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Investigation of Genes Involved in Osteosarcoma Development

by

Kolja Eppert

A Thesis submitted in conformity with the requirements for the degree of Masters of Science
Graduate Department of Molecular and Medical Genetics
University of Toronto

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Dedication

For my parents

Inge and Franz
Abstract – Many genes involved in osteosarcoma development have yet to be described. The chromosomal regions commonly altered in osteosarcoma mark the location of further tumor suppressor genes and oncogenes. The study of these regions may yield further understanding of known cancer genes and the isolation of novel ones. I therefore had two objectives: i) the investigation of 12q13-15, a region commonly amplified in osteosarcoma and ii) the isolation of novel genes involved in osteosarcoma pathogenesis. Three genes located at 12q13-15 are most likely to be driving amplification: SAS, CDK4 and MDM2. Analysis of gene amplification and expression indicated that the region is more frequently amplified in the parosteol subtype of osteosarcoma and that SAS may be the most critical gene. In addition, the analysis of gene expression in osteosarcoma tumor samples isolated a number of genes potentially involved in tumor development and progression, including the von Willebrand factor gene.
There are a number of people I would like to thank:

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### Abbreviations

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<th>Definition</th>
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<tbody>
<tr>
<td>AS</td>
<td>-asparagine synthetase</td>
</tr>
<tr>
<td>BRCA2</td>
<td>-early onset breast carcinoma gene 2</td>
</tr>
<tr>
<td>bp</td>
<td>-base pair</td>
</tr>
<tr>
<td>CAK</td>
<td>-cdk activating kinase</td>
</tr>
<tr>
<td>CDK2</td>
<td>-cyclin dependant kinase 2 gene</td>
</tr>
<tr>
<td>CDK4</td>
<td>-cyclin dependant kinase 4 gene</td>
</tr>
<tr>
<td>CGH</td>
<td>-comparative genomic hybridization</td>
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<tr>
<td>DM</td>
<td>-double minute chromosomes</td>
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<tr>
<td>EST</td>
<td>-expressed sequence tag</td>
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<tr>
<td>HSR</td>
<td>-homogeneously staining regions</td>
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<tr>
<td>KAI1</td>
<td>- 'kang ai', prostate cancer antimetastasis gene</td>
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<tr>
<td>LOH</td>
<td>-loss of heterozygosity</td>
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<tr>
<td>MADR2</td>
<td>-mothers against DPP gene</td>
</tr>
<tr>
<td>MDM2</td>
<td>-murine double minute gene</td>
</tr>
<tr>
<td>MFH</td>
<td>-malignant fibrous histiocytosis</td>
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<tr>
<td>OSA</td>
<td>-osteosarcoma</td>
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<tr>
<td>PCR</td>
<td>-polymerase chain reaction</td>
</tr>
<tr>
<td>RACE</td>
<td>-rapid amplification of cDNA ends technique</td>
</tr>
<tr>
<td>Rb</td>
<td>-retinoblastoma gene</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>-reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>RDA</td>
<td>-representational difference analysis</td>
</tr>
<tr>
<td>SAGE</td>
<td>-serial analysis of gene expression</td>
</tr>
<tr>
<td>SAS</td>
<td>-sarcoma amplified sequence gene</td>
</tr>
<tr>
<td>SSCP</td>
<td>-single strand conformation polymorphism</td>
</tr>
<tr>
<td>THC</td>
<td>-tentative human consensus sequences</td>
</tr>
<tr>
<td>UV</td>
<td>-ultraviolet light</td>
</tr>
<tr>
<td>vWF</td>
<td>-von Willebrand factor</td>
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CHAPTER 1

Introduction
Purpose and Rationale

It is generally accepted that neoplasia develops through a multistep process involving an acquisition of genetic alterations leading to malignancy (1). Although a great deal of progress has been made in identifying the genes that are altered in tumors, our comprehension of the pathogenesis of neoplasia is still far from complete and many of the genes involved have yet to be described. Benefits that would accrue from a more thorough understanding of neoplastic development include better management of patient care. For example, the treatment of osteosarcoma (aggressive bone producing tumors) is limited by the lack of good prognostic markers and would benefit from the identification of the genes involved in tumor progression and development. The molecular alterations that drive the progression of a tumor involve different types of genes: mainly genes that normally inhibit tumor development (tumor suppressor genes), genes that have a positive effect (proto-oncogenes) and those involved in DNA repair. The different types of neoplastic genes have different modes of action and therefore different mechanisms are involved in their alteration. Tumor suppressors are generally inactivated through deletions of chromosomal regions (leading to a loss of heterozygosity, LOH) and point mutations, while proto-oncogenes become oncogenic through chromosomal amplification, translocation or point mutation.

The study of the commonly altered chromosomal regions in osteosarcoma tumor cells, marking the location of tumor suppressor genes and oncogenes, may yield further understanding of known cancer genes and the isolation of novel ones applicable to osteosarcoma and cancer in general. Towards this end, I had two objectives. First, I investigated region 12q13-15, which is commonly amplified in osteosarcoma. Specifically, cyclin dependant kinase 4 (CDK4), SAS and MDM2, genes located in 12q13-15, were examined for DNA amplification and gene expression.
Second, I isolated novel genes involved in osteosarcoma pathogenesis through the examination of gene expression in osteosarcoma tumor samples.

Osteosarcoma

Osteosarcomas are tumors of mesenchymal origin that most commonly affect patients between the ages of 10 and 20 years (2,3). In general, osteosarcomas are aggressive tumors; patients develop pulmonary metastases within 9 months and succumb to the disease within a further 6 months. With the advent of treatment protocols involving aggressive surgery and neoadjuvant chemotherapy, the 5 year survival rates have risen from 19.7% (prior to 1971) to 60% (4). However, 40% of the patients will still develop metastases and succumb to the disease, indicating that at least some of the patients could benefit from more aggressive therapy. At present, the available prognostic indicators are ineffective in identifying these high risk patients. Subsequently, all patients are treated in a similar fashion, with a balance being struck between the benefits and detrimental effects of treatment. A better understanding of the pathogenesis of osteosarcoma, especially at the molecular level, promises to yield new prognostic markers.

While few of the genes involved in the pathogenesis of osteosarcoma are known, the tumor suppressor genes p53 and Rb, and the proto-oncogenes CDK4, MDM2, and possibly c-myc have been implicated in their development. These tumor suppressor genes and oncogenes are commonly altered in multiple types of cancers (i.e. p53 is the most commonly mutated gene in cancer). Studies using osteosarcoma have contributed a great deal to our understanding of p53, Rb, and MDM2 (5-16). Due to the aggressive nature of osteosarcoma and the common nature of the neoplastic genes involved, it is an excellent system for the study of genes involved in cancer.
Understanding the reasons underlying the aggressive nature of osteosarcoma in primary growth and metastasis may provide universal insight into the nature of neoplastic development.

In this introduction, I will discuss: i) the known genetic alterations in osteosarcoma; ii) the examination of known genes implicated in osteosarcoma progression and development; and iii) the isolation of novel genes potentially involved in osteosarcoma pathogenesis.

I. Genetic Changes in Osteosarcoma

Karyotypic analyses of osteosarcomas have identified many complex chromosomal alterations including translocations, duplications, deletions, LOH, and amplifications (17-21). Due to the mechanisms of activation of oncogenes (amplification) and the inactivation of tumor suppressor genes (LOH and mutation), these chromosomal abnormalities mark areas that may contain tumor suppressor genes and oncogenes which are altered during progression. For example, in the case of colorectal tumorigenesis a detailed model of genetic alterations involved in initiation and progression has been proposed involving the loss of chromosome 5q (adenomatous polyposis coli tumor suppressor gene), DNA hypomethylation, ras oncogene activation, loss of 18q (DCC tumor suppressor gene), and loss of 17p (p53 tumor suppressor gene) from normal epithelium, through adenoma, to carcinoma (1). However, despite its aggressive nature, relatively little is known about the genes associated with osteosarcoma initiation and progression. Therefore, its phenotype and wealth of alterations make it a good system to study. The altered regions provide evidence for the involvement of further genes in osteosarcoma development and progression and can be used to assist in the isolation of these genes.
A. Deletions and LOH in Osteosarcoma

Chromosomal regions that are commonly deleted or show LOH are thought to mark potential sites of new tumor suppressor genes. The Knudson Two-Hit hypothesis states that both wildtype copies of a tumor suppressor gene must be inactivated for a tumor to develop (22). The loss of function of the either copy of the gene may arise from nondisjunction, nondisjunction and duplication, mitotic recombination, gene conversion, deletion or point mutation. It is likely that if one event were due to a deletion the other event would be a point mutation. This is because two deletion events (or replacement of the wildtype region with a copy of the abnormal region as in duplication or recombination) could deprive the cell of the other genes located in the same region, presumably resulting in the death of the cell if the deletions were large enough to include many key genes. Since tumor suppressor gene inactivation often involves deletions and LOH, chromosomal regions containing these genes may exhibit a frequency of LOH and deletion that is substantially higher than background levels.

