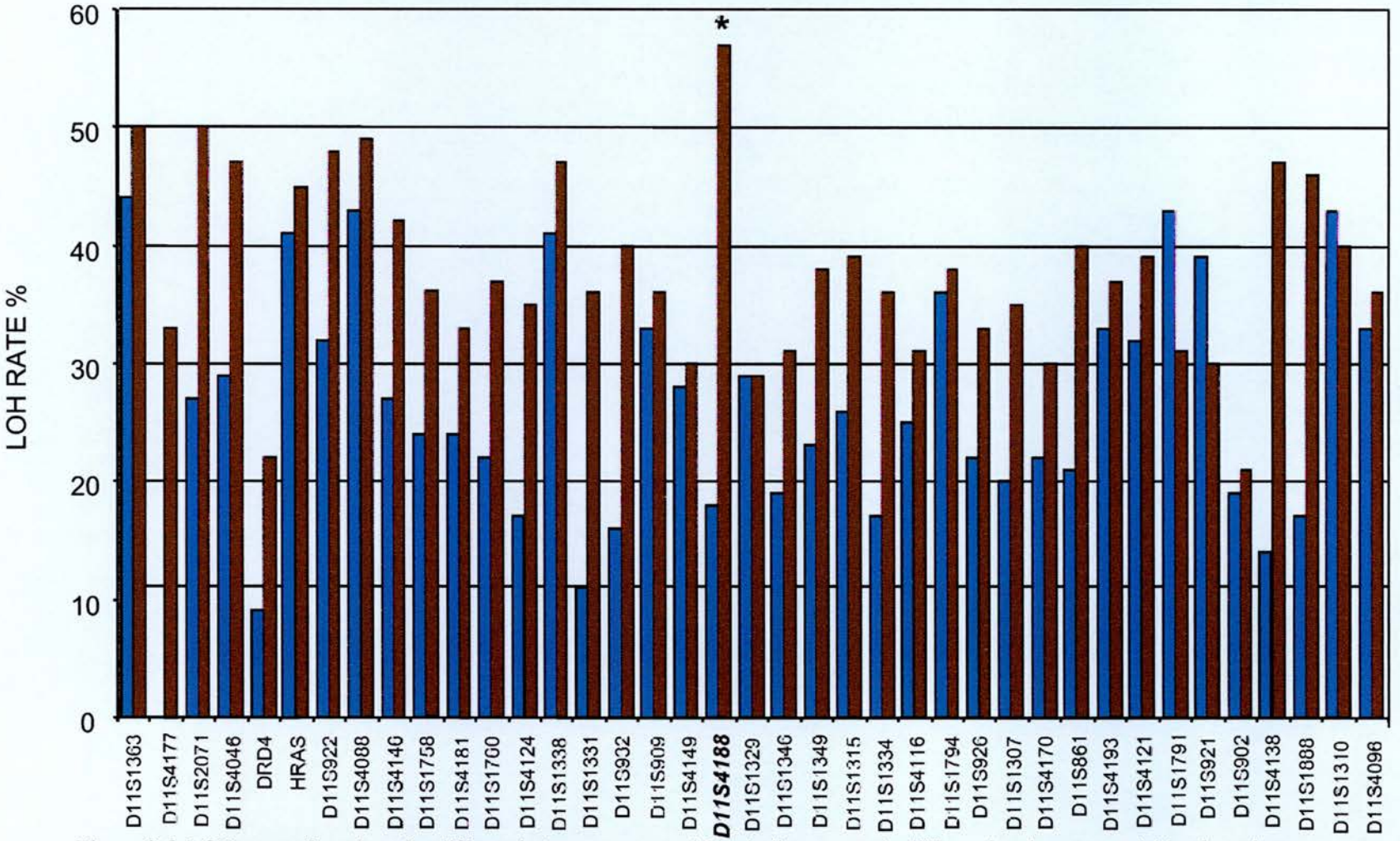


Figure 8.6. LOH rates of markers for differentiation status. A red box indicates poorly differentiated tumours. A blue box shows tumours that are well or moderately differentiated. An * and bolded italics indicate significance <p=0.01

8.1.5 Survival analysis

Taking a cut-off of $p<0.05$ as significant, none of the 39 markers associates with survival when those patients with LOH and follow-up of >2 years are analysed (Table 8.5). There are however two markers with p values very close to significance, D11S4121 and D11S4188.

Marker	Association with adverse survival (p value)
D11S4121	p=0.0508
D11S4188	p=0.0535
D11S932	p=0.1044
D11S922	p=0.1064

Table 8.5 Correlation of marker LOH with adverse survival

LOH at D11S4121 is almost significantly associated with adverse survival (Figure 8.7). The median survival for those with LOH was 4 years as compared to 8.3 years for those with no LOH. At 5 years, patients survival with LOH was only 30% versus 65% for those without.

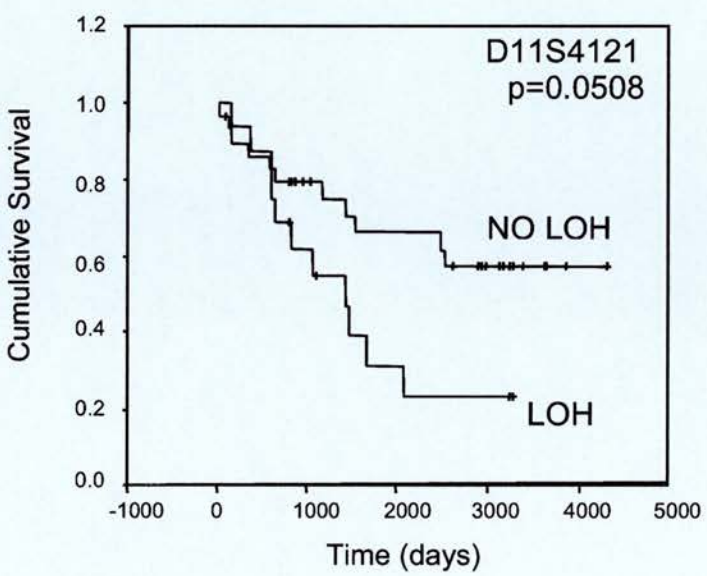


Figure 8.7
Kaplan-Meier survival curve and log-rank analysis for association with D11S4121 status.

Allelic loss at D11S4188 and survival was also correlated very near to significance (Figure 8.8). Patients with LOH at D11S4188 showed an approx. 28% survival rate

as compared to 70% for those without. The median survival of patients with LOH at this locus was 3 years, less than half of the 8 years seen for those with none.

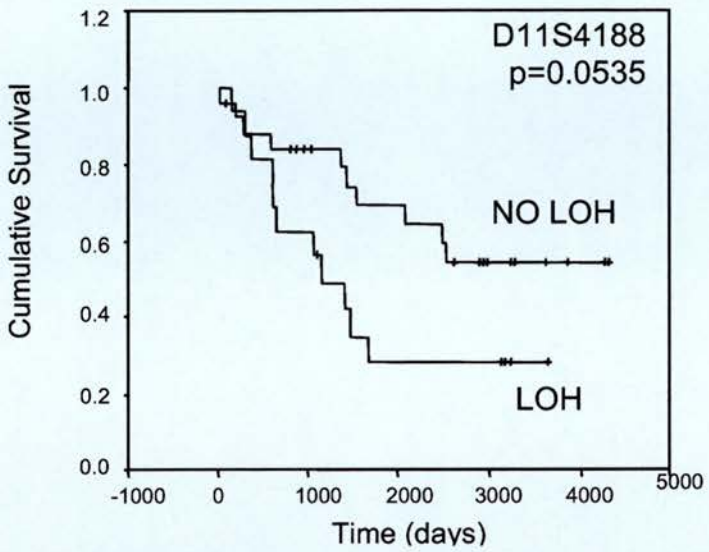


Figure 8.8
Kaplan-Meier survival curve and log-rank analysis for association with D11S4188 status.

8.1.6 Analysis of LOH along 11p15 - overview

High levels of LOH (>35%) were detected for 12 markers of 39 along chromosome 11p15. Figure 8.9 shows a scale diagram of marker positions along 11p15 from telomere to 11p15.1. Marker positions, distance between markers, LOH rate, histopathological correlations, mean peak ratios (R value) and determined heterozygosity rates are indicated.

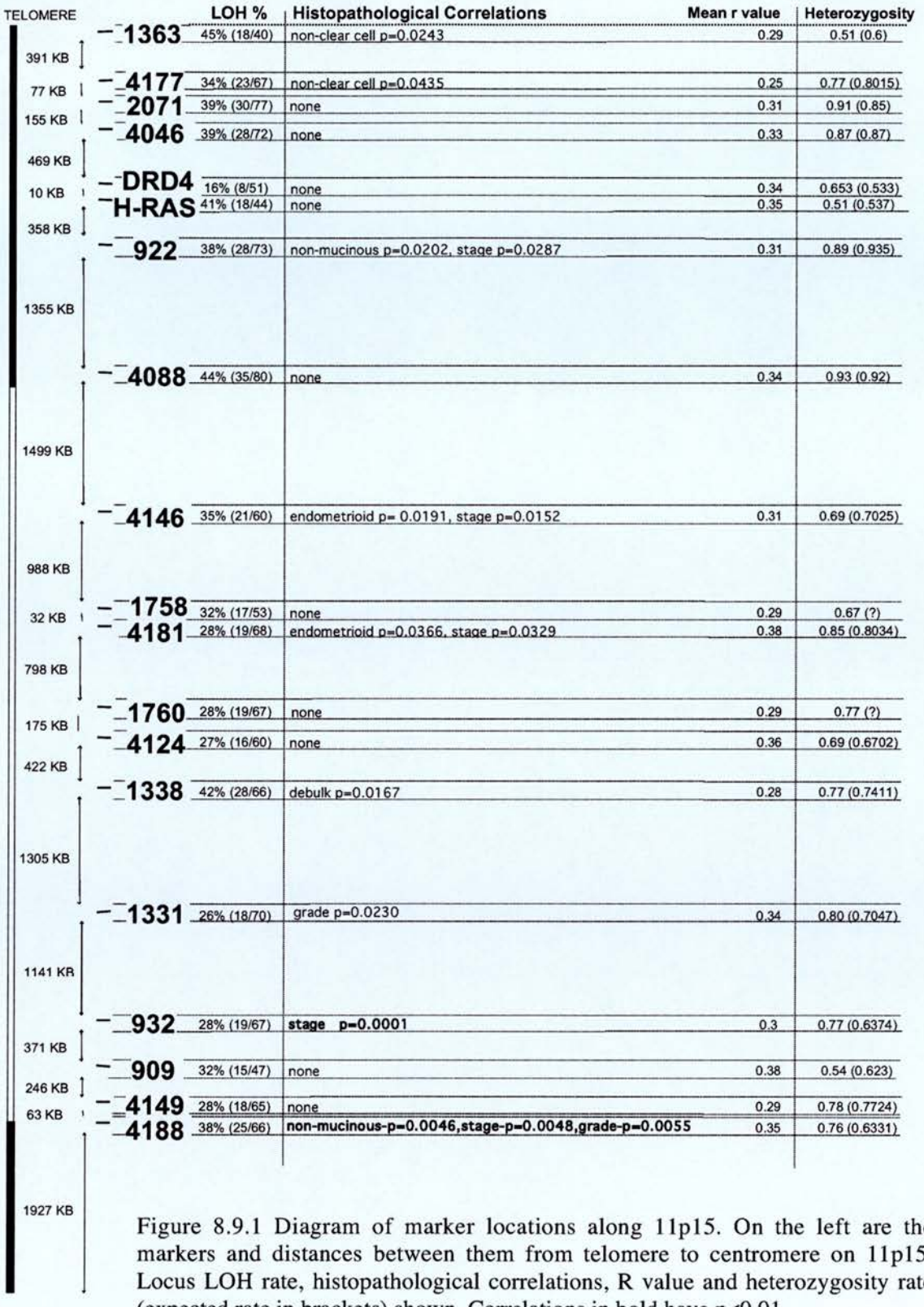


Figure 8.9.1 Diagram of marker locations along 11p15. On the left are the markers and distances between them from telomere to centromere on 11p15. Locus LOH rate, histopathological correlations, R value and heterozygosity rate (expected rate in brackets) shown. Correlations in bold have p<0.01

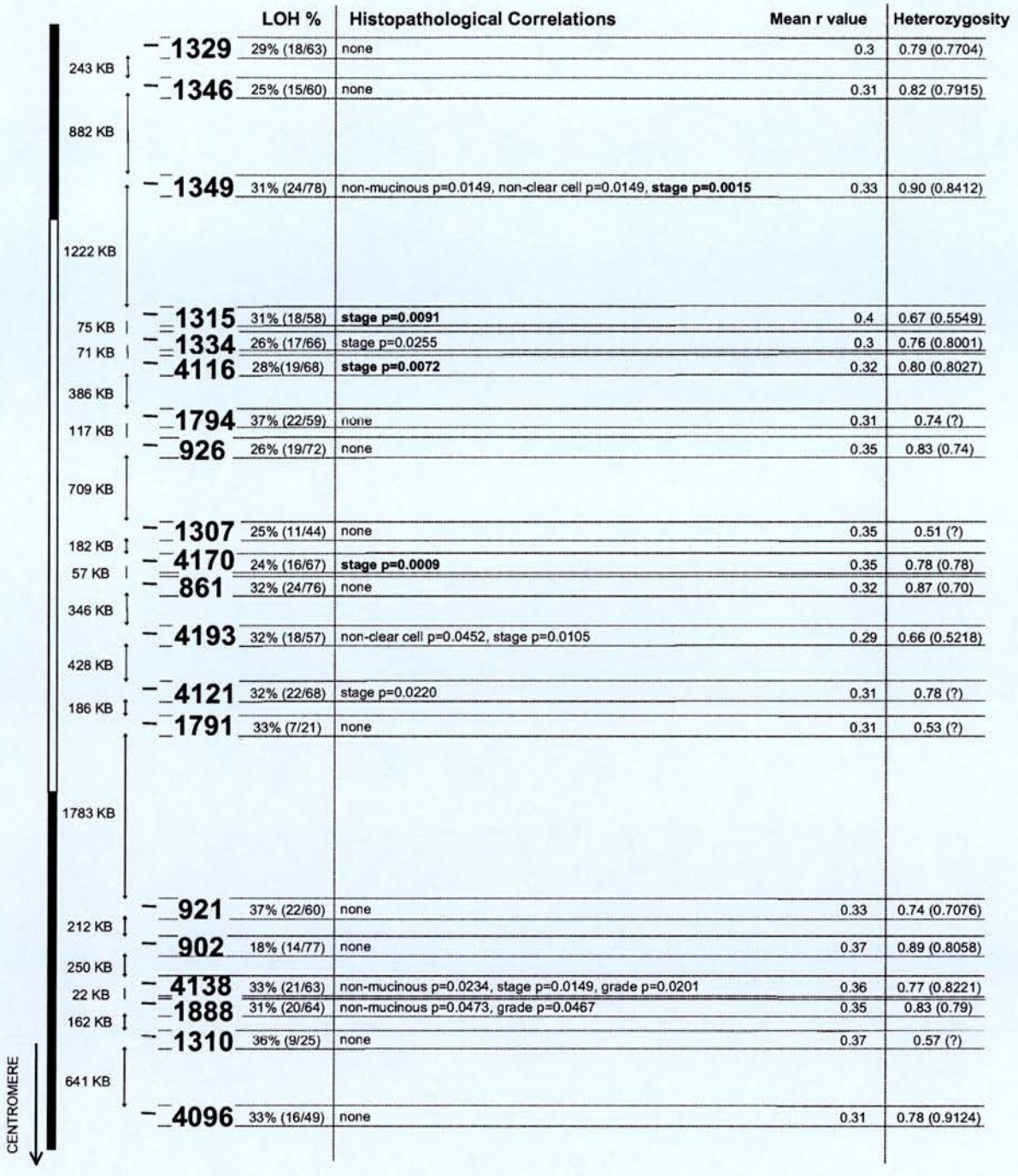


Figure 8.9.2 Diagram of marker locations along 11p15. On the left are the markers and distances between them from telomere to centromere on 11p15. Locus LOH rate, histopathological correlations, R value and heterozygosity rate (expected rate in brackets) shown. Correlations p<0.01 are in bold.