i. Tumor suppressor genes in osteosarcoma

Two lines of evidence indicated the involvement of tumor suppressor genes in cancer (22-25). Familial cancers such as retinoblastoma are often associated with the loss of specific chromosomal regions (i.e. 13q14 in retinoblastoma). In addition, cell fusion experiments by Harris and Stanbridge showed that the introduction of chromosomes from non-tumorigenic cells into tumorigenic cells could suppress the neoplastic phenotype (26-29). At present, two tumor suppressor genes (Rb and p53) have been found to be involved in osteosarcoma progression with evidence for the existence of possibly four others (9,10,18-20,30). The isolation of the retinoblastoma gene (Rb) is an excellent example of the use of cytogenetic evidence to isolate a
tumor suppressor gene. The study of retinoblastoma has been vital to the development of many of concepts concerning the genetics of tumor suppressor genes and I will therefore discuss in detail.

a. Retinoblastoma, Rb: a prototypical tumor suppressor gene

Retinoblastoma, an eye tumor that occurs in infancy, is a well-characterized example of a human tumor that results from the inactivation of both copies of a tumor suppressor gene (Rb) (22,31). It occurs in two forms: sporadic and hereditary. The latter form is inherited as an autosomal dominant trait that generally occurs in infancy and tends to be bilateral. In contrast, the sporadic form occurs later and is usually unilateral. The differences in epidemiology of the two forms of retinoblastoma led Knudson (22) to propose the two-hit model of carcinogenesis which is the paradigm of tumor suppressor gene genetics; two mutational events must occur that eliminate wildtype activity from both copies of a tumor suppressor gene present in a cell. Patients with the heritable form of retinoblastoma have inherited an Rb mutation which is therefore present in all of their somatic cells. The second 'hit' occurs somatically and results in tumor development. Since only a single sporadic mutational event per cell is required, malignant cells can often arise in both eyes, resulting in bilateral disease. However, for sporadic retinoblastoma to develop a retinal cell must be 'hit' twice, a much less likely occurrence. Therefore, it occurs later (30 months, heritable Rb occurs at 14 months) and in a unilateral fashion. Deletions of 13q were seen in patients with retinoblastoma, indicating that the target gene of at least one of the two 'hits' resided there.
Cytogenetic evidence for Rb at 13q14

The first evidence that indicated the presence of an Rb gene were deletions of 13q14 found in a small number (5%) of retinoblastoma patients (32,33) and in tumors from patients with normal constitutional karyotypes (34). The patients which showed a constitutional deletion of 13q14 also had a deficiency in the serum enzyme esterase D (located at 13q14) which resulted in 50% of the enzyme activity found in normal patients (34-36) due to the loss of one copy of the gene. Although these varied in size, they all included 13q14 and therefore defined a region that possibly contained the Rb locus. In addition, Strong (37) described an interesting kindred that provided further evidence. All the members of this family had an interstitial deletion of 13q14, including the affected patients. However, all of the unaffected family members possessed a translocated region on chromosome 3 that contained 13q14. Furthermore, the linkage between esterase D and Rb (and the existence of isozymes of esterase D) was used to support the recessive nature of Rb, even in cases without obvious deletions; it was shown that in 4 out of 6 retinoblastoma tumors there was LOH of esterase D (5). Further evidence for the existence of a tumor suppressor gene located at 13q14 was provided by restriction fragment length polymorphism (RFLP) analysis, which showed that up to 70% of informative retinoblastoma tumors exhibited LOH at 13q14 (38-40) and that the mutant allele linked to retinoblastoma was always the one retained (41).

Rb and Osteosarcoma

Patients who survive the heritable form of Rb (but not the sporadic form) have an increased risk of developing non-ocular tumors, most commonly sarcomas (42-46). More specifically, these individuals have a 1000 times greater risk of developing osteosarcoma than
unaffected infants, with correspondingly higher risks if treated with radiation (42,47). These secondary osteosarcomas are clinically indistinguishable from sporadic osteosarcoma.

Genetic analyses of sporadic osteosarcoma tumors have identified alterations in the Rb gene. Three of 13 osteosarcomas studied by Friend (48) exhibited homozygous deletions and Toguchida (49) found alterations in 13 of 30 tumor samples. Wunder (10) found 6 of 14 sporadic osteosarcomas had alterations in the Rb gene while Araki (30) found structural alterations in 8 of 23 osteosarcomas. Notably, Wunder found alterations only in high grade osteosarcomas (40%) (10). Additional evidence supporting the role of Rb in osteosarcoma was provided in a study by Huang (12) in which osteosarcoma cell lines were transfected with the Rb gene. Cells which expressed Rb (with no native Rb) reverted to non transformed phenotype, while cells with a native copy of Rb were unaffected by the transfected gene. Taken together, these observations support the role of Rb as a tumor suppressor gene, and its involvement in osteosarcoma development.

Rb function

Rb plays an important role in cell cycle control, more specifically in the link between cell cycle control enzymes and the transcriptional machinery necessary for progression (50,51). Regulation of Rb is at the hub of many signal pathways that regulate cell growth, including p53 and TGF-β responses, DNA damage response, and cell contact inhibition pathways (52-58). These regulatory pathways are commonly altered in tumors and are involved in the neoplastic process. In the mammalian cell cycle, a restriction point (R point) exists where the cell commits to continue through the cell cycle and this is where Rb seems to have its major function. Before this point the cell requires serum mitogens and after this point cell cycle progression is serum-
independent. Preceding the R point, Rb is hypophosphorylated while after the R point Rb is hyperphosphorylated until the end of M phase. The phosphorylation of Rb prevents it from inhibiting E2F (a transcription factor which regulates the expression of genes involved in cell growth) and therefore allows E2F to promote the expression of genes such as c-myc, cdc-2, dihydrofolate reductase, thymidine kinase and E2F-1, which in turn drive the cell through the cell cycle (59,60).

b. p53: tumor suppressor gene

In addition to Rb, the tumor suppressor gene p53, located on 17q13, is also implicated in osteosarcoma progression. It is the most widely studied tumor suppressor gene and is found to be mutated in many disparate tumors such as lung cancer, breast cancer, colon cancer and sarcomas. Originally discovered as a protein that associates with the large T antigen of SV40, it was thought to be an oncogene (61,62). However, in 1990, Lane showed that the mutant forms of p53 with transforming activity function to inactivate wild-type p53 in trans (63).

p53 has many functions but its overall role seems to be as a 'guardian of the genome'. When a cell has sustained major DNA damage, p53 serves a key role in the decision to commit to apoptosis or halt the cell cycle in order to allow for repair of the damage. Without these options, the cell would continue to undergo cell division and may acquire further mutations.

There are two lines of evidence implicating p53 in osteosarcoma development. First, in families with germline p53 mutations (Li-Fraumani families) there is an increased incidence of osteosarcoma and other tumors. Secondly, molecular studies have shown that approximately 30% of osteosarcomas have p53 mutations (9,64-68).
ii. Other Tumor Suppressor Genes Implicated in Osteosarcoma

There have been two major studies of LOH and deletions in osteosarcomas, one of which used allelotype analysis on 37 tumors (20) while the other used comparative genomic hybridization on 11 samples (18). Comparative genomic hybridization (CGH) is a relatively new technique which involves hybridizing sample DNA and normal DNA (labeled with different fluorescent dyes) against a metaphase chromosomal spread of normal DNA and comparing the ratio of the signal intensity. Allelotype analysis identified four regions that exhibited a high frequency of LOH: 3q, 13q, 17p, 18q. However, comparative genomic hybridization demonstrated additional regions with a high frequency of deletion: 2q, 3q, 3p, 6q, 8p, 9p, 10p, 11p, 13q, 15q, 16p. Known tumor suppressor genes map to several of these loci (69-100). The following regions have already been shown to contain tumor suppressor genes:

(i) 9p - A tumor suppressor gene (p16) that is involved in many tumor types has already been mapped to 9p and may be responsible for the LOH (101). P16, the familial melanoma tumor suppressor, functions to inhibit the cyclin dependent kinase CDK4, a gene commonly amplified in osteosarcomas (101-103). As yet, mutations in p16 have not been identified in osteosarcomas but there is another mechanism for alteration of p16 activity: expression can be altered by aberrant methylation in the promoter region (104-106). A similar situation occurs in gastric carcinomas where there is LOH of 9p without mutations in p16. Furthermore, in a study by Puig et al, malignant melanomas exhibited a minimal deleted region on 9p outside the p16 locus(107). These studies indicate the potential for another tumor suppressor gene located on 9p.