8.2 Minimising Regions of Loss on 11p15.

Of the eighty-seven tumours used in our study, 45 samples showed partial loss along the chromosome at four or less regions. Seventeen tumours had LOH at only one region, the rest of the chromosome being heterozygous or uninformative. A further 8 tumours showed loss of two distinct regions. Thirteen and eight had allele imbalance at three and four areas respectively.

As previously mentioned, 21 samples lost the majority of 11p15 and 8 had no loss at all. The remaining 13 showed patterns of loss known as harlequinning. These samples have bands of loss and retention all the way down the chromosome arm and are difficult to interpret for distinct regions of loss.

Figure 8.9 shows those 45 samples with partial losses. Deletions are shaded and where a locus is uninformative and separates loss from a heterozygous marker, this locus is included in the deletion. Analysis of the 45 samples with partial loss identified eight or nine potential shortest region of loss (SRO's). All are supported by patient samples with only one or two areas of allele loss.

The minimal regions identified encompass from 77KB to 1927KB. Of the 19.7MB spanned by the 39 markers, these SROs represent 7.5MB, minimising our candidate TSG containing area to 38%.

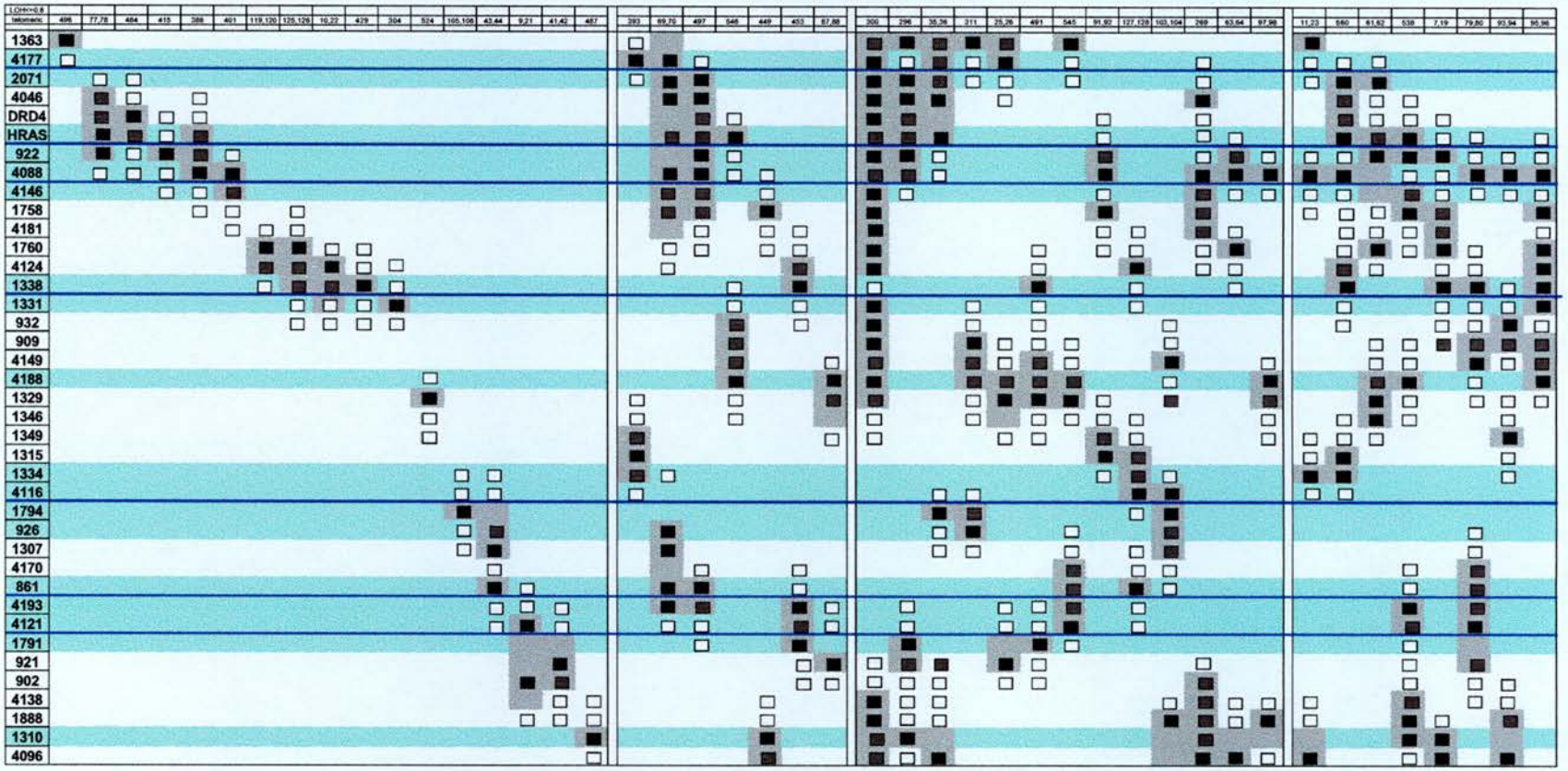


Figure 8.10. Minimal regions of loss in patient samples. A white box= retention of locus, a hashed box = uniformative, a black box= LOH. Grey areas show regions of loss, ranging from one (on left) to four (on right). Blue shading shows minimal losses, highlighted by blue lines.

8.3 Genes within minimal regions of loss

Chromosome 11p15 is very gene rich and the SROs contain varied numbers of candidate genes from 4 to 21 (Figures 8.11-8.13). Of the 160 genes contained within this entire region, our SROs contain 56% of these genes, minimising the number of candidates to 89.

The eight distinct regions are focussed around markers with particularly high LOH rates, apart from region VI, with a lower LOH rate of 33%. The smallest SRO, Region I, spans from D11S4177 to D11S2071 and contains the gene PSMD13, which was identified by cDNA-RDA to be upregulated in the growth suppressed clone 11OH2.1 (Figure 8.11). Region II spans from the H-RAS gene to D11S922 and again this region contains a candidate TSG identified from our difference analysis experiments, CTSD. Region III spans from D11S4088, a marker with high LOH, to D11S4146. This region contains three genes previously associated with cancer progression, including the well studied CDKN1C/p57Kip. Region IV spans from D11S1338, which has a very high LOH and an apparent association with early stage, to D11S1331 (Figure 8.12). The high LOH rate at D11S1338 suggests this marker lies very near an important cancer-associated gene. Spanning from D11S4149 to D11S1329, region V is the largest of our regions, however due to the very significant associations of D11S4188 with stage, grade and histology, this strongly suggests the positioning of a TSG near to this marker.

Region VI is a small region containing only four known genes, spanning form D11S1334 to D11S1794 (Figure 8.13). The high LOH rate of 37% at D11S1794, positioned within the gene ARNTL, indicates this gene may play a role in the progression of ovarian cancers. Regions VII and VIII, spanning from D11S861-D11S4193 and D11S4121-D11S1791 respectively, both encompass markers that do not reach the arbitrary LOH threshold of 35%. The regions have however been lost in multiple samples suggesting a role for one of the included genes in a small number of cancers. Finally region IX, spanning D11S1888 to D11S4096 contains 16 known genes, including candidate cancer associated gene TSG101. This region focuses around the marker D11S1310, situated within an intron of the DELGEF gene (a gene involved in deafness).

Candidate oncogenes and TSGs within these SROs are highlighted. Loss of the TSGs has previously been implicated in various cancers. These oncogenes are shown because they may be excluded from the 89 genes within the regions as they are unlikely to act as TSGs.

LOH rates for the individual regions range from 35% to 49%. Of the nine individual regions pinpointed in Figures 8.10-12, 6 have an LOH rate of >40%. Clinicopathological associations were calculated for each of the regions (Table 8.6). Of the nine regions, 5 are associated with clinicopathological variables. None are associated with survival. It is interesting to see that although some of the markers contained in the SROs have strong individual associations with variables, when they are included into a region these correlations are lost. This, to me, provides more support that gene(s) of interest may lie very near to those markers with significant associations.

Region	Defining Markers	LOH rate	Histopathological associations
I	D11S4177-D11S2071	46%	Non-clear cell p=0.0305, endo and serous p=0.0347
II	H-RAS-D11S922	46%	Non-mucinous p=0.0349
III	D11S4088-D11S4146	49%	None
IV	D11S1338-D11S1331	43%	Debulk status p=0.0395
V	D11S4149-D11S1329	47%	Non-mucinous p=0.0294
VI	D11S4116-D11S1794	37%	Non-mucinous p=0.0467
VII	D11S861-D11S4193	35%	None
VIII	D11S4121-D11S1791	38%	None
IX	D11S1888-D11S4096	42%	None

Table 8.6 Clinicopathological associations of 11p15 SROs. Region number, span, overall LOH rate and correlations with clinical variables shown. Tested with Fisher's exact test.

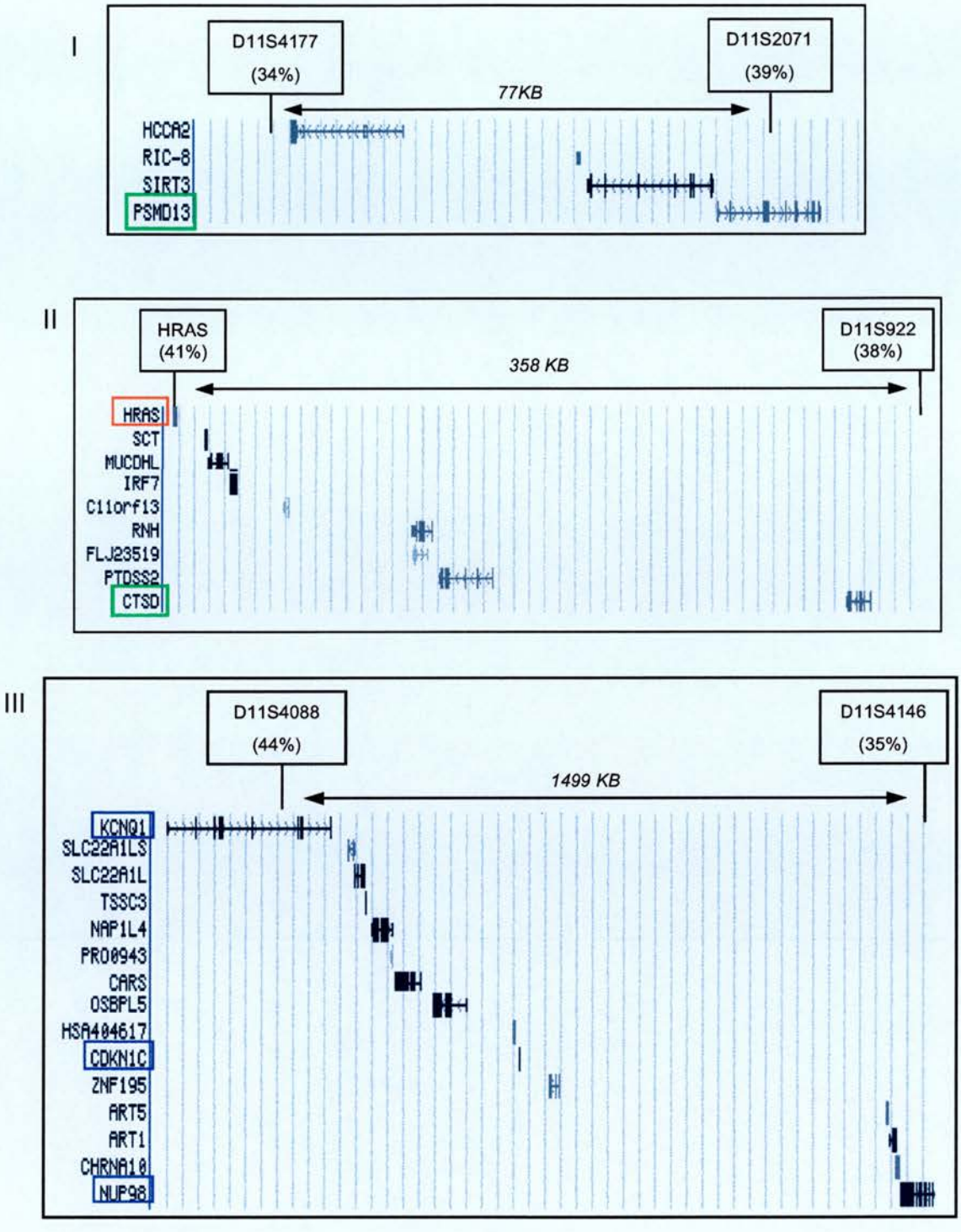


Figure 8.11 Genes within SROs, regions I-III. Taken from UCSC Genome Browser - June 2002. Distances between markers are shown. Green boxes show genes identified by our difference analysis techniques. Red boxes show oncogenes previously implicated in cancers. Blue boxes show implicated TSGs.