(ii) 13q - This chromosomal region appears to contain a number of tumor suppressor genes involved in multiple tumor types. The retinoblastoma (Rb) tumor suppressor gene maps to 13q and is involved in osteosarcoma development. In addition, BRCA2 (a hereditary breast
cancer tumor suppressor) maps to 13q (108). While there is evidence for at least two tumor suppressor genes in osteosarcoma, BRCA2 has not been implicated in osteosarcoma progression to date (109).

(iii) 17p - Allelic loss of 17p has been shown to occur in a large number of tumor types. The region contains the tumor suppressor gene p53 (17p13), the most commonly mutated gene in cancer. While p53 may account for the LOH at 17p13, there is evidence for the presence of a second tumor suppressor gene: 39/57 osteosarcomas had LOH on 17p, of which 19 lacked detectable p53 mutations (9). Either mutations that were not detected or a second tumor suppressor gene is the target of allelic loss.

(iv) 18q - LOH of 18q is seen in cancer of the colon, breast, prostate, pancreas, bladder, lung, esophagus and stomach (94-100). Three tumor suppressor genes have been found to map to 18q: DCC (Deleted in Colorectal Carcinoma), DPC4 (Deleted in Pancreatic Cancer, locus 4), and MADR2 (Mothers against DPP) (94,97,110). All of these genes have been shown to be altered in colon cancer (94,110,111). In addition, DPC4 is homozygously deleted in 30% of pancreatic tumors and mutated in 22% of pancreatic tumors without homozygous deletions (97). Loss or reduction of DCC expression was found in 17 of 25 osteosarcoma (112) indicating a possible role in osteosarcoma development. As yet, there is no direct evidence that MADR2 is involved in osteosarcoma. As part of my graduate work, I examined 76 sarcomas, including 35 osteosarcomas, by SSCP (covering 44% of MADR2, including highly conserved regions) and no mutations were found (110).
B. DNA Amplification

Osteosarcomas also contain regions of DNA that are frequently amplified (17-19,21). Gene amplification results in the accrual of multiple copies of genes, which is often associated with increased mRNA expression. Regions of amplification are marked by the presence of double minute chromosomes (DMs) and homogeneously staining regions (HSRs) within chromosomes and can range in size from 100 to more than 1000 kilobases (113).

Amplification is a common mechanism for the increased expression of proto-oncogenes, resulting in their deregulation and thereby providing a selective growth advantage to cancer cells. In humans, proto-oncogenes have been found to be amplified in many solid tumors such as neuroblastoma, breast cancer, brain tumors, and small cell lung carcinoma (24,114). The importance of amplified regions in cancer cells is supported by three lines of evidence: 1) amplified regions of DNA are only maintained in mammalian cells if they provide a growth advantage for the cell, 2) amplification units frequently include proto-oncogenes, and 3) the amplification or increased expression of a proto-oncogene can correlate with a specific phenotype (25).

Oncogenes, derived from mutation of normal cellular genes (proto-oncogenes), act to promote or positively regulate cell growth in a dominant fashion and therefore impart a selective growth advantage to tumor cells (25). Although there over 60 proto-oncogenes presently known, they generally act through only three biochemical mechanisms: phosphorylation of proteins (on serine, tyrosine or threonine), regulation of the transcription of genes (i.e. myc), or transmission of GTPase signals (i.e. ras) (24,25).

Oncogenes have been shown to arise from the deregulation of proto-oncogenes through multiple mechanisms: constitutive (unregulated) activity resulting from point mutations or
translocations (i.e. mutant c-ras that sensitivity to GTPase activating proteins), excess amount of gene product (i.e. myc amplification), or the formation of fusion proteins produced from translocations that join different genes (i.e. EWS linked to FLI1 in Ewing's sarcoma) (24,113,114). The oncogenes seen to be involved in osteosarcoma development, CDK4 and MDM2, are included in the 12q12-15 region which is commonly amplified in sarcomas (Figure 1-1). Both these genes can be deregulated by over-expression of their RNA, which can result from DNA amplification.

i. Genes Located at 12q12-15

a. CDK4

CDK4 was first isolated in a screen for members of the protein-serine kinase family (115). It is the catalytic subunit of the cyclin D/CDK4 complex, which functions to phosphorylate Rb during the G1 phase of the cell cycle in order to drive the cell through the G1/S checkpoint (7,53,54,57,68,116-118). CDK4 is regulated by cdk inhibitors (cdki) such as p15, p16, p21 and p27 and it requires phosphorylation at threonine 172 by cyclin activating kinase (CAK) to be catalytically active (8,50,52-56,58,102,119-123). Increased expression of CDK4 has been observed to coincide with amplification of the 12q13 amplicon, providing a mechanism for the deregulation of CDK4 activity (124-126).

There is genetic support for CDK4 having a role in tumor development. It is included in the 12q13-15 region which is commonly amplified in tumors. In gliomas, the tumor suppressor p16, which inhibits CDK4 activity, is also seen to be mutated. In the majority of gliomas either p16, CDK4 or Rb is altered. The frequency of genetic alteration of any of these genes is much
Figure 1-1. The 12q12-15 amplified region.
MDM2- a p53 associated protein
HMGIC- an architectural transcription factor gene
SAS- Sarcoma Amplified Sequence, member of transmembrane 4 superfamily of genes
CDK4- Cyclin Dependant Kinase 4
GADD153 (CHOP)- a DNA damage inducible transcription factor involved in chromosomal rearrangements in liposarcoma
GLI- a transcription factor in the Sonic hedgehog-Patched signaling pathway
A2MR- α-2 Macroglobulin Receptor
lower in gliomas that have an alteration of either of the other two genes (127-129). This indicates that these genes serve a similar role in tumor development and that the loss of either tumor suppressor gene p16 or Rb, or the amplification of CDK4 is sufficient to inactivate that cell cycle regulatory pathway (127-129). Furthermore, a p16-insensitive CDK4 mutant has been found in a human melanoma, a type of tumor with a high frequency of p16 mutations (130).

b. MDM2

MDM2 was originally identified as a gene that was amplified in double minute chromosomes in a tumorigenic mouse fibroblast cell line and whose gene product induced tumorigenicity when overexpressed in non-transformed mouse cells (131). It has also been shown that MDM2 overproduction results in the immortalization of primary rat embryo fibroblasts (131). The MDM2 oncoprotein regulates cell growth through interactions with multiple gene targets: p53, Rb and E2F/DP1 (15,16,132-134). MDM2 seems to negatively regulate the tumor suppressor gene p53 in a regulatory feedback loop (132-135). p53 protein induces the transcription of MDM2. MDM2 may then act to inhibit the transactivation capacity of p53 through binding its transactivation domain. Expression of MDM2 can abrogate the growth suppression of transformed cells by wild-type p53. In addition, recent studies have indicated that the binding to MDM2 regulates p53 protein levels through the enhancement of proteasome-dependent p53 degradation (14,123,136). In studies using osteosarcomas, it was found that MDM2 physically interacts with the Rb tumor suppressor gene and inhibits Rb growth regulation (16). Also, MDM2 was found to stimulate the activities of the E2F1 and DP1 transcription factors which are S phase promoting genes that are negatively regulated by Rb (15).
Oliner et al (1992) mapped the human MDM2 gene to the 12q13-14 region which led them to examine sarcomas for alterations in the gene. They found MDM2 was amplified in 17 of 47 sarcomas, including 3 of 11 osteosarcomas. MDM2 was amplified in both sarcomas and gliomas and was the gene most consistently included in the 12q amplicon found in these cells, indicating that it is probably providing a growth advantage to these cells. However, evidence to the contrary includes studies which have examined sarcoma and glioma tumours that are amplified for markers in 12q13-15 but not amplified for MDM2, indicating that another gene may be responsible for driving the amplicon (126,137). Amplification can provide a mechanism for increasing MDM2 mRNA expression, which then increases the amount of protein present, resulting MDM2 deregulation and oncogenic activity.

c. CDK2

CDK2 was cloned as a gene that could complement mutations in the budding yeast cdc28 gene (138). Like CDK4, it phosphorylates Rb to regulate progression of the cell cycle (52-54,139,140). CDK2 forms a complex with cyclin E and is most active during G1 and early S phase. Cyclin E is then degraded in early S phase at which time newly synthesized cyclin A activates cdk2. CDK2 is negatively regulated by the cdki genes p21 and p27 and is activated through phosphorylation by CAK (50,52-55,120-122,133,134).