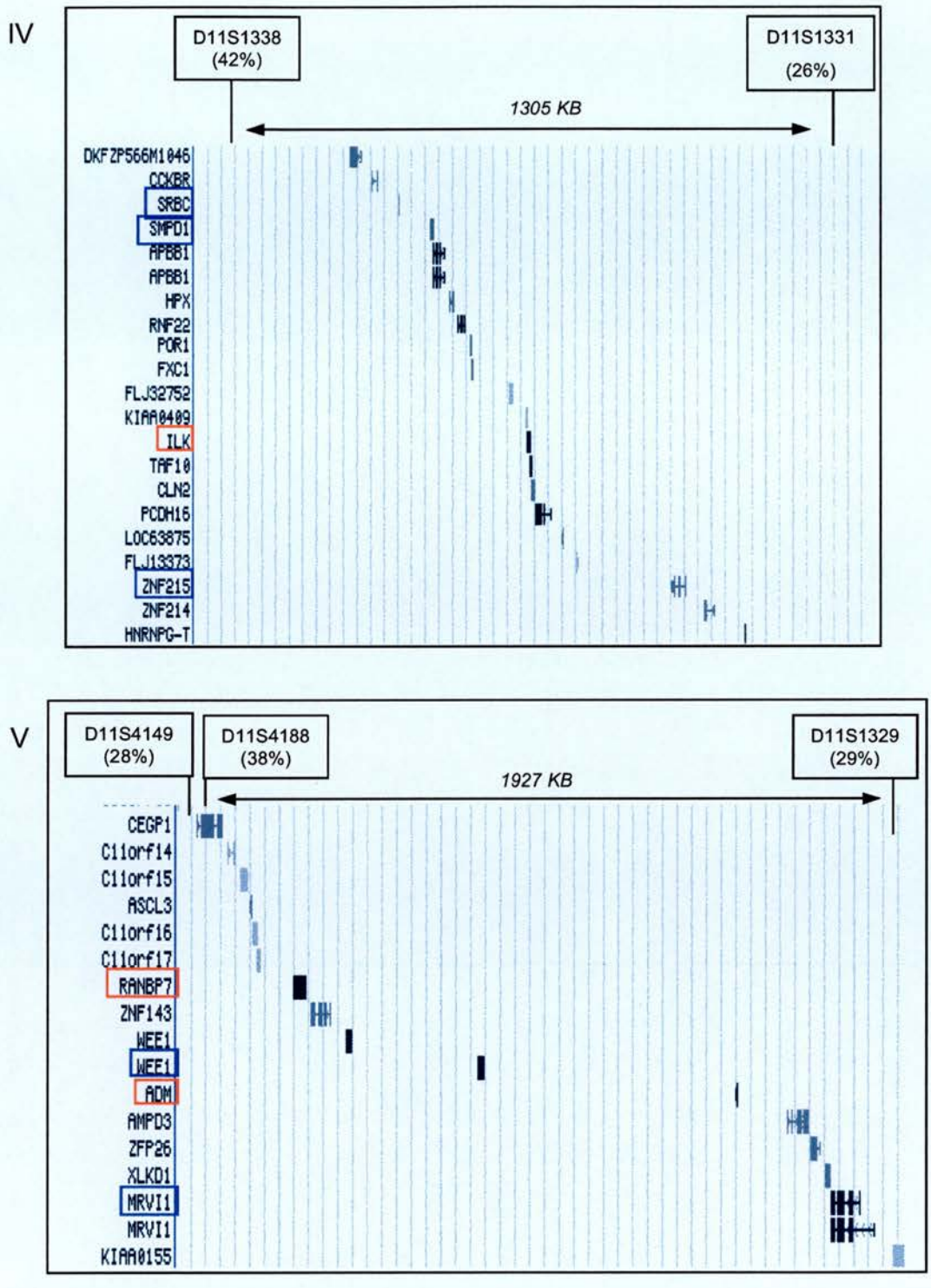


Figure 8.12 Genes within SROs, regions IV and V. Taken from UCSC Genome Browser - June 2002. Distances between markers are shown. Red boxes show oncogenes previously implicated in cancers. Blue boxes show implicated TSGs.

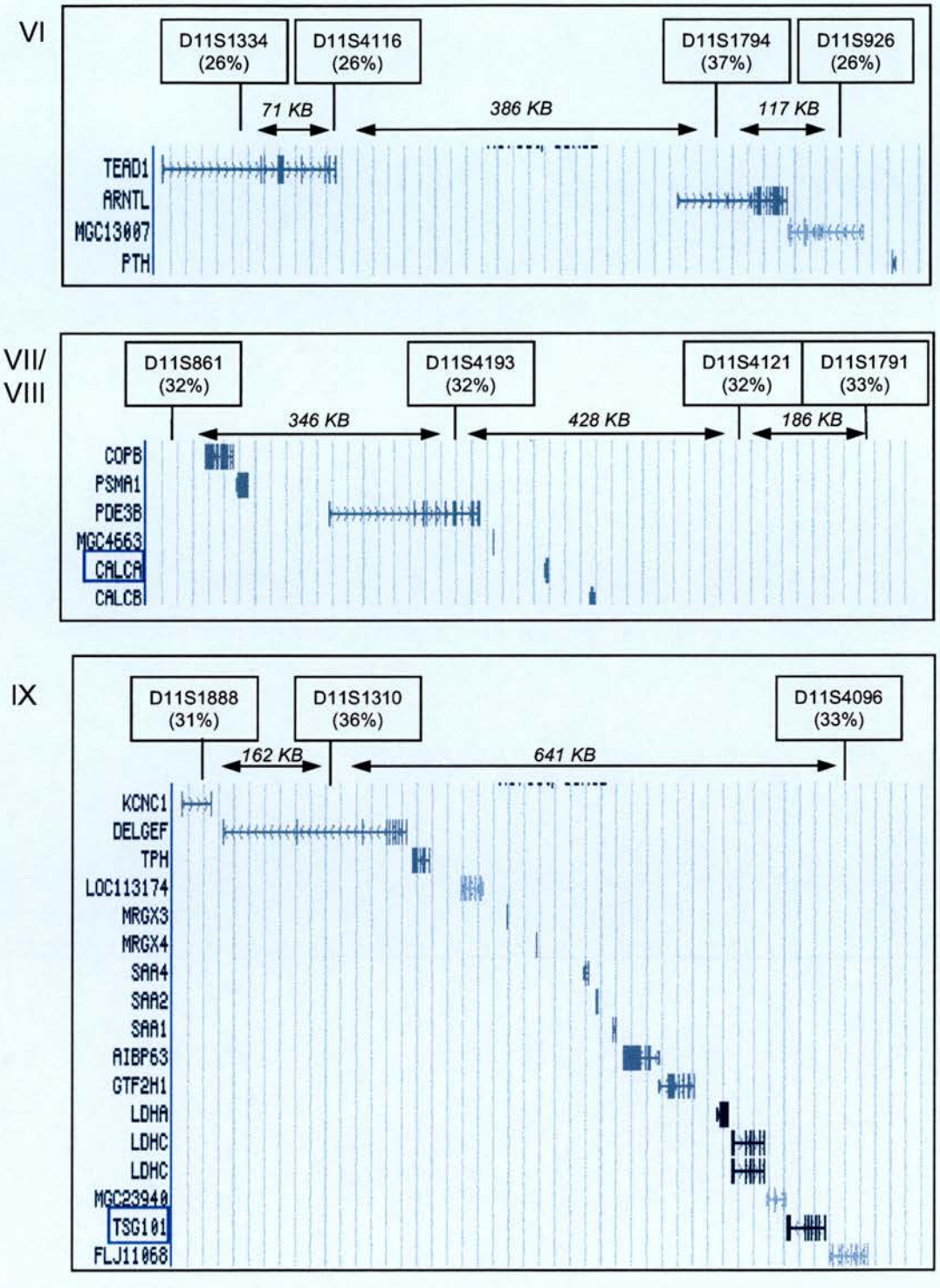


Figure 8.13 Genes within SROs, regions VI - IX. Taken from UCSC Genome Browser - June 2002. Distances between markers are shown. Red boxes show oncogenes previously implicated in cancers. Blue boxes show implicated TSGs.

There are markers that lie outwith these eight regions of loss which are strongly correlated with clinicopathological features. Markers D11S932, D11S1349, D11S1315 and D11S4170 are all correlated with advanced FIGO stage with a significance of about p=0.001.

Whether these associations are due to markers being near to candidate TSGs or as a result of the whole arm loss which has been commonly seen was analysed. By removing 21 samples that have whole arm loss from our data set, many markers lost their associations with advanced stage (Table 8.7).

Loss of markers outwith the nine identified SROs may be lost as part of complete 11p LOH rather than being lost individually or in a discrete region. Those markers that showed clinicopathological associations often lose these when samples with complete region LOH are removed. This suggests that the mechanism of LOH at these loci may be due to whole arm allele loss. Markers within SROs also lose certain clinicopathological associations but there are patient samples only with loss at these regions suggesting that these minimal regions are involved in ovarian cancer.

MARKER	CLINICOPATHOLOGICAL ASSOCIATION
D11S1363	None
D11S4177	None
D11S2071	None
D11S4046	None
DRD4	None
HRAS	None
D11S922	None
D11S4088	None
D11S4146	Endometrioid p=0.0385
D11S1758	None
D11S4181	None
D11S1760	None
D11S4124	None
D11S1338	Early stage p=0.0185
D11S1331	None
D11S932	None
D11S909	None
D11S4149	Clear-cell p=0.0371
D11S4188	Grade p=0.0292
D11S1329	None
D11S1346	None
D11S1349	None
D11S1315	None
D11S1334	None
D11S4116	None
D11S1794	None
D11S926	None
D11S1307	None
D11S4170	None
D11S861	None
D11S4193	None
D11S4121	None
D11S1791	Early stage p=0.0192
D11S921	None
D11S902	None
D11S4138	None
D11S1888	None
D11S1310	None
D11S4096	None

Table 8.7 Clinicopathological associations with marker LOH. Patient samples with complete 11p15 region LOH have been removed from analysis. Two-tailed Fisher's exact test used.

8.4 LOH at 11p15- a literature review

As discussed in the introduction, many groups have carried out LOH studies on chromosome 11p in multiple cancer types. The main areas of interest are at 11p15.5 and 11p13 so these are the most commonly studied areas.

A comparison of our identified regions of LOH along 11p15 and those previously reported in Breast cancer, Lung cancer and Wilm's tumours is shown in Figure 8.14. Direct comparison of these studies is complicated because most authors used different markers, as well as a different number and order of markers. In order to

overcome these problems I have plotted the markers used in the most up to date order possible.

Many of these studies used a minimal number of microsatellite markers with the maximum, to date, being 17 in breast cancer. Those markers used also span short regions of 11p15, focussing around the 11p15.5 region spanning D11S1363-D11S932. There are three common regions of LOH detected in these tumours. The first spans between D11S2071 to just distal of D11S922. The second region is focussed around the area from D11S4088 to D11S4181. The third region common in breast cancer, Wilm's tumour and in our ovarian samples is centred on the marker D11S1338. Only three studies, of the nine, use markers that are centromeric of this point. Four of our regions of loss have not been previously reported.

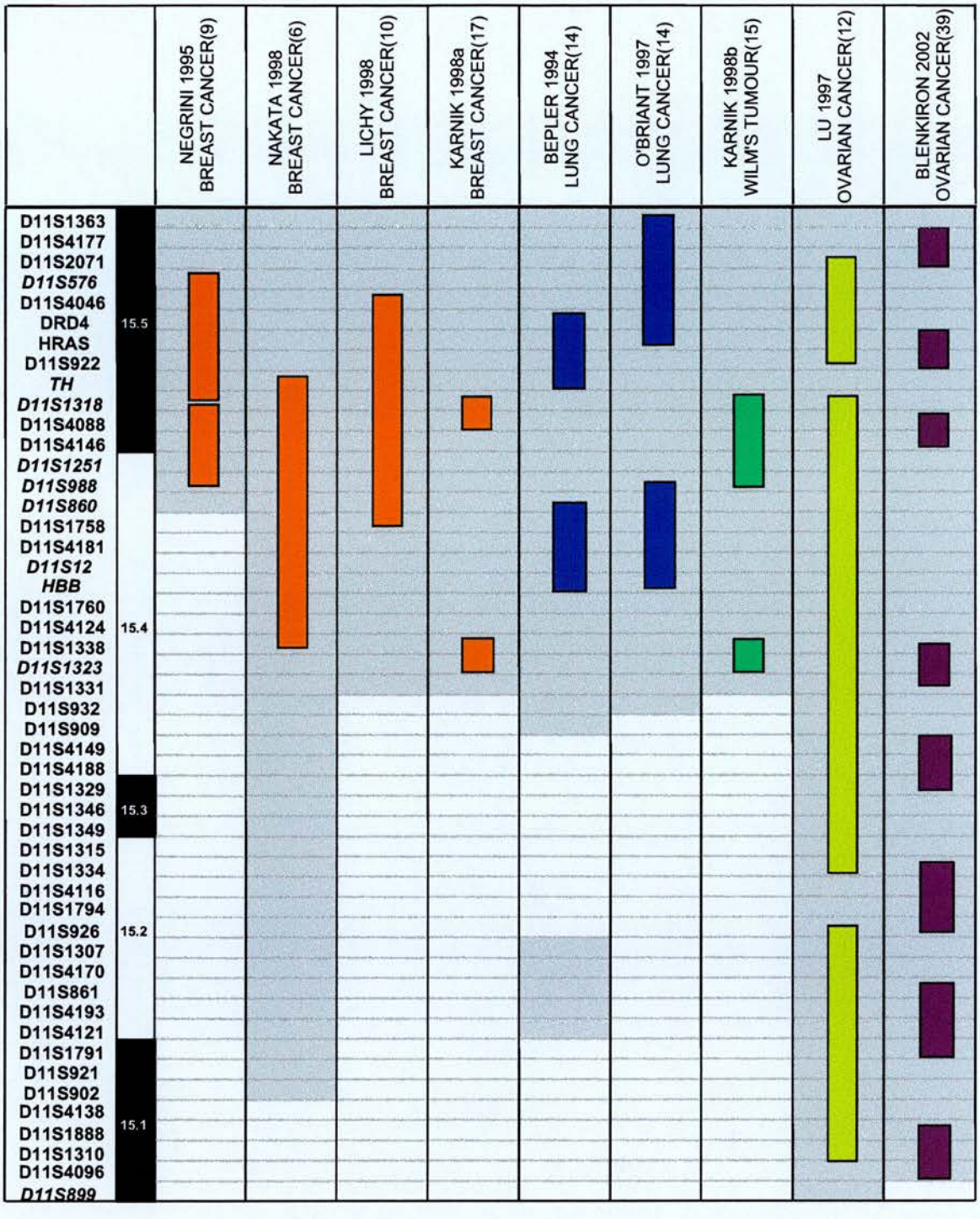


Figure 8.14 Regions of LOH on 11p15 in cancers. Author name, cancer type and year shown. Number in brackets is number of markers used in study. Grey area shows region encompassed by markers. Coloured blocks are regions of LOH. Markers seen on LHS, those in Italics were not used in our study. Our regions of loss shown on RHS.

9. DISCUSSION

9.1 Revertant analysis

Independent transfers of neo tagged chromosome 11 into the ovarian cancer cell-line OVCAR3 generated a series of growth and tumorigenicity suppressed clones.

Microsatellite mapping of MMCT hybrids 11OH1.1, 11OH2.1, 11OH2.2, 11OHX1.1, 11OHX2.1 and 11OHX3.1 with a further eight markers was done to determine the extent of chromosome 11p15 transferred in these clones. This mapping showed that all clones, except 11OHX2.1 had transferred at least the ~9.3Mb region at 11p15.5-p15.4 and also the ~2.6Mb region surrounding marker D11S926 at 11p15.2. This further mapping minimised the regions in which potential TSGs could lie. A highly tumorigenic xenograft of OVCAR3 generated the line OHX. This underwent independent chromosome 11 transfers to generate the 11OHX series. Clone 11OHX2.1 was found to have a less suppressed phenotype than the other 11OHX clones. This clone showed transfer of only the region at D11S926, suggesting that one or more genes involved in the growth suppression lies out-with this transferred region to account for the phenotypic differences seen between clones. This would point to the more telomeric region, at 11p15.5-p15.3, as harbouring functional TSGs.

The traditional approach of minimising candidate TSG harbouring regions following on from MMCT is to perform revertant analysis of the hybrids. Revertants are hypothesised to have lost sections of the transferred chromosome, which carry TSGs, and therefore re-acquire the parental tumorigenic phenotype.

Our initial strategy in identifying these TSG loci was therefore to passage some of our clones ten times with the aim of generating revertants. From four of the hybrids, 11OHX1.1, 11OHX2.1, 11OHX2.2 and 11OHX3.1, we generated 48 passaged clones. These were densely microsatellite mapped at 11p15 and then compared with the parental hybrids to determine regions of chromosome loss. We found that the majority of clones did not lose any of the eight 11p15 markers used. Fourteen clones did however lose the marker D11S926 on the transferred chromosome, positioned at 11p15.3. These clones would need to be functionally assessed for growth characteristics to determine whether the loss of this region leads to an altered growth phenotype.

Through further mapping of the MMCT hybrids we were able to determine the region of chromosome 11p15 transferred within each clone. This mapping allowed us to minimise the candidate 11p15 TSG harbouring regions to two areas spanning ptel-D11S932 and D11S569-D11S419 (Figure 9.1).

We did not find sufficient information from the revertant mapping method to identify minimal candidate TSG containing regions. This standard approach was therefore unsuccessful for us so an alternative was needed, and led to the use of difference analysis methodology. We carried out cDNA-RDA to compare expression differences between the hybrid 11OH2.1 and a parental derivative OVCAR3. The revertant analysis and cDNA-RDA were carried out in parallel in anticipation of these problems.

Revertant analysis has been relatively unsuccessful in minimising regions, although there are notable exceptions. For example the prostate cancer associated metastasis suppressor KAI1 was identified by this method (Kawana *et al.*, 1997). This technique has proven successful in that we were able to generate revertants with chromosome loss at 11p15, so if time allowed I may choose to continue with further revertant analysis. Initially, I would repeat the passaging and use all of the hybrid clones, including 11OH2.1, probably passaging fifteen or more times as we have seen that chromosome loss after only ten passages is rare. I would also use more microsatellite markers, as there may actually be small fragments of loss that we have not detected using only eight markers. If this were successful the next step would be to transfer small subchromosomal fragments of DNA, which map to those regions of loss associated with reversion.

If a small region harbouring only a few candidate genes was found, those candidates within this interval would then be analysed for expression in tumours and further assessed for mutation or methylation.

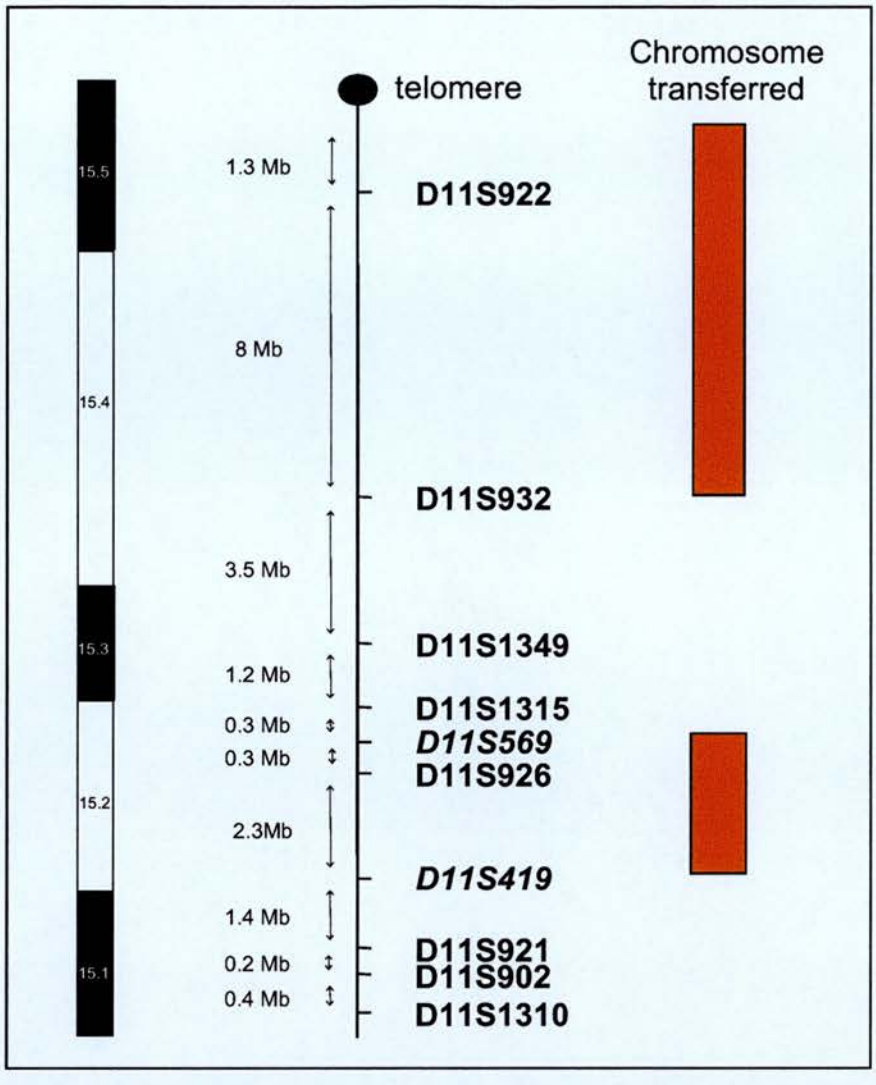


Figure 9.1 Chromosomal regions harbouring candidate 11p15 TSGs. Red boxes indicate the regions transferred. Markers in *Italics* were previously mapped.

As the hybrid 11OH2.1 was chosen for use in the difference analyses, I decided to fine map 11p15 in this clone. In all, 19 polymorphic markers spanning 19.1Mb from ptel-11p15.1 were mapped, with interval spacing of between 200Kb and 3Mb. I found that the complete 11ptel-11p15.4 (D11S1363-D11S4188) region was retained, as was 2.8Mb section from D11S4116-D11S419 (p15.2) (see Figure 3.5). Therefore if there are any functional 11p15 TSGs, they must reside within these regions.

9.2 Difference analysis methods.

The comparison of gene expression between ovarian cancer cell-line OHN and Chr 11 transferred hybrid 11OH2.1 was performed using three difference analysis techniques, DD-RT-PCR, cDNA-RDA and HDFA.

DD-RT-PCR, as a method, produced the fewest differentially expressed candidate genes. In all the technique identified 18 products with altered expression between OHN and 11OH2.1. The second method is cDNA-RDA, which couples subtractive hybridisation with kinetic enrichment. cDNA-RDA was carried out five times, two to identify candidate TSGs and three in the reciprocal direction to identify those genes upregulated in OHN and potentially oncogenic. Products from cDNA-RDA were subcloned into bacterial plasmids and individual colonies picked for sequencing. The number of independent transcripts that can be identified should increase with the number of colonies sequenced. We sequenced, in total, 572 transcripts that represented 231 individual genes.

The final method used was the hybridisation of OHN and 11OH2.1 RNA to Clontech 1.2K arrays. Three arrays were hybridised, each carrying 1,176 clones. Prior to validation, differentially expressed products identified by these arrays were subjected to strict inclusion criteria, which minimised the data set to a total of 72 genes.

DD-RT-PCR and cDNA-RDA are examples of open systems of expression analysis, that is they are capable of identifying all genes within a transcriptome that may be differentially expressed, including novel ones (Green *et al.*, 2001). It is not therefore surprising that these methods each pinpointed novel genes. A microarray is limited by its clone content and is therefore a closed system. One advantage of the microarray system however, is that all genes contained on the chip are assayed whether differentially expressed or unaltered. As we are aiming to identify genes which are differentially regulated along one chromosome this is useful as those chromosome 11 genes which do not show expression differences can be disregarded as candidates.

Considering that, in all, 310 individual genes were identified as differentially expressed, overlap between the methods was minimal. No genes were identified by all three methods. Only 10 genes were identified by two independent methods.

Due to the false positive rates reported for these difference analysis methods, further validation using quantitative RT-PCR was performed for the majority of the identified candidates. Of the 321 genes in total, 178 were assessed for validity by quantitative RT-PCR. Genes to be validated were chosen due to their cellular function, abundance of transcripts (in the case of cDNA-RDA) and importantly their position within the genome. All chromosome 11 genes identified as upregulated in growth suppressed hybrid 11OH2.1 were subjected to Light Cycler quantitative RT-PCR.

Of the 178 genes assayed (100 up and 78 downregulated), only 17 were confirmed to be differentially expressed >2-fold between OHN and 11OH2.1. Of these two represent the same gene and one is a mouse contaminant from the chromosome transfer method. This murine sequence, MMLV, was found by cDNA-RDA and served as a useful positive control for the cDNA-RDA method. Other murine transcripts were identified by DD-RT-PCR but not further validated. HDFA represents only human sequences so murine transcripts are not assayed.

9.2.1 Validated Candidate TSGs

Eleven human genes were found to be upregulated in response to transfer of chromosome 11 into 11OH2.1. These include three structural keratins and four chromosome 11 products. A substantial enrichment in chromosome 11 candidates was seen after Light Cycler validation. The non-chromosome 11 located products fall into two functional categories. These are proteins involved in cellular structure and then those associated with the normal trafficking and degradation of proteins. These non-chromosome 11 products are downregulated in response to the chromosome transfer and could well be involved as downstream factors in the growth suppression phenotype.

9.2.2 Genes with roles in cellular structure

Disruption of genes involved in cell structure within tumours may promote invasion and metastasis. Tumour invasion involves in the upregulation of cell surface receptors, intercellular adhesion molecules and signalling proteins (Skubitz, 2002). A

metastatic cell may also increase expression of genes that degrade the existing ECM, such as matrix metalloproteases and collagenases.

Our difference analysis techniques identified three upregulated keratins, KRT5, KRT8 and KRT19. The upregulation of these genes may effect an increase in cell structure and stability.

Keratins 8 and 19 are expressed normally in simple epithelial cells suggesting that the reversion back to a more OSE-like state, such as in the 11OH2.1 cell-line, may cause their upregulation. KRT8 and KRT 19 are positively co-expressed according to the SAGE and NCI60 data available from CGAP (http://cgap.nci.nih.gov/). Keratin 5 is involved in the regulation of cell size and shape and it has been reported as decreased in expression between rat normal bladder cells (100%) and highly metastatic bladder cell lines (20%) supporting the expression difference we have found (Nan *et al.*, 1993). CGAP data shows an association of KRT5 co-expression with SLPI, a gene commonly over expressed in ovarian tumours. This association suggests that expression of KRT5 may simply be an effect of the growth alteration rather than a direct cause, which is consistent with our observation.

Tumour cells have highly upregulated levels of matrix metalloproteases (MMP) which promote degradation of the ECM and assist in cell motility and metastasis (fantastic review- (Egeblad and Werb, 2002)). MMPs are upregulated in multiple tumour types including ovarian cancer (Herrera *et al.*, 2002). We identified TIMP2, which is an inhibitor of matrix metalloproteases, particularly MMP2 (Wang *et al.*, 2002). TIMP2 has been shown to inhibit invasion in cells derived from a serous ovarian tumour, probably acting by inhibition of MMP2 (Yoshida *et al.*, 2001). In ovarian cancer effusions, that is cells which have migrated away from the main bulk of the tumour, TIMP2 expression is decreased with a concomitant increase in MMP2, strongly suggesting that loss of TIMP2 is involved in the invasive phenotype (Davidson *et al.*, 2001).

We also identified EGFR as upregulated in the growth suppressed hybrid. This discovery is paradoxical as EGFR is frequently found to be upregulated in tumours and is known to stimulate tumour growth and invasion. A decrease in EGFR expression decreases adhesion on laminin-1, which would promote invasion, but conversely lowers MMP9 activity (Alper *et al.*, 2001). The introduction of

chromosome 11 into the suppressed hybrid causes increase in expression of EGFR but the mechanism is, at this point, obscure. CGAP data show a positive correlation of EGFR expression with the expression of chromosome 11p15.5 gene LMO1 (LIM domain protein). It may be the introduction of a functional LMO1 that is increasing expression of EGFR.

KRT8, KRT19 and TIMP2 have been identified as differentially expressed in ovarian cancers by expression analyses previously (see Figure 1.3). KRT8 is normally reported as upregulated in ovarian tumours rather than the decrease in expression that we have detected. KRT19 and TIMP2 have been seen as up and down regulated depending upon the experimental situation. This variation may be due to the use of different techniques and samples. We can however say that in this context, these genes are upregulated with growth suppression.

9.2.3 Genes involved in Protein degradation/trafficking

Proteins involved in the degradation and trafficking of specific molecules are vital for normal cell cycle regulation.

HM13/SPP is a protease involved in the proteolysis of signal peptides after cleavage from a preproprotein. It acts as a presenilin-type aspartyl protease (Weihofen *et al.*, 2002). Signal peptide peptidase activities are associated with cell regulation and signalling. SPP is also vital in antigen processing for MHC immune response (Weihofen *et al.*, 2002). Loss of this protein in tumours may hypothetically allow the cancer to evade the host immune defences or have a role in the dysregulation of cell signalling. SAGE data from the CGAP project shows a significant decrease in expression of SPP in ovarian tumours as compared to their normal counterpart by virtual Northern.

BENE encodes a proteolipid involved in raft mediated trafficking in endothelial cells (de Marco *et al.*, 2001). BENE is involved in raft reorganisation and may be important in the transport of GPI anchored proteins between the plasma membrane and the internal compartments (Alonso and Millan, 2001). MAPK and PLCγ signalling cascades initiate within these lipid rafts. BENE also interacts with candidate TSG Caveolin-1 and co-localises with CD59, interestingly encoded from a

gene on 11p13 (Wiechen *et al.*, 2001). It could be hypothesised that loss of BENE in tumours alters signalling and intracellular transport.

Neither SPP nor BENE have been described as downregulated in ovarian tumours previously.