d. SAS

SAS (sarcoma amplified sequence) was cloned from a region of amplified DNA in a malignant fibrous histiocytoma and was classified as a member of the transmembrane protein 4 superfamily (141,142). Although the function of SAS is unknown, the data available on
transmembrane 4 family genes indicates a possible role in signal transduction and growth control (142). We had previously examined osteosarcoma samples for DNA amplification and found SAS to be amplified in 10/28 (36%) samples (143). When evaluated for tumor subtypes, SAS was amplified in all 7 surface (parosteol -- generally low grade) osteosarcomas in contrast to 2 of 15 intramedullary (generally high grade) osteosarcomas. This indicated that SAS, or the another gene in the 12q12-15 region which is co-amplified with SAS, may play a role in the pathogenesis of osteosarcoma subtypes(143).

ii. Amplified Regions in Osteosarcoma

In addition to the 12q12-15 amplified region, there are other regions of amplification which provides evidence for the involvement of possibly 10 more proto-oncogenes in the pathogenesis of osteosarcoma. CGH studies have identified ten commonly amplified regions in osteosarcoma tissue samples: 1q(q21-q31), 3q(q25-qter), 4q(q27-q32), 6p, 8q(cen-q13), 8q(q23), 11q(q14-q23), 17p(p11-p12), Xp(cen-p21) and Xq(q25-qter) (17,18).

II. 12q12-15 Amplicon in Osteosarcoma

The 12q12-15 region has been extensively studied in soft tissue sarcoma and glioma. The amplified regions at 12q12-15 were first believed to be a single amplicon detected in a malignant fibrous histiocytoma and since found in tumor samples and cell lines from bone and soft tissue sarcomas, astrocytomas, and gliomas (6,67,125,126,137,141,144-146). This region is known to contain: MDM2 (6), CDK4 (125), SAS (141), CHOP/GADD153 (137), GLI (147,148), A2MR (147), HMGIC (128), CDK2 (149) and two additional uncharacterized genes mapping near
CDK4/SAS (Figure 1-1) (150). The determination of the gene target(s) of amplification of this region may expand our understanding of osteosarcoma pathogenesis.

While any of the genes in this amplicon, with the possible exception of A2MR, may contribute to osteosarcoma progression, CDK4 and SAS are most consistently amplified and therefore are the most likely targets of amplification. However, these studies have not indicated which of the two genes may be more critical. In addition, in some cases only MDM2 is amplified, complicating the interpretation of which genes may provide the selective growth advantage driving the amplicon. Recent studies have shown that the 12q15 amplified region may consist of 2 discontinuous regions, one containing MDM2 and the other containing CDK4 and SAS (13,151-154). This raises the possibility that MDM2 and CDK4/SAS are separate targets of amplification. Furthermore, HMGIC, an architectural transcription factor located between MDM2 and CDK4/SAS, has been shown to be independently amplified and may be the target of a third amplicon (128). Although CDK2 has been mapped to 12q13, whether it is amplified or, if so, which amplified region it is included in has yet to be determined. In order to investigate the role SAS, CDK4, MDM2 and CDK2 play in osteosarcoma progression and neoplastic growth, I elected to examine osteosarcoma tumor specimens for the DNA amplification and mRNA expression status of these genes.
III. Isolation of Additional Genes Involved in Cancer

A. Isolation Strategies

In addition to the investigation of known genes, the novel tumor suppressor genes and oncogenes in frequently altered chromosomal regions must be isolated and described. The mechanisms outlined in previous sections for the inactivation of tumor suppressor genes and activation of oncogenes can lead to the altered expression of such genes. Therefore, the isolation of these genes can be accomplished through the analysis of mRNA expression in either cell lines or tumor samples through the use of various subtractive or PCR based techniques. Samples can include both cell lines and tumor samples. The use of direct tumor samples is the most suitable approach due to the availability of different stages and grades of tumors. Not only does this allow for the isolation of genes involved in tumor development, but also genes that determine grade (i.e. low or high grade) and genes involved in progression from early to late stages. In order to assign genetic relevance to the differentially expressed genes that are isolated, they can be mapped to see if their chromosomal location corresponds to a commonly altered region.

Currently, there are five main approaches used for the screening of differentially expressed genes: differential hybridization (also known as plus/minus hybridization), subtractive hybridization, representational difference analysis (RDA), serial analysis of gene expression (SAGE) and differential display polymerase chain reaction (DDPCR). Due to the use of RNA from actual tumor samples, the isolation technique must meet the following 2 requirements. First, it must be able to compare multiple samples simultaneously in order to identify the most common genetic alterations (to compensate for the presence of a high background of non-specific
genetic alterations in tumor samples). Secondly, it must require very little RNA since the tumor samples are a limited resource (unlike cell line RNA).

i. Differential screening

Differential screening is the most basic technique and perhaps the least sensitive (155-157). It involves the comparison of mRNA from two samples by the hybridization of a cDNA library with two sets of probes, each generated from the mRNA of one of the samples being analyzed. Differences in hybridization of a plaque by the two different probes indicates a potentially differentially expressed gene. Limitations of this technique include a high background and the ability to detect only abundant mRNAs.

ii. Subtractive hybridization

Subtractive hybridization is a more sophisticated technique based upon the same principles as differential hybridization (155-157). The RNA is isolated from the sample of interest and reverse transcribed using polyT primer to produce cDNA from only the polyA RNA (alternatively, RNA polymerase can be used to produce cRNA from cDNA libraries). This is then hybridized to an excess of subtraction driver mRNA from the second sample. In addition, sense cRNA generated by RNA polymerase or denatured double stranded cDNA can be used. The cDNA from genes expressed in both samples hybridize while the cDNA from genes expressed only in the first sample remain single stranded, which can be separated from the hybridized cDNA for cloning. Limitations of this technique include the need for a large amount of RNA (at least 10 ug/sample), that it is labor intensive and that it is capable of identifying differentially expressed genes in only one of the two samples. The sample providing the driver RNA must be tested in
turn using RNA from the first sample as driver RNA. The benefits are that it is a well established technique capable of detecting rare mRNAs. An example of the isolation of a gene potentially involved in cancer through subtractive hybridization is the cyclin dependent kinase inhibitor p21/waf1/cip1, a gene induced by p53 (158).

iii. Representational difference analysis

Representational difference analysis (RDA) of cDNA is the latest advance in subtractive based technology (Figure 1-2) (159-162). It incorporates both subtractive steps and PCR amplification to increase the power of the technique. Originally developed to identify differences between genomes, the technique has been adapted by Hubank and Schatz to detect differences in gene expression (160). The first step involves decreasing the complexity of the genome by digesting the cDNA with four-cutting enzyme in order to produce short pieces that act as representations of the full length cDNA. Short oligos are attached to the cDNA and are used as binding sites for primers in a PCR reaction to amplify the material. The linkers are removed and new linkers are added to the cDNA that will act as the tester (not the driver) in the subtractive step. The tester cDNA is then hybridized with a large excess of driver cDNA and a PCR is performed on the mix. Since only the tester cDNA has primer binding sites, fragments that are present in the tester but not the driver will amplify exponentially. However, due to the large excess of driver cDNA, a fragment that is present in both samples will hybridize in a heterozygous fashion and contain a primer binding site only at one end. Therefore, it will amplify linearly. If the fragment is only present in the driver, it will not amplify at all. Further rounds of hybridization and amplification result in a small number of differentially expressed products that are visible on an agarose gel with ethidium bromide stain. The advantages of this technique are the use of PCR
Figure 1-2. Representational Difference Analysis
to minimize the amount of initial cDNA required (2 ug) and the speed with which it is possible to generate a result. Unlike the previous two subtractive techniques, it is possible to generate a result in 3 weeks. In addition, by altering the ratio of driver to tester it is possible to detect very small difference in expression (2 fold).