9.2.4 Chromosome 11 candidates

Four genes were validated as candidate Chr 11 TSGs. These are PSMD13, CTSD, RPL27A and CRYAB. Interestingly three of these genes are positioned at 11p15, which has been shown to have high rates of LOH (as reviewed in introduction-section 1.7). All four genes lie within the region of Chr 11 transferred into 11OH2.1.

9.2.4.1 Proteasome subunit PSMD13

PSMD13 is a 19S proteasome subunit and forms part of the lid structure of this complex (Figure 9.2). The 19S subunit acts to regulate the function of the 20S proteasome and is essential for the degradation of multiubiquitinated proteins. PSMD13 has a yeast homologue named Rpn9 which is needed for stability/efficient assembly of the 26S proteasome (Takeuchi *et al.*, 1999). The protein also appears to be important in the degradation of multiubiquitinated proteins through its recruitment of subunit 5a to the 19S proteasome and may also be required for efficient heat shock response. A temperature sensitive mutant of Rpn9 arrests at G2/M when at the permissive temperature (Takeuchi and Toh-e, 2001). Loss of this protein in yeast causes the slow degradation of Pds1p for which the human homologue is the oncogene PTTG (Zou *et al.*, 1999).

The 19S lid-complex consisting of twelve protein subunits can act independently of Rad23 in Nucleotide excision repair (NER) (Russell *et al.*, 1999). The loss of expression of Rpn9 may alter this activity.

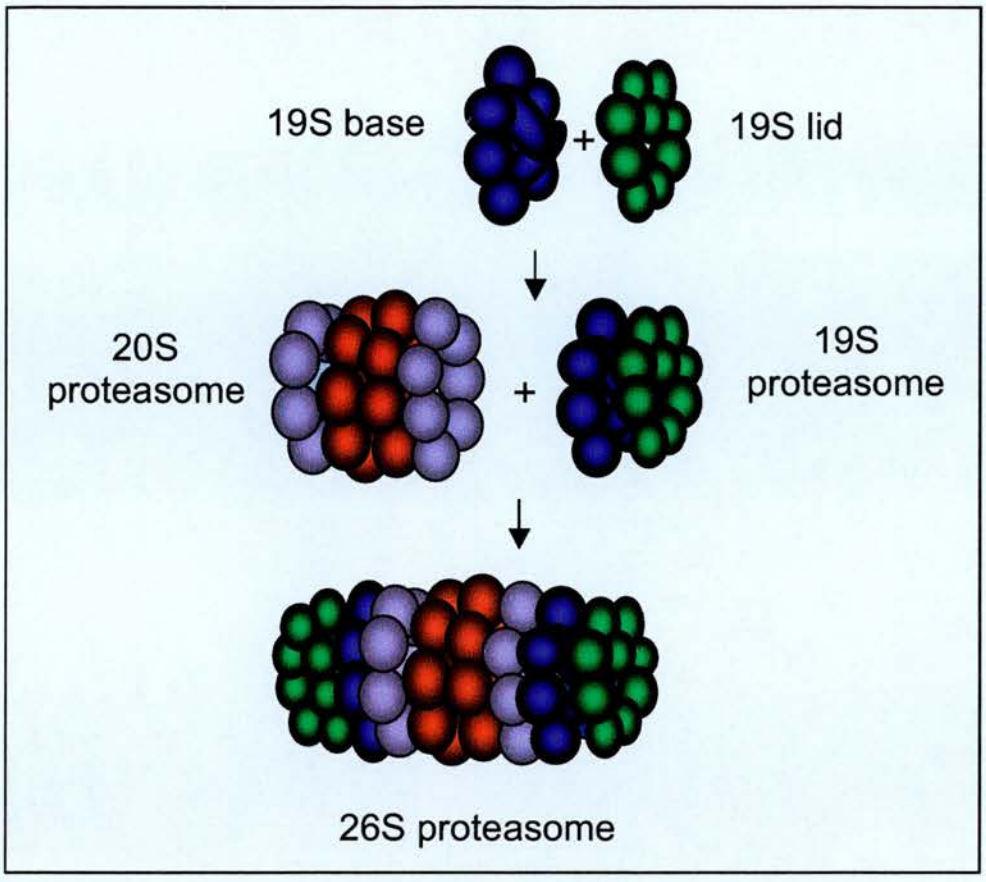


Figure 9.2 Structure of the 26S proteasome. The 26S proteasome degrades multiubiquitinated proteins and consists of one 20S subunit and two 19S regulatory subunits. The 19S subunit is made up of a lid and a base complex. PSMD13 is a component of the 19S lid.

The yeast rpn9 subunit interacts with multiple proteins, including other 19S subunits, DNA repair proteins and replication associated proteins (Figure 9.3). Many proteins interact with multiple subunits of the 19S proteasome such as repair protein Rad23 and deubiquitinase USP6. There are however proteins which appears to interact with Rpn9 exclusively, of all the proteasome subunits. Interestingly these proteins are involved in transcription, DNA repair and more curiously the aromatase cytochrome CYP19/ARO1.

The effects of these interactions are however unknown. Aromatase is essential for the production of estrogen from its precursors. In breast cancers aromatase inhibitors are seen to decrease proliferation and activate apoptosis (Sasano *et al.*, 1999). Overexpression in mice of the aromatase transcript causes increased hyperplasia in mammary glands as well as an associated decreases in apoptosis and in expression of

TSGs p53 and Rb (Kirma *et al.*, 2001). If Rpn9 has a negative regulatory effect upon aromatase activity it could be postulated that knockout of expression would be advantageous for the tumour. Another interesting protein with which Rpn9 interacts is Ribonucleotide Reductase, M1 (RRM1) which in humans also lies on 11p15.5. The activity of RRM1 correlates significantly with the rate of DNA synthesis and is constitutively expressed in cycling cells.

The human form of PSMD13/p40.5/TSAP13 has been identified as downregulated with decreased tumorigenicity/increased apoptosis as instigated by p53/p21waf signalling in the cell-line K562 (Roperch *et al.*, 1999). The 19S subunit regulates activity of the whole 26S proteasome complex and has other nonproteolytic functions within the cell. It has been seen to play a role in efficient activated, not basal, transcription (Ferdous *et al.*, 2002). The human PSMD13 gene is not well characterised in the literature and its loss has not previously been implicated in cancer progression.

The interaction between PSMD13 and other proteins, as seen in yeast and humans, suggests that it may have a non-proteolytic role in the cell. Transcription, DNA repair, DNA replication, estrogen regulation and heat shock response are all pathways in which

this protein may normally function. Its disruption in ovarian cancers may cause dysregulation of these cellular pathways. Further validation of PSMD13 gene expression in a panel of tumours and cell-lines confirmed loss of expression in all ovarian cancer cell-lines as compared to normal OSE. Of 18 primary HOV tumours, decreased expression was detected in four (22%).

The transfection of full-length PSMD13 into an OVCAR3 clonal line was unsuccessful and needs to be repeated in order to prove functionality of this protein in tumours.

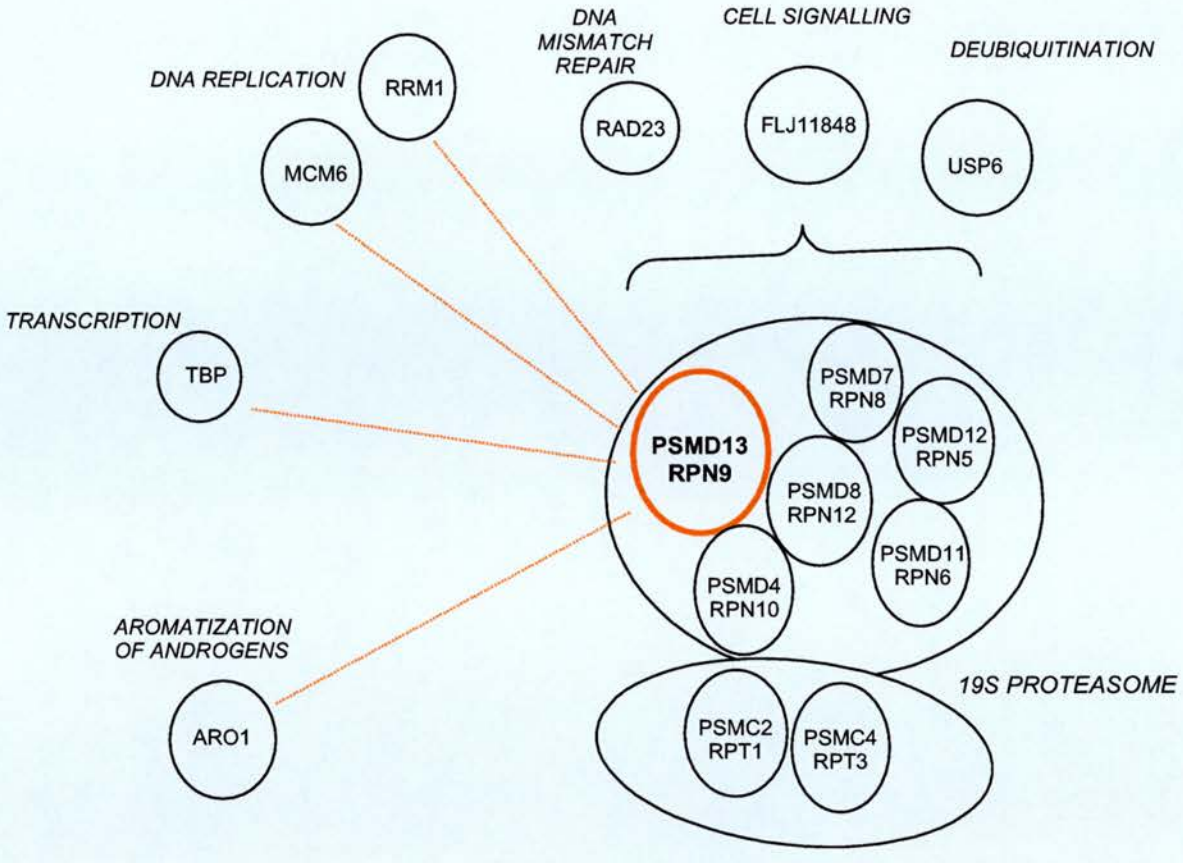


Figure 9.3 PSMD13 (Rpn9) protein interactions in yeast. Taken from http://genome-www.stanford.edu/Saccharomyces/. All proteins shown interact with PSMD13 (Rpn9). RAD23, FLJ11848 and USP6 also interact with other components of the 19S proteasome.

9.2.4.2 Protease Cathepsin D; CTSD

Moving down the chromosome, CTSD, at 11p15.5, encodes for a lysosomal aspartyl protease. Unlike PSMD13, the human form of this gene has been widely studied, particularly for its involvement in the progression of breast and ovarian cancers. Historically, in ovarian cancers, expression of CTSD was determined to be a poor prognostic factor of survival (Scambia *et al.*, 1991; Scambia *et al.*, 1994; Shaheen *et al.*, 1995). These observations correlated with the consistent view that CTSD expression is a prognostic factor in breast cancers (Fitzgibbons *et al.*, 2000). More recent IHC studies, however, have shown expression of CTSD in the epithelial cells and stroma of tumours as a favourable prognostic factor for survival in advanced EOC (Baekelandt *et al.*, 1999b). Furthermore, overexpression of CTSD inhibits growth of colon, liver and ovarian cancer cell-lines in vitro (Wu *et al.*, 1998). Knockout of CTSD in mice showed it to be essential for the degradation of proteins

involved in cell growth and tissue homeostasis (Tsukuba *et al.*, 2000). Secretion of CTSD in prostate carcinomas cells has also been shown to induce production of angiostatin, a potent angiogenesis inhibitor (Morikawa *et al.*, 2000).

CTSD is a mediator of IFN-γ and TNF-α induced apoptosis (Wu *et al.*, 1998). This activity is associated with an increase in p53 activity and transcription (Kagedal *et al.*, 2001). p53, estradiol and retinoic acid induce expression of CTSD. This complex regulation of CTSD reflects its many normal roles in the cell including protein degradation, antigen processing and induction of apoptosis.

We have shown that CTSD is upregulated in the growth suppressed chromosome 11 hybrid 11OH2.1. Analysis of CTSD mRNA expression in a panel of ovarian, colon and breast cancer cell-lines and HOV tumours showed clear downregulation of this gene in all lines tested. SKOV3 cells, which we have found to have robust expression of CTSD do not show increased mRNA expression in response to estradiol (Hua *et al.*, 1995). This suggests a defect in the control of CTSD that may be due to promoter dysfunction.

Clinicopathological analysis of expression in 18 HOV samples showed that very low expression of CTSD in patients (<6%) correlated significantly with poor survival. Expression <20% correlated with poor differentiation status of tumours. Furthermore, the availability of a good antibody allowed IHC analysis of 48 ovarian tumours, which showed a trend towards association of very low protein expression with adverse survival (p=0.0847). Loss of protein expression significantly correlated with a serous histology. This data shows that CTSD expression is a clinical prognostic factor for the most common histological sub-type of EOC although further IHC is required to confirm this.

CTSD protein expression in lysates from 22 cancer cell-lines was also assayed. Western blots were done using an antibody that detects all forms of the CTSD protein. Three different molecular weight bands were found which are believed to correspond to the 52kDa proenzyme, the cleaved 34kDa active form of CTSD, and a smaller unknown protein of approx. 30kDa.

Bazzett et al analysed the presence of CTSD proteins in the sera of ovarian cancer patients and found multiple bands ranging from 24-60kDa (Bazzett *et al.*, 1999). In control sera they however found a major band at 34kDa and two minor bands of 27

and 48kDa in half of the samples. These control bands correspond with our data. Our analysis used mini-gel westerns, which are less accurate of determining band sizes due to decreased resolution. The largest band at 48-52kDa was seen in four ovarian cancer cell-lines, PEO23, PEO4, SKOV3, and OVCAR5 and was also seen in the estrogen treated breast cancer cell-lines MDA, MCF7, ZR75. This band may represent the uncleaved pro-CTSD that has been shown to increase proliferation and decrease sensitivity to chemotherapy of tumours (Bazzett *et al.*, 1999). Interestingly, the platinum sensitive isogenic pairs of PEO23 and PEO4 do not exhibit expression of this 52kDa form confirming these findings. In the breast cancer lines we tested, CTSD mRNA expression may have been induced by their estrogen treatment, leading to high protein levels of all three CTSD isoforms. All of the cell-lines expressed the activated 34kDa CTSD isoform to some extent. Leukaemia cell-lines K562 and Jurkat however, showed very low levels of expression. No normal OSE lysate was available, so expression in these cell-lines cannot be compared to normal levels. The western blots did however confirm the upregulation of CTSD in 11OH2.1 cells for the mature 34kDa protein and the smaller 27-30kDa protein. We also detected a strong correlation between mRNA and 34kDa protein levels. This suggests that the low mRNA expression seen by quantitative RT-PCR in the HOVs may relate directly to protein expression.