A major disadvantage of RDA is common to all of the subtractive based techniques: it is only possible to compare one sample versus one other sample. When using tumor samples it is important to be able to compare multiple samples versus each other in order to eliminate the random genetic differences that arise when neoplasia develops. In addition, the reaction must be repeated with the tester and driver RNA switched in order to isolate both under and overexpressing genes. This results in the need for large amounts of RNA when comparing tumor specimens.

iv. Serial analysis of gene expression

Serial analysis of gene expression (SAGE) is a very recent advance in gene expression studies (Figure 1-3) (163-167). It is based upon the principle that 9 basepairs at a defined position is sufficient to identify a gene. Poly A RNA (2.5-5 ug) is converted to cDNA with biotinylated poly T primers. A 4-base cutter is used to cleave the cDNA and the 3' most end of the cDNA is bound to streptavidin beads. Linkers are added to the free end of the cDNA. The linkers reintroduces the site for the first enzyme and also introduces a recognition site for a type IIS restriction endonuclease which cuts up to 20 base pairs away. When digested with the second enzyme the linker and a short region of the original cDNA is all that remains (the 'tag'). These fragments are ligated together, amplified with primers specific to the linkers, and then cut with the first enzyme. This results in pairs of 9 base pair tags attached end to end. These are ligated
Cleave with anchoring enzyme (AE)
Bind to streptavidin beads

Divide in half
Ligate to linkers (A+B)

Cleave with tagging enzyme (TE)
Blunt end

Ligate and amplify with primers A and B

Digitag
Cleave with anchoring enzyme
Isolate ditags
Concatenate and clone

Figure 1-3. Serial Analysis of Gene Expression (SAGE)

Adapted from Velculescu, et al '95
together to produce long concatamers which are then cloned and sequenced. The frequency of occurrence of the gene's tag is indicative of its expression level.

SAGE is a very powerful technique which has been successfully used to study gene expression in yeast, to investigate expressed genes in pancreatic and colorectal cancer versus normal colon tissue, for the expression analysis of p53-dependant growth regulation, and to study p53-induced apoptosis (163-167). Its benefits include the ability to produce a catalog of the expressed genes in a sample and their expression levels, which can then be empirically compared between different samples to identify differentially expressed genes. It is limited in the study of sparse samples due to the high RNA requirement (5ug polyA RNA = 100ug total RNA) which precludes the examination of small tumor samples.

v. Differential display

Differential display is radically different from the techniques discussed above. It is a PCR based technique that involves comparing RNA fingerprints of multiple samples in order to screen for uniquely expressed genes (Figure 1-4) (168-172). It is based upon a degenerate reverse transcriptase - PCR reaction that amplifies a specific subset of mRNAs in a sample for each primer set. The genes that constitute that subset are a random (yet consistent) selection of the total mRNA population. The PCR products corresponding to those mRNAs can be separated by acrylamide gel electrophoresis and visualized through the incorporation of $^{33}\text{P}$ or $^{35}\text{S}$ dATP in the PCR reaction. Multiple samples can be run along side each other and examined for the presence of unique bands, which correspond to uniquely expressed gene mRNAs.

A particular mRNA will be amplified in a differential display if its 3' end corresponds to the T12XY primer and there is an acceptable binding site for the random primer within 1000
Figure 1-4. Differential Display PCR (DDPCR)
bases of the 3' end. There are two estimates regarding the number of primer pairs necessary for the visualization of all the mRNA species in each sample. Liang and Pardee determined that approximately 20 different 10mers plus 12 polyT primers would be necessary based upon the following facts: the 10mers bind as 6mers and therefore there is a 5% chance of a primer binding within the first 500 bp upstream of the T12XY primer. Bauer et al (173) take a similar approach. However, they did statistical analysis (based upon the 10mers binding as 6mers and the size limit of the PCR products was 500 bp) and determined that a gene would show at least one DDPCR band if 25 10mers were used (confidence level of 95%).

The differential display technique has already been successfully used to isolate novel expressed genes in a wide range of sample types. For example, Bauer et al (173) have used the technique to analyze regenerating mouse liver. They used the full range of primer pairs (12 TxyYZ and 26 10mers) and therefore visualized the complete mRNA of normal and regenerating mouse liver. Out of the 38000 bands they produced, they identified 70 gene fragments that were differentially expressed. In another case, Liang and Pardee (172) have investigated the differences in expression between human breast cancer cells and mammary epithelial cells (using 2 TxyYZ and 3 10mers) and identified 3 uniquely expressed gene fragments. In yet another case, chronic cardiac rejection of transplanted hearts was analyzed using the technique to identify 5 upregulated genes (174). Three of the genes show homology to known genes but the remaining two represent novel genes that are possibly involved in cardiac rejection. In addition, differential display has been successfully used to identify genes involved in glucose response, fibroblast growth factor response, the effect of exposure to ionizing radiation on squamous carcinoma cell lines, differentiation of the mammalian brain, and the isolation of genes whose expression is induced by Rb.
Recently, examples of studies that utilize DDPCR to isolate genes involved in cancer progression have been published. The majority of these studies compared cell line RNAs and normal tissue versus cell line RNAs. In the case of Uchiyama (175), however, normal brain tissue and glioma tumor samples were compared using DDPCR and 20 novel sequences and one known sequence (an isoform of the kinesin heavy chain (KHC) gene) were found.

Differential display has some excellent advantages over more traditional subtraction based methods that make it a superior choice for the analysis of tumor samples. The amount of RNA necessary to perform DDPCR is minimal compared with subtractive techniques. An analysis of all of the mRNAs expressed in a tissue can be accomplished using 6 ug of total RNA (25ng per primer pair, of which 12 T12YZ and 25 10mers would be required). However, it is not necessary to do a complete analysis to find potentially important genes and therefore even 1 ug will allow for the detection of 1/6th (on average) of the genes differentially expressed in a sample. In comparison, 20 ug of RNA would be required in order to compare two samples for genes unique to either using subtractive hybridization.

A second advantage of differential display is the ability to compare multiple sample simultaneously (i.e. 40 samples could be run simultaneously on one gel and compared). Since multiple samples can be compared with each other in one reaction, differential display is ideal for the examination of large numbers of tumor and normal samples. An important genetic change (i.e. loss of tumor suppressor expression) may not be present in the majority of the tumor samples and could therefore be overlooked by the analysis of only a handful of specimens. Using differential display to examine numerous samples at a time could pick up these changes in the pattern of expression.
Consequently, DDPCR is the optimal approach for the screening of differentially expressed genes in osteosarcoma. The technique meets the requirements of working with RNA from tumor samples. First, it is able to compare multiple samples simultaneously in order to identify the most common genetic alterations (to compensate for the presence of a high background of non-specific genetic alterations in tumor samples) and secondly, it requires very little RNA.

B. DDPCR and Tumor Samples

As described previously, the mechanisms of activation and inactivation of oncogenes and tumor suppressor genes, respectively, can alter their expression, allowing for the isolation of these genes through the examination of gene expression in tumor samples. Although many techniques exist for the comparison of gene expression, DDPCR was the most advantageous: it is sensitive, capable of comparing multiple specimens at once and uses very little RNA. I therefore elected to use DDPCR for the analysis of mRNA expression in tumor samples and cell lines. The osteosarcoma samples included high grade primary tumors with corresponding metastasis samples, low grade tumors, and high grade tumors without metastasis. The examination of samples of varying grade and stage allowed for the isolation of novel cancer genes involved in the determination of grade, initial development, and progression from early to late stages of osteosarcoma. These genes may highlight new biological pathways that will increase our understanding of the pathogenesis of osteosarcoma and cancer in general, providing new prognostic markers or novel therapeutic targets.
CHAPTER 2

Materials and Methods
I. Tumor Samples

Sarcoma tissue was obtained at biopsy, resection of primary tumor, and resection of metastatic tumor. After tumor removal, a pathologist selected a portion of viable tumor based on examination of an adjacent section. Samples were taken wholly from within the tumor mass to avoid any possible contamination by surrounding normal tissue and immediately flash frozen in liquid nitrogen to prevent RNA degradation. The diagnosis of osteosarcoma was determined histologically. The location of the tumor was assessed radiographically and by histological and gross analysis. The lesion was classified as extraskeletal, intramedullary, surface, or surface with intramedullary involvement. The tumours were graded I, II, or III as per a modified criteria of that described by Dahlin (357).

II. RNA and DNA Extraction

The tumors were crushed in a Brinkmann Retsch crusher. DNA were extracted using standard procedures and quantitated by spectrophotometric determination. The RNA was extracted using the Trizol reagent method (GibcoBRL). The mRNA for use with DDPCR was treated with DNase in a 300 ul reaction volume containing 10mM MgCl₂, 1mM DTT, 12 units DNase, 24 units RNasin. After 20 minutes at 37°C, 35 ul of NaOAc was added to stop the reaction and the DNase was removed with phenol/CHCl₃ extractions and ethanol precipitation.
III. CDK4, CDK2, SAS - RNA Expression and DNA Amplification

A. Quantitation

Total cellular RNA was obtained from 27 osteosarcoma samples, reversed transcribed into cDNA by reverse transcriptase (MMLVRT) and amplified for either cdk4, cdk2 or SAS and asparagine synthetase. 200ng of RNA was reversed transcribed with 40 units MMLVRT in 8ul volume for 1 hour at 37°C. After denaturing the reverse transcriptase at 95°C, 4ul cDNA was added to 8ul of PCR mix, which includes 0.5mM MgCl2-ckd4/1.5mM MgCl2-SAS/1.5mM MgCl2-cdk2, 1 unit Taq polymerase, and 1.9uM of each primer (cdk4/AS or SAS/AS):

- cdk4RNA1 5’CATGTAGACCAGGACCTAAGG, cdk4RNA2 5’GGAGGTCGGTACCAGAGTG, SASRNA1 5’CCCTGTATCAACAAGATTATG, SASRNA2 5’CAGGGCTTCTCGTCTGAATG, AS1 5’ACATTGAAGCACTCCGCGAC, AS4 5’CCTGAGGTTGTTCM’C’CACAG, or 1uM of cdk2/AS primers: cdk2RNA1 5’GAATTCTGGCAACAGATCTTC, cdk2RNA2 5’AGGTTTAAGGTCTCGGTGGA.

The reaction was denatured at 94°C for 30 seconds, annealed for 30 seconds at 48°C (60°C for cdk2) and extended at 72°C for 1 minute in a Perkin-Elmer Cetus 4800 DNA Thermocycler. The cdk4, cdk2, SAS and AS RNA specific primers were chosen to amplify a region containing at least one intron, thereby minimising the effects of contaminating DNA.

B. PCR for DNA Amplification

MDM2 amplification was assessed by Southern blot or PCR analysis as previously described for SAS (143). CDK2 was co-amplified with AS by PCR under similar conditions with the following changes: 60°C annealing temperature, a 12 ul reaction volume containing 50 ng
DNA, 1.1 mM MgCl₂ and 1 uM of each primer. CDK4 was also assayed with AS except in a 20 ul reaction volume with 1.6 mM MgCl₂, 1.1 uM of each primer and in a Perkin-Elmer Cetus 9600 Thermocycler with denaturation at 94°C for 15 seconds, annealing for 15 seconds at 60°C and extension at 72°C for 20 seconds. Primers are as follows: CDK4DNA1 5’ CTGAGAATGGCTACCTCTC, CDK4DNA2 5’ AGCCACCTCACGAACTGTGC; CDK2DNA1 5’ TATCTGTTCCAGCTGCTCCA, CDK2DNA2 5’ CATGGGTGTAAGTACGAACAG; AS1 (see above), AS5 5’ TGCAACTTTGCCATTTGGCT.

In order to ensure that each PCR analysis was quantitative, a range of PCR cycles (generally 22-28) was examined for each sample to ensure that the reaction was in the logarithmic phase of amplification (the phase when the amount of product corresponds to the amount of the initial template). Asparagine synthetase (AS) was co-amplified along with cdk4, cdk2, and SAS in order to act as an internal control for the PCR reaction. The results were corrected against cell line DNA or RNA included in each experiment in order to control for variations between each set of PCR reactions. The products were run on 12% polyacrylamide gels, stained with ethidium bromide and photographed for quantitative densitometry using a Molecular Dynamics densitometer.

IV. Differential Display

A. DDPCR Samples

Five progression sets (12 samples), each consisting of 2 or more samples from primary and metastatic tumor specimens, 3 high grade tumors that did not metastasize, 4 low grade
tumors, 7 metastatic samples, 1 osteoblast culture and 8 cell lines.

B. DDPCR reaction

Total RNA (350ng) was reverse transcribed into cDNA in a 70 ul reaction which contained 50 mM TrisHCl, 75 mM KCl, 3mM MgCl₂, 10mM DTT, 2.55 uM T12XY primer, 120 uM dNTP (total), 350 units MMLV reverse transcriptase, and 35 units RNasin. The reaction was incubated for 1 hour at 37°C, and then 4 minutes at 94°C in order to inactivate the RT. Four ul of cDNA was used in a 12ul reaction which contained 50mM KCl, 10mM TrisHCl, 0.01% gelatin, 1.2mM MgCl₂, 2uM dNTP (total), 2.5 uM T12XY primer, 1 uM random 10mer primer, 1 unit Taq, and 1uCi ³³P. The PCR was cycled for 33 and 36 cycles at 94°C for 20 seconds, 37°C for 30 seconds, 72°C for 20 seconds in a Perkin Elmer 9600 PCR machine. To the 12 ul PCR reaction 4 ul of sucrose dye (40% w:v sucrose, 0.25% xylene cyanol, 0.25% bromophenol blue) was added. 2 ul was loaded onto 6% acrylamide (29:1 acryl:bis) gels (large sequencing type gels) and run at 60W until the bromophenol blue dye ran out the bottom of the gel. The gels were dried and exposed to film overnight.

C. Reamplification of DDPCR Bands

Bands were selected and excised from the dried acrylamide gel. In addition to the band of interest, an adjacent section of the gel (a lane without the band) was also excised to control for the specific reamplification. 50 ul of H₂O was added to the gel fragment, incubated at 37°C for a minimum of 3 hours, and then used for template in a reamplification reaction. The reamplification reaction consisted of 50mM KCl, 10mM Tris HCl, 0.01% gelatin, 1.2mM Mg, 0.45uM T₁₂XY primer, 0.45uM random 10mer primer, 1 unit Taq, and 1.3ul DNA template in a 20 ul reaction.
The PCR consisted of 15 seconds at 94°C, 20 seconds at 37°C, and 20 seconds at 72°C. The reaction was done in duplicate (at 25 and 35 cycles). The purpose was to only reamplify the main gene in the DDPCR band and to control for the inappropriate reamplification of miscellaneous genes present in the same portion of the DDPCR gel.

D. Purification and Sequencing of Reamplified DDPCR Bands

The reamplified bands were run on an agarose gel, excised and purified by the QIAquick gel extraction kit (Qiagen) as per the manufacturers instructions. The DNA was used as a template in a thermosequencing reaction (ThermoSequenase - Amersham). The Genbank sequence database, including the EST (expressed sequence tags) and STS (sequence tagged sites) databases, were searched for sequences similar to or identical to the sequenced DDPCR band using BLAST (nucleotide sequence comparison) and BLASTX (protein sequence comparison) programs. In addition, if there was an EST match for a novel gene the Tentative Human Concensus sequences (THC) database from the Institute for Genomic Research (TIGR) was checked. It is a compilation of overlapping EST sequences concatenated together to form longer, potentially full length, gene sequences and can therefore provide a great deal of sequence information while only starting with a very short sequence.

E. Primer sequences

T_{12}CC/AA/GG/GC/AG/GA/CG
KE5 5' CCT GAG ATG G
KE6 5' GGA CAC TGA G
KE7 5' CAC CTC AGT G
KE8 5' GGA CGT AGT C
KE9 5' CCT AGT CCA C
SKM 5' ACT GGC TGA G
KEP53 5' GTA GAG ATG G
KE5L 5' CCT GAG ATG GAG GAG TCG

V. Cell Culture Lines

5 osteosarcoma cell lines - HOS, KHOS, MG63, MNNG, SAOS2; 2 breast carcinoma cell lines MDA231, MCF7, and a lymphoblast cell line 7666 (purchased from ATCC - grown according to their specifications).

OB3P2 - an osteoblast culture (grown for 2 passages) from collagenase treated bone fragments. Osteoblast phenotype was confirmed by alkaline phosphatase activity.

VI. vWF RT PCR Quantitation

Total cellular RNA was reversed transcribed into cDNA by reverse transcriptase (MMLVRT) and amplified for vWF and asparagine synthetase. 200ng of RNA was reversed transcribed with 40 units MMLVRT in 8ul volume for 1 hour at 37°C. The cDNA was amplified using AS1 5'ACATTGAAGCACTCCGCGAC, AS4 5'CCTGAGGTTGTTCTTCACAG, vWF F1 5'TAAGTCTGAAGTAGGAGTGG, and vWF 5'AGAGCAGCAGGAGCAGCAGTGTGTTACAG primers. The vWF and AS RNA specific primers were chosen to amplify a region containing at least one intron, thereby minimizing the effects of contaminating DNA.

In order to ensure that each PCR analysis was quantitative, a range of PCR cycles (generally 22-28) was examined for each sample to ensure that the reaction was in the logarithmic
phase of amplification (the phase when the amount of product corresponds to the amount of the initial template). Asparagine synthetase (AS) was co-amplified along vWF in order to act as an internal control for the PCR reaction. The results were corrected against cell line RNA included in each experiment in order to control for variations between each set of PCR reactions. The products were run on 12% polyacrylamide gels, stained with ethidium bromide and photographed for quantitative densitometry using a Molecular Dynamics densitometer.
CHAPTER 3

Co-amplification and Overexpression of CDK4, SAS and MDM2 in Human Osteosarcomas

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The 12q12-15 region, commonly amplified in osteosarcoma, is known to contain many genes of which SAS, CDK4 and MDM2 are the most likely candidates for providing the growth advantage that drives the chromosomal amplification of this region. DNA amplification and mRNA expression status of SAS, CDK4, and MDM2 were examined in osteosarcoma tumor specimens in order to explore the involvement of these genes in osteosarcoma growth and neoplasia in general.