The analysis of CTSD mRNA and protein expression in multiple ovarian cell-lines and HOVs has shown that this gene is downregulated in the majority of tumours. Clinicopathological associations suggest this gene may be important in the progression of ovarian cancers. To prove that CTSD acts as a TSG in ovarian tumours analysis of patient samples for evidence of mutation or methylation is required. Full-length sense transfections of CTSD are underway and will hopefully prove that this gene is responsible for functional suppression of the cells.

9.2.4.3 Ribosomal protein L27A: RPL27A

RPL27A lies distal to CTSD at 11p15.4. This protein forms a rRNA binding subunit of the 60S ribosome that is essential for translation (Kusuda *et al.*, 1999). Very few studies have been done to determine to function of this protein out-with or even in conjunction with its ribosomal activities. We have identified this gene as upregulated

2.3-fold upon introduction of chromosome 11 into OVCAR3 cells. CGAP virtual Northern data shows a significant reduction in expression of RPL27A in ovarian tumours as compared to normal ovary. Ovarian cell-line expression of RPL27A was lower than expression in normal OSE for all samples but one (PEO4). This low expression was also seen for the breast cancer cell-lines MDA and ZR75 although we could not compare expression to that of normal breast tissue due to lack of resources. HOV tumour expression of RPL27A was variable with six of the eighteen patients having loss of expression (33%). Although expression of RPL27A did not correlate with any clinicopathological parameters, we found a striking association with expression of PSMD13 ($p<0.0001$). This correlation was seen for all samples, including colon and breast cancer lines tested, suggesting global co-regulation of these genes. This co-expression of two genes that are separated on the chromosome by many other genes, including CTSD, is very interesting. Both genes code for proteins that are involved in normal housekeeping roles as components of multisubunit complexes. Translation is often coupled directly to protein degradation to remove miscoded proteins from a cell.

Permanent protein complexes often have co-ordinated expression of their subunits. It is therefore interesting that in the case of the RPL27A and PSMD13, we have not seen a concomitant increase in expression of other ribosomal and proteasomal proteins. This may suggest the regulatory importance of these subunits within these multiprotein complexes. Whether the induction of expression in 11OH2.1 of RPL27A and PSMD13 is parallel or due to one regulating expression of the other should be further studied by transfection.

SAGE data from the CGAP project showed a positive correlation between expression of PSMD13 and two ribosomal subunits, RPL19 and RPS5 supporting our ribosome/proteasome correlation.

9.2.4.4 Crystallin alpha-B: CRYAB

The fourth chromosome 11 positioned gene validated as upregulated in the growth suppressed hybrid 11OH2.1 is CRYAB. This gene is positioned at 11q23.1, just within the region transferred, and is upregulated 5.2-fold. LOH at 11q22-23 is

common in ovarian tumours (Koike *et al.*, 1999). The gene also lies within a region associated with paragangliomas (Baysal *et al.*, 1999).

CRYAB codes for a small heat shock protein, which is induced by cell stress and also functions as a chaperone in the transport of unfolded proteins to the degradation machinery. The CRYAB protein is abundantly expressed in the lens, the tissue in which it has been mostly studied. The mouse knockout of CRYAB displays hyperproliferative lens epithelial cells, which also become genomically unstable (Andley *et al.*, 2001). This data along with the decrease in expression of CRYAB in testicular and breast cancers as compared to their normal tissues supports a role for the gene as a tumour suppressor (Klemenz *et al.*, 1994; Takashi *et al.*, 1998). CGAP EST data showed a significant decrease in expression of CRYAB in ovarian tumours as opposed to normal ovarian tissue.

We found that expression of CRYAB was reduced in all 15 ovarian cell-lines tested. Furthermore there was almost no expression in the breast and colon cancer cell-lines examined. HOV expression of CRYAB was variable but 10 of 18 (55%) had decreased expression as compared to the normal OSE. Low tumour expression of CRYAB was significantly associated with adverse survival and with a serous histology. These clinicopathological associations further support the role of CRYAB as a candidate tumour suppressor gene.

9.2.5 Validated candidate oncogenes

From the Light Cycler analysis of 78 genes we validated only four as actually down regulated upon introduction of Chr 11 into the suppressed hybrid 11OH2.1. These genes are hypothesised to be downregulated by functional Chr 11 TSG(s) as part of the growth suppression pathway. Two of these genes have been previously characterised, RALDH2 and IGFBP2. The other two represent DpnII fragments that do not code for functional proteins as determined by in silico translation, and named CB1 and CB2. The massive downregulation of these two non-translated sequences by the transfer of Chr 11 is due to an unknown mechanism. Why any TSG would downregulate genes that do not code for a functional protein is unclear. It may be possible that these regions of DNA contain sequences similar to regulatory elements that inadvertently affect transcription of the gene.

RALDH2 codes for an enzyme with tissue specific activity, which is vital for the conversion of trans-retinal to retinoic acid (Napoli, 1999). Paradoxically, all-trans-retinoic acid is used as a treatment for solid tumours because it blocks cell cycle progression and enhanced cisplatin induced apoptosis (Wu *et al.*, 1997).

If RALDH2 acts as an oncogene, ovarian cancer cell-line expression would be expected to be higher than in normal OSE. Our panel of cell-lines however generally showed very low expression of RALDH2 except for line 59M. Five of eighteen HOV tumours showed overexpression of the gene. There was a slight trend towards the association of high tumour expression and poor survival. This would need to be further analysed with a larger sample set. Knockout of expression of RALDH2 by antisense transfection in OVCAR3 derived cells did not appear to suppress in vitro growth. In all, our data suggests that overexpression of RALDH2 is contextual and probably not involved as a mechanism for the progression of ovarian tumours.

Insulin-like growth factor binding protein 2, IGFBP2, is involved in the regulation of growth factor IGF-I. It has been well researched in tumours and increased serum levels have been detected in patients with malignancies of the colon, lung, ovary, adrenal gland, CNS and prostate (Hoeflich *et al.*, 2001). Serum IGFBP2 levels in ovarian cancer patients correlates with cancer antigen 125 (CA125) (Flyvbjerg *et al.*, 1997). Expression of IGFBP2 increases in chemotherapy resistant ovarian cell-lines as compared to those that are sensitive (Sakamoto *et al.*, 2001). Interestingly, CTSD acts as an IGFBP protease and therefore modifies the action of IGF-I in prostate carcinoma cells (Conover *et al.*, 1995). CTSD also degrades IGF-I and –II suggesting it has a general role in the regulation of this proliferation enhancing and anti-apoptotic system.

Although IGFBP2 protein is commonly reported as overexpressed in ovarian tumours, we have not seen this at the mRNA level. All of the cancer cell-lines and the majority of HOVs had less IGFBP2 expression than the normal OSE. Analysis of protein expression in these cell-lines and also on tumour sections would be interesting to see if there is an inverse correlation between CTSD and IGFBP2 protein expression.

Increased expression of IGFBP2 in ovarian tumours has been reported (Hough *et al.*, 2001). We have detected suppression of this transcript upon chromosome 11 transfer.

We have not however, found global overexpression of IGFBP2 mRNA in ovarian cell-lines and HOVs, therefore suggesting that again, suppression of this gene may be contextual.

9.2.6 Summary of difference analysis

Using three difference analysis techniques, we have successfully identified and validated eleven candidate TSGS. Four of these eleven reside on chromosome 11, all within published areas of common LOH. Our approach has been successful in minimising candidates from hundreds of genes within the regions transferred into the growth suppressed hybrid 11OH2.1 to just four.

All eleven are good candidates as ovarian cancer associated genes. They each perform normal role within the cell which if dysregulated may lead to a neoplastic phenotype (Table 9.1).

The four genes we have identified on Chr 11 are all good candidates and should be further analysed for their roles in ovarian cancer progression.

FUNCTION \ GENE	PSMD13	RPL27A	CTSD	CRYAB	TIMP2	SPP/HM13	KERATINS	BENE	EGFR
DNA repair	■			■					
Signalling						■		■	■
Invasion/cell structure			■		■		■		■
Apoptosis	■		■		■				
Immune response	■					■			
Transcription/ translation	■	■							■
General proliferation			■						■
Angiogenesis			■						
Protein degradation	■		■	■	■	■			

Table 9.1 Normal functions within a cell of our candidate TSGs. The red blocks show an association of the gene(s) with that function.

9.3 LOH analysis at 11p15

9.3.1 LOH overview

LOH at 11p15 has been identified multiple times in ovarian cancers, suggesting the presence of TSG(s) with in the region. The identification of three 11p15 situated genes by expression difference analyses led to the construction of a dense LOH map in this area. I aimed to minimise regions of LOH on 11p15 in ovarian cancers and to hopefully correlate these with genes identified by our functional analyses.

To do this I used 39 polymorphic microsatellite markers and 87 paired samples from patients with EOC. By calculating the ratio of allele loss between the tumour and its normal counterpart (ratio < 0.6), I was able to determine distinct regions of loss within these tumours. Traditionally, LOH rates are deemed as significant if ≥35% (Shelling *et al.*, 1995). Twelve of the 39 individual markers, according to these criteria, had significant LOH rates ranging from 35% to 44%. Marker loss on 11p15 was biased towards those tumours of advanced stage suggesting a mechanism of complete arm loss in these generally more genetically unstable tumours.

By analysing individual regions of loss in patient samples I was able to minimise the regions harbouring candidate TSGs to 39% (7.5Mb) of the original 19.1Mb, as characterised by nine SROs.

9.3.2 Shortest Regions of Overlap

A large number of patient samples showed LOH throughout the 11p15 region (24%). This suggests that complete loss of 11p may be an important mechanism in inactivating TSGs and suggests that there may be more than one TSG contained within the region. This mechanism of loss makes the minimisation of regions more complicated and also requires a large number of patient samples to be successful. The use of 39 markers in a dense map makes the interpretation of data more complicated than with fewer more distantly spaced markers. This is due to the large number of samples with complex 'harlequin' patterns. To minimise regions of loss and overcome this problem, I analysed only those samples with four or less individual regions of loss.

I was able to identify nine individual SROs. These regions span from 77Kb to 1927Kb and contain from 3 to 20 genes. In contrast to the most comprehensive LOH

analysis of 11p15 in ovarian cancer, by Lu in 1997 (using 12 markers), we have been successful in minimising the LOH regions (Lu *et al.*, 1997). Figure 9.4 summarises our SROs, showing the size and LOH rate of each region and highlighting any interesting genes within that interval.

Of the nine regions, six have LOH rates >40%. These regions are lost in more ovarian cancers and therefore most interesting to further investigate.

Clinicopathological analysis of the SROs showed no associations with histology, grade, FIGO stage or survival ($p>0.01$). Some individual markers within these regions however, e.g. D11S4188, were strongly associated with variables. This difference leads us to ask whether we should use regions or individual markers to interpret LOH data. Regions are important in minimising sections of chromosome to analyse. Once minimised, I believe the markers within these regions, and their clinicopathological associations should then be taken into account.

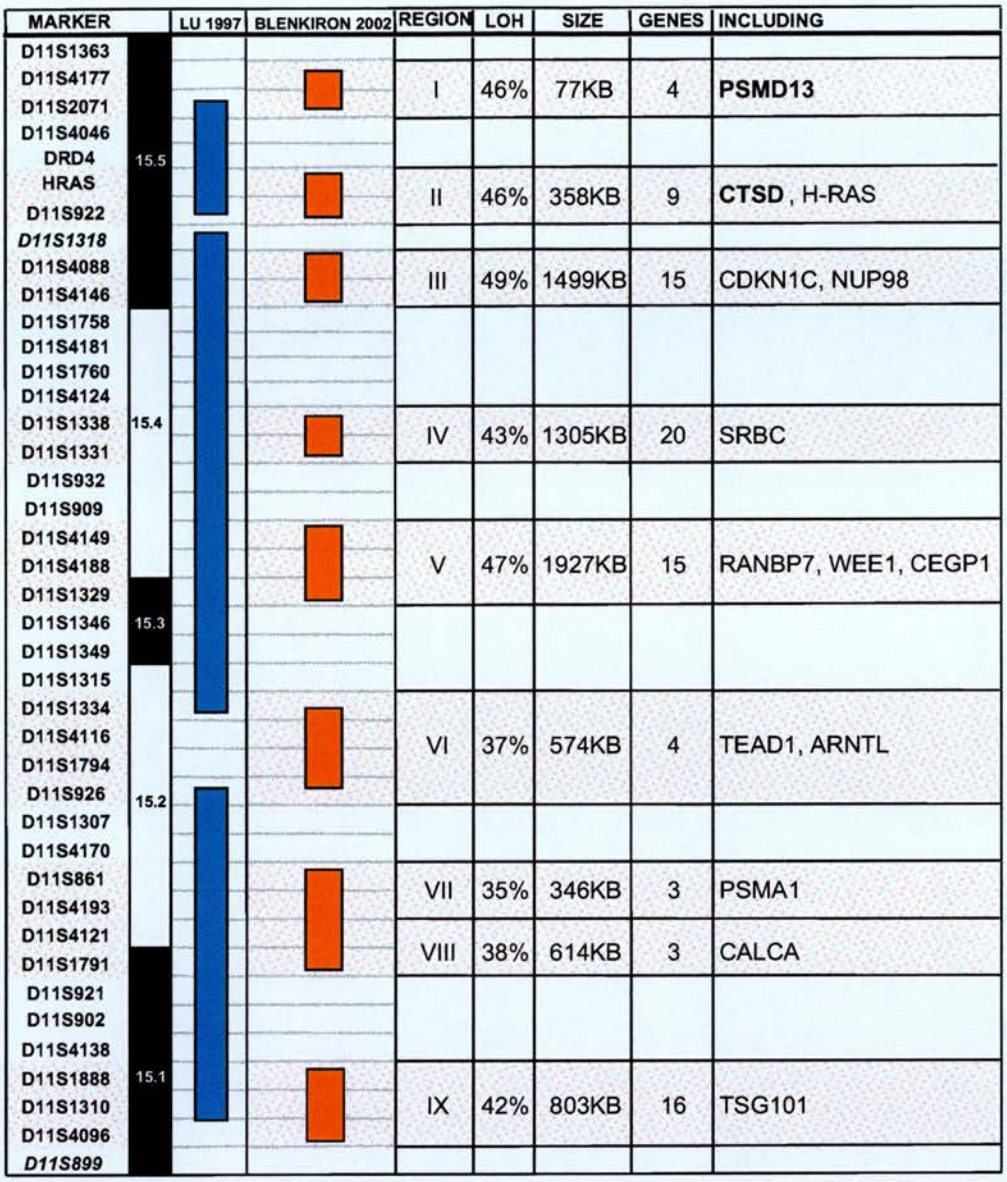


Figure 9.4 Minimal regions of LOH on 11p15 in ovarian cancers. Overview of the nine regions of LOH, their size, overall LOH rate, number of genes within the region and interesting gene candidates. Compared to Lu et al 1997, which was the most comprehensive analysis prior to this work.