I. Results

A. Co-amplification of CDK4, SAS, and MDM2 in Surface Osteosarcoma

I initially examined 24 primary osteosarcoma (OSA) tumor specimens (6 surface, 16 intramedullary and 2 extra-skeletal), as well as 4 metastases for gene amplification. CDK4 was amplified in 7/28 (25%) tumors, including 6/6 surface and 1/2 extraskeletal OSA but not 16 intramedullary tumors or 4 metastasis (Table 2-1, Figure 3-1). Each of the 7 tumors with multiple copies of CDK4 also exhibited SAS amplification. In addition, two intramedullary tumors had amplification of SAS alone. In 4/5 surface tumors and 1/2 extraskeletal OSA, MDM2 was also found to be amplified. There was no evidence of CDK2 amplification in any tumors. A comparison of the two main types of OSA indicated that amplification of CDK4, SAS and MDM2 occurred more frequently in surface tumors than the more common intramedullary type (p=0.00001, p=0.0004, p=0.0008 respectively).
B. Expression of SAS, CDK4 and MDM2 in Osteosarcoma

In the 9 cases exhibiting gene amplification, each amplified gene had a correspondingly elevated mRNA level (Figure 3-2, Table 3-2). For each gene, the lowest mRNA level associated with gene amplification was used as the cut-off point for defining overexpression: CDK4 - 3.4; SAS - 2.3; and MDM2 - 2.2. These levels were chosen in order to avoid an arbitrarily low definition of overexpression which might lead to overestimating the number of tumors with high level gene expression. CDK4 and SAS were co-amplified and overexpressed in 1 extraskeletal OSA as well as all 6 surface tumors (Table 3-2). MDM2 was also amplified and overexpressed in the extraskeletal tumor and 2/3 surface tumors. For comparison, CDK4 mRNA level was also measured in three short-term cultures of proliferating human osteoblasts and was found to be consistently low relative to the tumors, with an average expression of 0.7.

In the two intramedullary tumors with SAS amplification the mRNA was also elevated. In addition, four tumors had increased SAS gene expression without amplification. One of these intramedullary tumors overexpressed both SAS and CDK4, while another had elevated SAS and MDM2. CDK2 had a consistently low mRNA level in all samples.

C. Amplification and Overexpression in Tumor Progression Specimens

For 8 cases, I analyzed at least two sequential tumor samples from each patient representing different surgical procedures including biopsy or resection of the primary tumor and metastasectomy (Table 3-3). In addition, 4 unrelated metastases were examined for amplification (Table 3-2). Amplification did not appear to be associated specifically with metastatic disease; gene amplification was generally observed in those lesions that also had amplification in the primary specimen.
<table>
<thead>
<tr>
<th>Type of osteosarcoma</th>
<th>CDK4 amplification</th>
<th>High CDK4 mRNA expression</th>
<th>SAS amplification</th>
<th>High SAS mRNA expression</th>
<th>MDM2 amplification</th>
<th>High MDM2 mRNA expression</th>
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Note: (i) mRNA levels used to determine overexpression: cdk4, 3.43; SAS, 2.3; MDM2 2.25
(ii) amplified and overexpressed values are **bolded**
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Note:  
(i) DNA levels are relative to placenta  
(ii) mRNA levels relative to MCF-7 used to determine gene overexpression: CDK4, 3.43; SAS, 2.3; MDM2 2.25
Figure 3-1. Quantitative PCR analysis of (22-28 cycles) CDK4 and CDK2 DNA amplification in human osteosarcomas. AS (asparagine synthetase) was used as an internal control and DNA copy number was normalized against placenta as described in Materials and Methods. Samples with a two-fold or greater increase in gene copy number relative to control unamplified DNA and indicated by * were considered to be amplified.
**Figure 3-2.** Quantitative RT-PCR analysis of CDK4, SAS and MDM2 mRNA expression in human osteosarcomas. AS was used as an internal control. The relative expression values were normalized against the tumor cell line MCF-7. Values indicated by * were considered to be over-expressed, as described in results. Upper numbers indicate PCR cycles.
II. Discussion

Gene amplification and over expression are generally thought to provide tumor cells with a selective growth advantage. For example, amplification of N-myc, c-myc, and neu have been associated with an adverse prognosis in neuroblastoma, small cell lung carcinoma and breast cancer respectively. Although amplification and overexpression of CDK4, MDM2 and SAS genes would also be expected to be associated with a more aggressive tumor phenotype, surface osteosarcoma (which are generally of lower histologic grade and slower growing) were found to be more likely to contain these molecular alterations than high grade intramedullary tumors. In addition, I did not observe amplification or overexpression in lung metastases unless it was also present in the primary tumor.

A. Amplification in Surface and Intramedullary Osteosarcoma

Because other studies have implicated MDM2 amplification in the progression of high grade osteosarcoma, the presence of amplification and overexpression of genes with potentially critical growth control functions in 6/6 surface OSA was surprising. These molecular changes are generally thought to provide tumor cells with a selective growth advantage, and would have therefore been expected in high grade intramedullary OSA or metastases. Intramedullary osteosarcoma are commonly associated with a poor prognosis despite aggressive treatment. In comparison, surface OSA are slower growing tumors which often present with a large mass, are generally of lower histologic grade, and therefore are usually treated with surgery alone because of their lower risk of systemic recurrence. As a result it would not have been unexpected to find these alterations in the more common high grade, intramedullary type of OSA which despite aggressive treatment has a high risk of metastasis.
Previous studies have shown 12q13-15 involvement in OSA to be uncommon. It is likely that the differences reported for the frequency of amplification of genes located at 12q13-15 are due to variations in the numbers of high grade, intramedullary tumors and parosteal (surface) tumors included in the study. Although not always stated, it is likely that these investigations involved the more common high grade, intramedullary tumors. MDM2 amplification has been found in only 4-7% of cases (176-178). In a study of 20 OSA, two (10%) tumors overexpressed CDK4 but in only one of these was the gene amplified (124). In another investigation, SAS was found to be amplified and overexpressed in 2/17 (12%) OSA (137). In one of these tumors CDK4 was also amplified and overexpressed, while in the other CDK4 and MDM2 were overexpressed without gene amplification.

However, other reports support the involvement of 12q13-15 amplification in the pathogenesis of surface OSA. In two CGH studies, Tarkkanen et al identified amplification of this region in 2/3 and 7/7 parosteal tumors, but in no other types of OSA (17,18). As well, MDM2 amplification has been found to be more common in surface tumors (2/8) than other types of OSA (3/67; p=0.085; (177)). Interestingly, soft tissue tumors of low grade or borderline malignancy have also been shown to have amplification of 12q13-15 sequences (179-181).

Involvement of 12q13-15 has been noted more frequently in soft tissue sarcomas. MDM2 has been studied most extensively, and amplification found in 12-38% of tumors (6,66,67,137,145,178,182). Although the majority of tumors with MDM2 amplification exhibited overexpression, in one study 17/62 tumors without amplification also had elevated MDM2 protein levels (67). In another report CDK4 amplification was identified in 9/64 (14%) cases and overexpression in 15 (23%; (124)). SAS was amplified in 9/24 (38%) soft tissue sarcomas in one investigation and 8/78 (10%) in another (137,145). Similar to our finding of 12q13-15
amplification in the specific subset of surface OSA, this amplicon has been identified most frequently in retroperitoneal malignant fibrous histiocytosis (MFH) and low grade liposarcomas (17,145,183). This suggests molecular specificity within certain histologic tumor types, as well as a possible predisposition for slow growing, large and lower grade tumors with less risk of metastases.

B. CDK4/CDK2 Amplification and Expression

Each of the three altered genes in this study has potentially critical functions that may be related to the development or progression of OSA. Several pieces of evidence support CDK4 as having an oncogenic function and make it an attractive target for amplification and overexpression in the 12q13-15 amplicon. CDK4 is normally involved in phosphorylation and inactivation of the retinoblastoma tumor suppressor protein (pRb) (7,116-118), leading to cell cycle progression (52-54,57,68). Therefore overexpression of CDK4 alone may result in loss of cell cycle control by normal regulatory signals, resulting in unchecked cellular proliferation. This is supported by studies showing that overexpression of CDK4 rescued fibroblasts from p53-mediated growth arrest, as well as from TGF-ß inhibition (7,184). This may occur by titrating out available CDK inhibitors, thereby allowing CDK2 activity to drive the cell cycle through the G1 phase. Other alterations in this pathway including mutations of the RB1 gene, overexpression of cyclin D, and less commonly inactivation of P16, a negative regulator of CDK4 activity, have all previously been implicated in sarcomas as well as other cancers (10,22,43,52,101,124,135,169,176,185,186).