9.3.2.1 Region I; 11p15.5 – D11S4177-D11S2071

Region I spans the smallest distance of 77Kb. The overall LOH is 46% and interestingly, PSMD13, identified as 2.3-fold upregulated in growth suppressed clone 11OH2.1, is contained within this region. Marker D11S2071 is situated within an intron of the PSMD13 gene and has an LOH rate of 39%. This marker is not significantly associated with any clinicopathological variables but there is a trend towards increased LOH in tumours of a serous or endometrioid histology, advanced

FIGO stage and advanced grade. Loss surrounding D11S2071 has been implicated in lung cancer region LOH11A (O'Briant and Bepler, 1997).

9.3.2.2 Region II; 11p15.5 –HRAS-D11S922

Region II spans 358Kb and encompasses 9 genes, including differentially expressed CTSD. LOH for this region is 46%. Marker D11S922 lies distal to the CTSD gene. As for D11S2071, loss at D11S922 appears to be more common in tumours of serous or endometrioid histology and of advanced FIGO stage. LOH at D11S922 trends towards an association with adverse survival in patients. It is also of note that low expression of CTSD in tumours was significantly associated with adverse survival in patients. D11S922 lies within regions of LOH identified in breast and lung cancers (see Figure 8.13).

9.3.2.3 Region III; 11p15.5-p15.4 – D11S4088-D11S4146

Region III spans 1499Kb and has the highest overall LOH of 49%. There are 15 genes within this region including the candidate TSGs KCNQ1 and CDKN1C. The region surrounding D11S4088 has been implicated in the progression of many cancers including breast, lung and Wilm's tumours (see Figure 8.13). This region is contained within a fragment of 2.5Mb, which suppresses embryonal tumours, supporting the presence of a TSG near this locus (Hoovers *et al.*, 1995). Karnik showed loss of a 500Kb region spanning D11S1318-D11S4088 in breast cancers and associated this loss to early events in malignancy and invasiveness (Karnik *et al.*, 1998a). We have found that LOH at D11S4088 is associated with all histological sub-types of EOC and there does not appear to be any bias towards either advanced stage or high grade disease. This suggests that loss at this marker may be an early step in ovarian cancer progression supporting the data found for breast cancers. D11S4088 is situated within a region containing imprinted genes including KCNC1, CDKN1C and SLC22A1L. These genes are imprinted by methylation in normal cells so LOH of the non-methylated allele would result in complete knockout of the gene. Imprinted genes are therefore more susceptible to inactivation and require only one-hit for this. However, hypomethylation of imprinted genes around D11S4088 has been detected in adult tumours, including breast, liver, colorectal and cervical (Scelfo

et al., 2002). This loss of imprinting (LOI) correlates with decreased expression of the genes suggesting that this mechanism acts by activating negative regulators of transcription. It is therefore possible that in ovarian tumours, LOI and LOH act in concert to knockout expression of one or more of the imprinted genes within this region.

9.3.2.4 Region IV; 11p15.4 – D11S1338-D11S1331

Region IV encompasses 1305Kb, and contains 20 genes. This region has an LOH rate of 43%. Marker D11S1338 (42% LOH) is lost in ovarian tumours of all histological types and, as for D11S4088, there is no bias seen for advanced stage and grade tumours. Loss at D11S1338 is however significantly associated with tumours that have not been debulked ($p<0.01$). This suggests an association with biologically more aggressive disease. Interestingly, when the 21 tumours that show loss of the majority of 11p15 are removed from the clinicopathological analyses, LOH at D11S1338 becomes associated with early stage tumours ($p=0.0185$). Why there should be a simultaneous association with irresectibility of these is unclear.

The ratio of alleles within a tumour as compared to its normal sample (R value) can be used a guide as to how recent this genetic event is in the progression of the tumour. If it is an early event in the tumour, the loss will often be more complete and a larger proportion of the tumour cells will have the genetic aberration (See Figure 9.5 for an explanation).

The average ratio of allele loss within tumour-normal pairs for D11S1338 is low ($r=0.28$), supporting the hypothesis that loss of this marker is an early event in progression.

LOH at D11S1338 has not been identified before in ovarian tumours but is common in esophageal cancers, leukaemia, melanoma, Wilm's tumour, and Breast cancers (Krskova-Honzatkova *et al.*, 2001; Lam *et al.*, 2002) (Goldberg *et al.*, 2000 ; Karnik *et al.*, 1998b; Karnik *et al.*, 1998a).

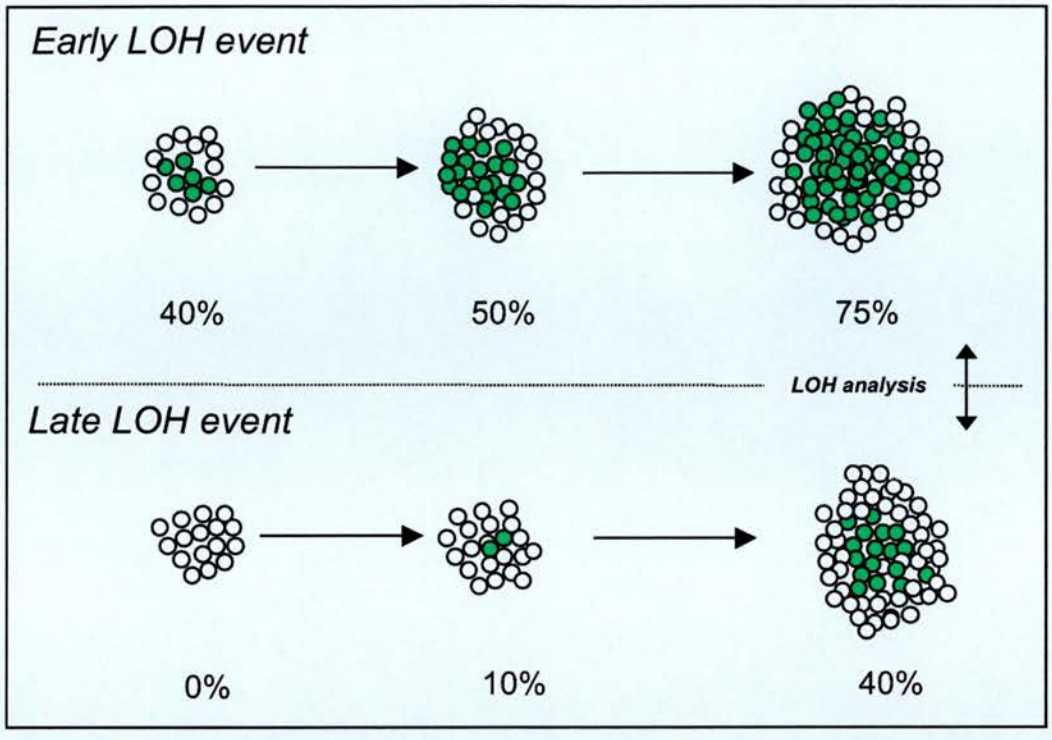


Figure 9.5 Accumulation of advantageous genetic events by a tumour. If LOH occurs early in the progression, as represented by a green cell, then this advantageous mutation will accumulate and account for a large proportion of the tumour mass. LOH analysis of a tumour with an early genetic event will show a larger allelic loss (75%- R=0.25) because the majority of cells have that lesion. Late LOH will be present in a smaller proportion of tumour cells so the R value will be higher (40% R=0.6).

In breast cancers, TSG candidate SRBC/PRKCDBP has been identified, located just distal to D11S1338. Down regulation of the protein kinase C binding protein SRBC is associated with hypermethylation of its promoter CpG island in 60% breast and 79% lung tumours (Xu *et al.*, 2001). The SRBC protein has unknown function but is known to interact with TSG BRCA1 and is postulated to play a role in cell-cycle control making it a good candidate.

9.3.2.5 Region V; 11p15.4-15.3 - D11S4149-D11S1329

Region V is the largest SRO, spanning 1927Kb. It contains 15 genes and has an overall LOH rate of 47%. The marker D11S4188, contained within this region, has an LOH rate of 38% whereas the surrounding two markers are lost in only 28-29% of patients. This suggests that a candidate TSG lies near to the D11S4188 marker. The region itself shows no significant associations with any clinicopathological parameters. D11S4188, however, is associated significantly with non-mucinous

histology, advanced stage and poor grade ($p<0.01$). LOH at D11S4188 also shows a trend towards association with adverse survival. These highly significant associations support the hypothesis that a candidate TSG lies very near to this marker.

Interestingly, loss at D11S4188 is common in the endometrioid sub-type (47% LOH) with a conversely low LOH rate in all other histologies (0-22%) suggesting that this gene may be important in the specific aetiology of endometrioid tumours.

D11S4188 is situated within an intron of the gene CEGP1. This gene was recently discovered and has yet to be characterised (Cichutek *et al.*, 2001). By homology, the protein has a signal peptide, a CUB domain, involved in developmental regulation, and EGF-like domains. The EGF super family members function as ECM components and as secreted signalling molecules and it is postulated that CEGP1 functions as the latter (Grimmond *et al.*, 2001). The gene is highly expressed in the mammary gland and by array profiling of breast tumours, expression was correlated with a survival advantage in patients (Saito-Hisaminato *et al.*, 2002; van 't Veer *et al.*, 2002).

The association of this marker with poor prognosis endometrioid tumours suggests this gene should be further analysed for a role in tumour development.

9.3.2.6 Region VI; 11p15.3-15.2 – D11S1334-D11S926

LOH at region VI is not as common as many of the others, at 37%. This region contains only four genes although it spans 574Kb. Individually, all of the markers within the region have LOH rates of only 26% except for marker D11S1794, which is lost in 37% tumours. D11S1794 lies within an intron of the ARNTL gene. The close proximity of other markers and genes suggests that within this region, transcription factor ARNTL is the best TSG candidate. The LOH rate of this gene is not as impressively high as for other markers but it may still be involved in the progression of a small number of ovarian tumours. LOH at D11S1794 is seen in all histological subtypes and, as for D11S4088 and D11S1338, there is no bias towards loss in advanced stage or poor grade tumours. Allele loss at D11S1794 may therefore, in a few tumours, be involved at an early point.

This region has not been previously identified as lost in cancers. This may be due to the majority of studies focussing on the 11p15.5-p15.4 region, or due to the minimal number of markers used within those studies.

9.3.2.7 Regions VII & VIII; 11p15.2 – D11S861-D11S4193 & D11S4121-D11S1791

My analysis of the SROs in these regions suggested that there may be two distinct regions of loss within this 960Kb interval. I am not however, fully confidant that these are separate so have grouped them together for discussion.

These regions encompass six genes and have LOH rates of 35-38%. The individual markers within this region only have LOH rates of 32-33%. Marker D11S4121 is just short of significance for an association with adverse survival in patients (p=0.0508). This marker lies nearest to CALCB and CALCA. Interestingly LOH at CALCA has previously been associated with adverse survival (Eccles *et al.*, 1992). This association suggests that D11S4121 may be lost in a small number of tumours with a poor prognosis.

9.3.2.8 Region IX; 11p15.1 - D11S1888-D11S4096

The final region, IX, spans three markers and 803Kb with an LOH rate of 42%. The three individual markers within the region have LOH rates of 31-36%. This suggests that in this area, regional loss is more important than looking at individual markers. Loss of region IX does not correlate with any clinicopathological variables.

The area surrounding marker D11S1310 was identified as a region of LOH by Lu et al (see Figure 9.4). This marker was central to a novel 4 cM region of LOH associated with high-grade non-mucinous tumours.

TSG101 lies within this SRO. This gene encodes for a cell cycle protein and is a candidate TSG in breast and ovarian cancers (Zhong *et al.*, 1998). Aberrant splicing of TSG101 is found in breast and ovarian cancers and appears normally regulated by p53 (Carney *et al.*, 1998; Moyret-Lalle *et al.*, 2001). This may therefore be the gene that is associated with LOH of region IX.

9.3.2.9 Summary of SROs

Of the nine SROs I have identified, there are six which are interesting and sufficiently relevant (i.e. high rates of LOH) to warrant further investigation. These are regions I, II, III, IV, V and IX. Interestingly, All of these regions (11p15.5-15.3 and 11p15.1) correlate to areas that have been identified as having high rates of LOH before. This data corresponds to the 11cM and 4cM regions identified by Lu et al previously, but we have been able to minimise the regions into smaller fragments for further analysis (Lu *et al.*, 1997) (Figure 9.4).

Region I contains PSMD13, which we identified as differentially expressed in the MMCT hybrid. Region II also contains a validated differentially expressed gene, CTSD. Region III has the highest rate of LOH, contains imprinted genes and appears to be lost at an early stage in tumour progression. Region IV includes candidate TSG SRBC that may also be involved at an early point in tumour development. Region V contains the marker D11S4188, which is commonly lost in endometrioid cancers with a poor prognosis, positioned within the gene CEGP1. Finally region IX, which includes the candidate TSG101, has an LOH rate of 42%. In all, these six regions contain 79 genes and encompass 5.9Mb. By analysing individual markers we can pinpoint loss even further within these regions.

There are five markers, one from each of the first five regions of LOH which appear to represent the most important areas of LOH along 11p15. The involvement of each of these markers in the progression of ovarian cancer is suggested in Figure 9.6.

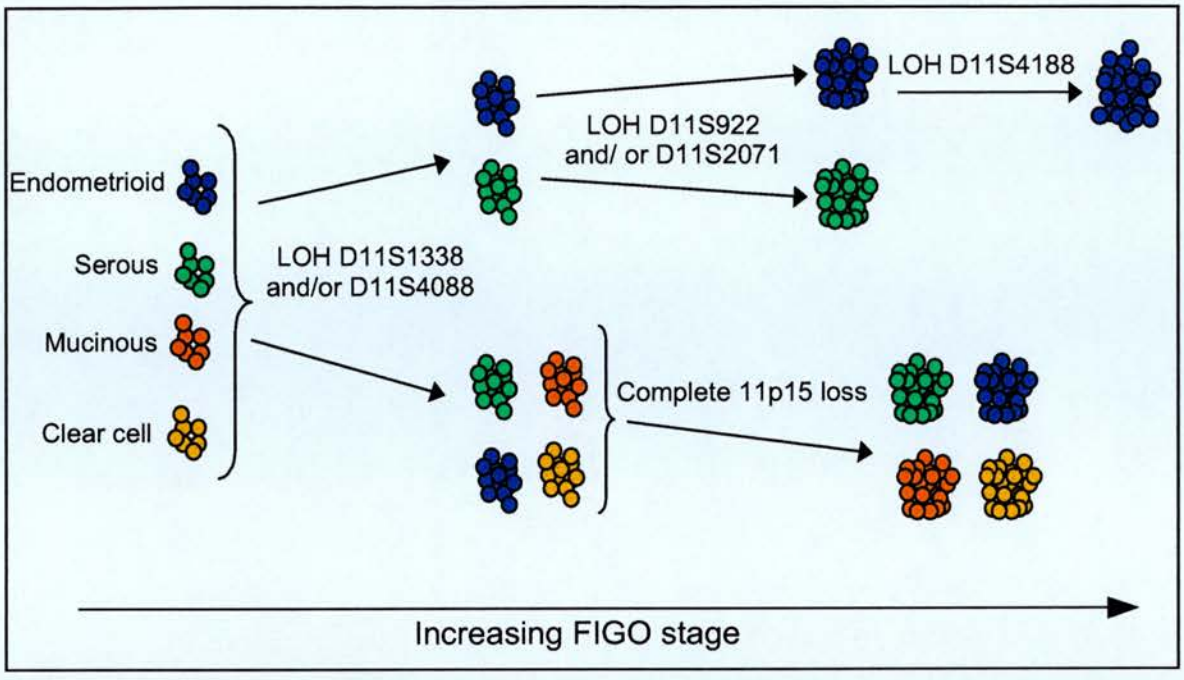


Figure 9.6 Model of 11p15 allelic loss in ovarian cancer. Accumulation of 11p15 marker LOH with increasing FIGO stage as associated with tumour histology.

9.4 Correlation of functional and positional data

MMCT of chromosome 11 into ovarian cancer cell-line OVCAR3 caused decreased growth and suppressed tumorigenicity. Revertant analysis of clones showed loss of one marker D11S926, which may or may not be associated with growth suppression. Further mapping of the clone 11OH2.1, which was used in the expression difference analyses determined the extent of 11p15 transfer.

Using difference analysis techniques coupled to functional suppression we have identified four candidate TSGs within 11ptel-q23. Three of these were positioned within a 9Mb region on 11p15.5-15.4.

Comprehensive LOH analysis of 11p15 in primary tumours identified nine regions of LOH, six of which I believe to be important in the progression of a sub-set of ovarian tumours. The identification of nine individual regions of loss explains why the mechanism of complete p arm loss is common in tumours.

Further mapping of the 11OH2.1 hybrid showed it had fragmented transfer of chromosome 11 in the ptel-p15.1 region. Using the same markers as used in the LOH analysis I have shown that there are two fragments of 11p15 in the hybrid 11OH2.1. The regions of chromosome transfer and the nine regions of LOH correspond well, except for region IX surrounding the marker D11S1310 (Figure 9.7). This shows that any of the other eight regions may harbour genes that are promoting the growth suppressed phenotype in clone 11OH2.1. This close correlation of chromosome retention in the 11OH2.1 clone and common regions of tumour LOH supports these as candidate TSG harbouring regions and validates the approach of integrating positional and functional analyses.

Since single genes are not commonly cloned via the MMCT approach, it may be possible that in vivo tumour suppression may be due to the effect of a large number of genes introduced by the chromosome transfer. The correlation of chromosome retention in microcell hybrids with common regions of LOH in primary tumours supports this idea.

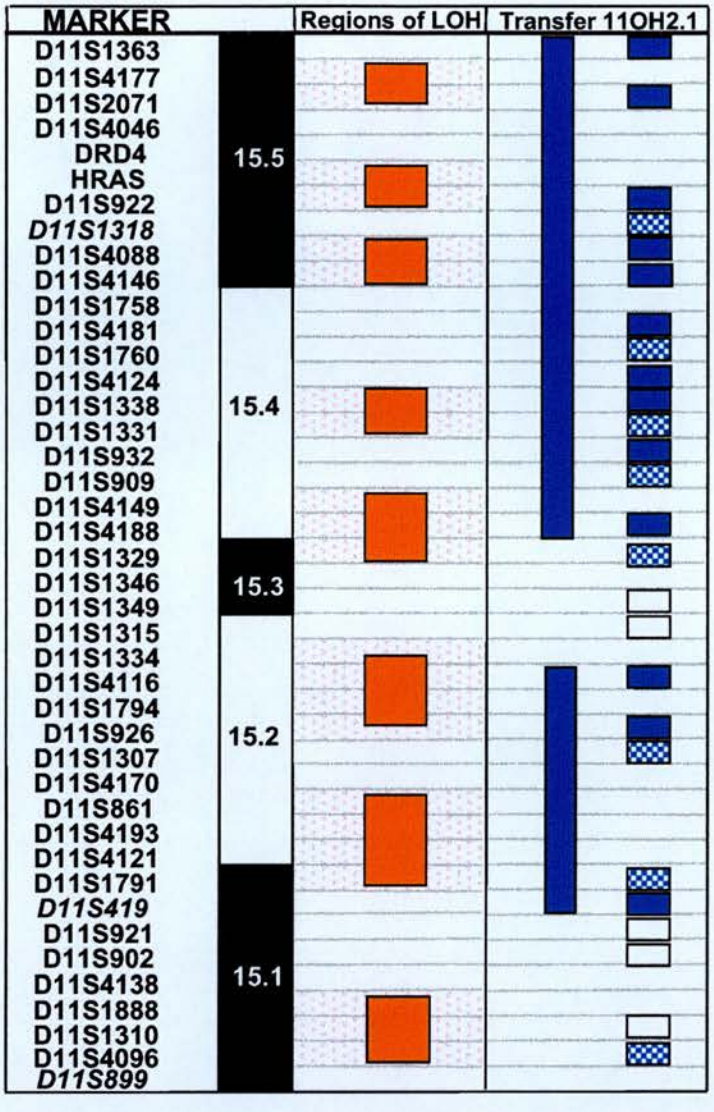


Figure 9.7 Comparison of 11p15 transfer and regions of LOH. Red bars= regions of LOH in relation to the markers shown from telomere down to 11p15.1. Blue bars= regions of 11p15 transferred in MMCT clone 11OH2.1. Not all markers were used in the mapping of 11OH2.1. A blue box= transfer, a white box= no transfer and a hatched box= an uninformative locus.

The integration of two methods of analysis have minimised the approx. 1500 genes along chromosome 11 to seven genes which may be involved in the growth suppression phenotype. Of these, three were identified by the functional approach coupled to difference analysis and four others were from a comprehensive LOH analysis of 11p15. Our integrated data is shown in Figure 9.8.

Two genes were validated as both differentially expressed in the suppressed microcell hybrid and within regions of high LOH. Another gene, RPL27A, was identified by difference analysis and correlated with a marker, LOH of which is

associated strongly with advanced stage (D11S932). RPL27A was not however identified within a region of common LOH.

CTSD and PSMD13 were both identified by difference analysis as upregulated in the growth suppressed clone 11OH2.1. Furthermore, these genes correlate with regions and markers with high rates of LOH. LOH of both are associated with tumours of a more advanced stage and of serous or endometrioid histology.

We have shown decreased expression of PSMD13 in 22% HOV tumours and 100% ovarian cancer cell-lines. LOH at marker D11S2071, situated within an intron of the PSMD13 gene is found in 39% of tumours with a bias towards those with a poor differentiation status. The correlation between PSMD13 and RPL27A expression in tumours and cell-lines suggests co-regulation of these genes. The marker nearest to the RPL27A gene, D11S932 does not show a high rate of LOH suggesting that it is either inactivated by an alternative mechanism or that it does not act as a tumour suppressor gene. It could be possible that PSMD13 directly affects transcription of RPL27A and this correlation should be further studied.

CTSD expression is convincingly decreased in 100% of HOVs and 100% ovarian cancer cell-lines. This clear downregulation in tumours, as well as CTSD being situated within a region of high LOH suggests CTSD as a very strong TSG candidate.

The association between loss of expression of CTSD in HOV samples and adverse survival suggests this gene is very important in the progression of ovarian tumours. We found decreased CTSD protein in some serous tumours which corresponds with LOH being more common in this histological subtype. Further immunohistochemical analysis of CTSD expression in tumours is underway.

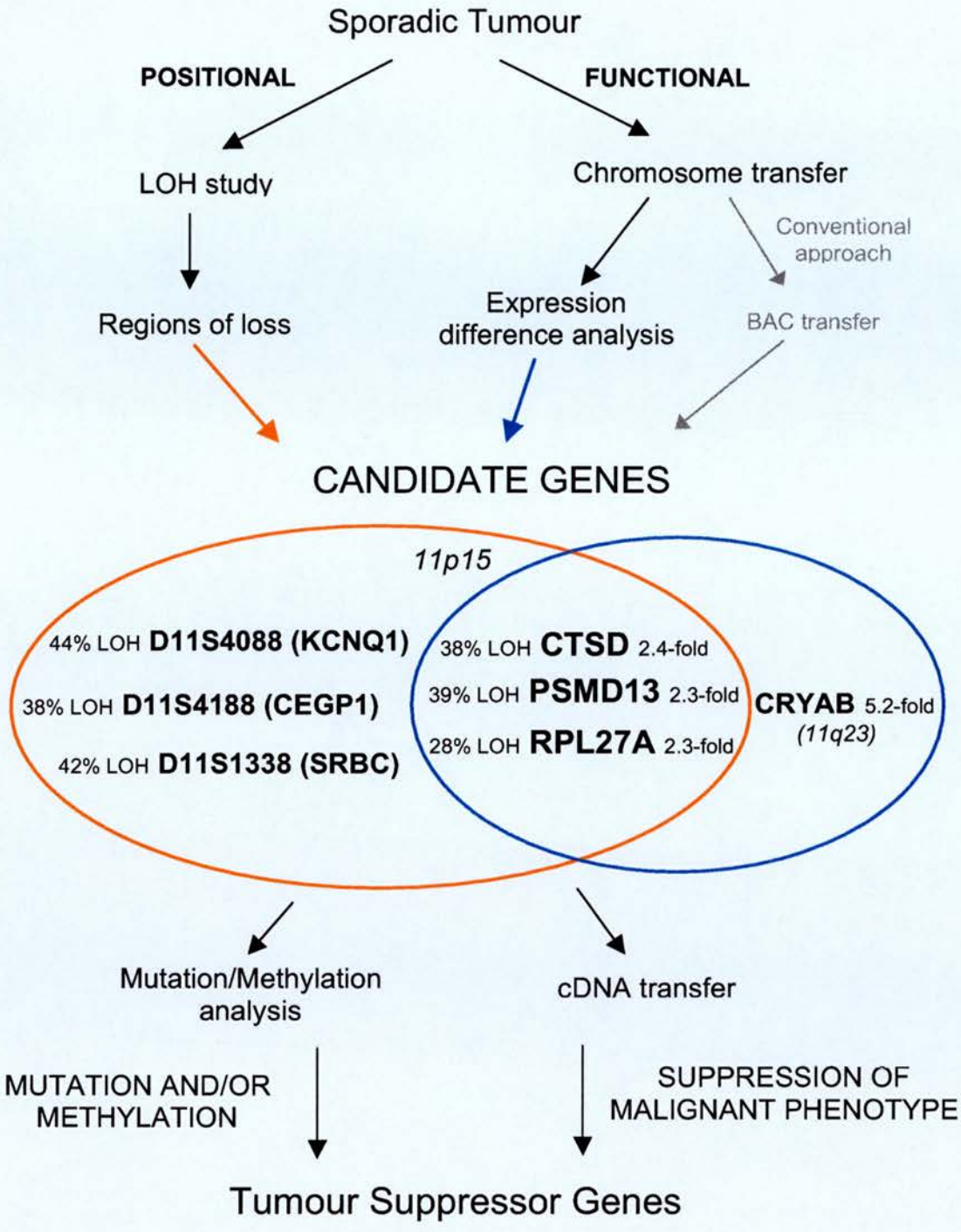


Figure 9.8 Integrated approach for identifying candidate Tumour Suppressor Genes. By combining a functional and positional approach we have identified genes that may be involved in the growth suppressed phenotype. Overlap between the methods shows three genes that should be further analysed for their roles in ovarian cancer progression.

Of the three 11p15 genes identified by difference analysis, concurrent LOH analysis has not excluded these from further analysis. The integrated data suggests both PSMD13 and CTSD as good TSG candidates. RPL27A does not reside in a minimal region of LOH but the co-expression with PSMD13 should be investigated.

CRYAB is positioned on 11q23 so was not included within the region analysed for LOH. This gene cannot therefore be excluded as a candidate functional growth-suppressor. Expression of CRYAB is decreased in 56% HOVs and 100% ovarian cancer cell-lines. This decrease in expression is associated with clinicopathological parameters of serous histology and adverse patient survival. CRYAB is therefore also a good TSG candidate.

I have identified nine regions of LOH, eight of which were transferred in the MMCT clone 11OH2.1. CTSD and PSMD13 are postulated to account for two of these regions. Difference analysis did not identify candidates from the other six. It may be that these regions are artefactual of the high overall rate of LOH along 11p15 and therefore do not actually harbour TSGs. Another possibility is that the methods of difference analysis are themselves limited. For example, arrays are limited by their clone content and cDNA-RDA is more successful for more abundant transcripts. The coupling of difference analysis techniques to MMCT is also limited in its ability to identify genes that are mutated because mutations may not affect the expression of the genes. Mutations may lead to truncated or missense transcripts, which are expressed at a normal level but affect the protein function.

The regions identified by LOH are also worthy of further analysis. Markers D11S4088 and D11S1338 in regions III and IV respectively, are lost in multiple tumour types, as already discussed. D11S4188 has not before been reported as lost in tumours. Along with the strong correlation of loss with aggressive endometrioid tumours, I think this will be an interesting region to study.

The coupling of these approaches has successfully minimised chromosome 11p15 TSG candidates to two, CTSD and PSMD13. The genes should be analysed for their potential roles as TSGs in ovarian cancer progression.

9.5 Summary and future directions

This study has succeeded in identifying a small number of candidate TSGs on 11p15. By combining two, normally distinct approaches we have been able to minimise the data to just two genes. These two genes, PSMD13 and CTSD have been shown to have decreased expression in ovarian cancer cell-lines and are both situated within regions of high LOH.

To identify whether these genes are true TSGs, we next need to show inactivating mutations and/or methylation. Mutation and methylation analysis of a panel of patient samples would show the mechanisms by which these genes are switched off in ovarian tumours.

In parallel, functional studies on these genes to prove they act upon growth suppression, both in vitro and in vivo, should be performed. By transfecting full-length sense transcripts into ovarian cancer cells that show very little endogenous expression, we will determine whether these genes have a functional effect upon growth and tumorigenicity.

We have found a clear correlation between expression of two of out four genes, PSMD13 and RPL27A. It would be interesting to see whether multiple gene knockout affects the phenotype of cancer cells in an additive manner.

The aim of this project was to identify candidate tumour suppressor genes from a large potential region of chromatin (Chr 11). We have been successful in doing that. Whether these genes will actually be confirmed as tumour suppressors awaits further investigation. These genes may hopefully be used in the future directly in the clinic whether in prevention, prognosis or treatment in order to improve the outcomes for women with ovarian cancer.

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