Similar to CDK4, CDK2 phosphorylates pRb at the G1/S transition to drive cell cycle progression and therefore could also provide tumor cells with a proliferative advantage (140,149).
Based on the above observation and the fact that CDK2 has also been mapped to 12q13-15, I questioned whether genetic alteration of this gene also occur in OSA. Neither CDK2 DNA amplification nor mRNA overexpression was found in any OSA, including those samples with amplification of other 12q13-15 genes. Recent studies have indicated that unlike CDK4, overexpression of CDK2 may not provide a growth advantage to neoplastic cells: constitutive CDK2 expression was not able to rescue cells from either p53 or TGF-β induced growth arrest (184,187).

C. MDM2 Amplification and Expression

MDM2 is transcriptionally activated by p53 and also binds and inactivates the p53 protein. This leads to down-regulation of p21 which normally binds cyclin/cdk complexes to block DNA replication and cell cycle progression. In this way, alterations of MDM2 can impact on both the p53 and pRb growth control mechanisms. As a result, it is not surprising that MDM2 is tumorigenic when overexpressed (135), and may be another target selected for by the 12q13-15 amplicon. In fact, it has been suggested that amplification and overexpression of MDM2 may have a similar effect to p53 mutations, which are known to occur in approximately 25% of high grade OSA. Studies of OSA (144), soft tissue sarcomas (66,188) and malignant gliomas (146) have shown that these are generally mutually exclusive events. However, one study of STS found a strong correlation between alterations of p53 and MDM2 in the same tumor and poor survival, suggesting that these two mutations may in fact have an additive effect (82). There is also evidence to suggest that MDM2 has transforming activity independent of p53 (189,190). MDM2 can also directly inactivate pRb, as well as activate the S-phase inducing transcription factors.
E2F1/DP1, possibly adding to the number of routes by which it can disrupt growth regulation (16).

D. SAS Amplification and Expression

Although the function of SAS in unknown, another member of the TM4SF gene family, KAI1, has been shown to suppress metastasis of prostate cancer cells injected into nude mice (191) without affecting the growth rate of the local tumor. More recently, KAI1 expression has been demonstrated to be downregulated during progression of human prostate cancer (192). Decreased expression was also associated with poor survival in patients with prostate, pancreatic, and non-small-cell lung cancers (192-195). If SAS similarly suppresses metastasis and yet provides an advantage to local tumor growth, its overexpression might help explain the generally low risk of metastasis associated with surface OSA.

E. Which Gene(s) Drives the 12q13-15 Amplicon?

SAS and CDK4 are tightly linked, which explains their frequent co-amplification in this and previous studies (196). In fact, expression of these two genes has rarely been found to be discordant. In support of this, all 6 surface tumors and 1 extraskeletal OSA had amplification and overexpression of both genes. Furthermore, the identification of a tumor (Case 10) with increased expression of both CDK4 and SAS, but without DNA amplification raises the possibility of a link between the transcriptional regulation of these two genes. Elevated gene expression usually occurs by transcriptional upregulation, with amplification being a late event associated with very high levels of mRNA expression (197). Increased expression of SAS and CDK4 may therefore be a preliminary step toward 12q13-15 amplification. Two intramedullary tumors were
identified which had amplification and overexpression of SAS alone, as well as two other high grade tumors with elevated SAS mRNA as the only alteration, indicating that this critical region does not always include CDK4 or MDM2. This also suggests that although its function is presently unknown, SAS, or possibly a nearby gene (OS4 (14)) likely plays an important role in OSA and may be the most critical gene in this amplicon.

Although MDM2 is known to be located several megabases from SAS and CDK4, it is often co-amplified with these genes, as seen in 4/5 surface OSA and 1/2 extraskeletal tumors in this study. However, discontinuities in the amplicon have been frequently identified with discrete regions of amplification localized around MDM2 as well as SAS/CDK4 and reduced amplification of the intervening sequences, suggesting that MDM2 and a gene near the CDK4/SAS area may separately contribute to tumor progression (13,151-154)

It is possible that the biological impact of these three genes depends on their pattern of involvement in a particular tumor. For instance, overexpression of CDK4 and MDM2 can lead to deregulation of the cell cycle and cell growth and tumor progression. In comparison, if SAS has a role similar to other members of the TM4SF family, it may counteract these effects by preventing tumor metastasis. These competing functions could help explain some of the clinical differences between high grade intramedullary OSA and the generally lower grade, surface tumors.
CHAPTER 4

Isolation of Novel Genes in Osteosarcoma
Although a number of the genes involved in osteosarcoma development and progression are known, the presence of many commonly altered chromosomal regions indicate the involvement of additional unidentified tumor suppressor genes and oncogenes. In addition, many of the biological pathways involved in osteosarcoma growth and metastasis have not been determined. The DDPCR analysis of changes in gene expression in osteosarcoma tumor samples, which can result from chromosomal alterations, may lead to the isolation of novel genes involved in neoplasia. The DDPCR assay was optimized for the detection of differential expression of genes using CDK4 expression (Chapter 3) as a positive control. DDPCR was then performed on a number of osteosarcoma tumor samples of varying stage and grade.

I. Results

A. Establishment of Basic DDPCR Conditions

The basic conditions for the reverse transcription step and PCR for optimal differential display were empirically determined using a modification of Liang and Pardee as follows (168-170,172,198). In order to minimize the depletion of tumor RNA stocks, 50ng of RNA per 10 ul cDNA reaction was determined to be optimal for the reproducible synthesis of cDNA, with 4 ul of this cDNA mix being used in the following 12 ul PCR reaction. Comparable to Liang and Pardee, the optimal concentration of dNTPs was found to be 2uM, significantly lower than conventional PCR reactions. The lower dNTP concentration allowed for better incorporation of radioactively labeled dATP and greater specificity in the PCR reaction. In addition, other experimental conditions such as PCR annealing temperature were altered as described in the Materials and Methods section.
i. Control Gene - CDK4

In order to have a positive control for the differential display analysis, DDPCR primers were designed that would bind to CDK4, a gene I have shown to be expressed at different levels in osteosarcoma (Chapter 3). T12GT was used since it complements the two nucleotides (AC) at the 3' end of CDK4 (before the poly A). A 10mer primer (KE5) that bound approximately 420 basepairs upstream of these T12 primers was chosen and PCR conditions (such as MgCl₂ concentration) were optimized. Initial attempts at DDPCR using these primers on samples with varying levels of CDK4 expression failed to give a definite CDK4 band. In order to amplify CDK4 in a more specific PCR reaction, an 18mer primer (KE5L) was made that consists of KE5 plus an additional 8bp of the CDK4 sequence at the 3' end of the primer. The PCR product from KE5 and KE5L would therefore be the same size, simplifying the isolation of a CDK4 band in DDPCRs using either primer. However, KE5L also failed to give a CDK4 band that varied in intensity consistent with CDK4 expression in samples.

The possibility existed that while a CDK4 band was being successfully amplified, it was saturating early in the PCR reaction and therefore did not display varied DDPCR band intensities among tumor samples with different levels of CDK4 mRNA expression (see lanes 5 and 12 on Figure 4-1). An experiment was performed using T12GT/KE5 over a range of PCR cycles (20-40) on the MCF7 cell line and tumor sample 45 (expresses CDK4 at 8 times the MCF7 level). At lower cycles (from 25 to 35), this revealed a band of the expected size that was substantially more intense in tumor 45 than in MCF7. As expected, there was no difference in the relative intensity of this putative CDK4 band between MCF7 and 45 in the reactions that had reached 40 cycles of amplification. This band was confirmed to be CDK4 by enzyme digestion (using SacI, PvuII, PstI, MspI, NcoI, NcoI+PvuII, and NcoI+PstI) and sequencing In addition, there were other
Figure 4-1. Differential Display of CDK4 in Two Samples.
DDPCR of MCF7 breast carcinoma cell line and osteosarcoma tumor sample #45 using Ti2GT/KE5 over a range of PCR cycles. Cdk4 is present in sample #45 at 8x the MCF7 level as independently determined by quantitative RT-PCR. +RT represents the presence of reverse transcriptase. -RT indicates no reverse transcriptase was added and all bands result from DNA contamination.
bands that were present only at certain cycle numbers. The range from 30-35 cycles gave the most stable results amongst samples (Figure 4-1).

In order to confirm the ability of DDPCR to differentiate between tumor samples with varied gene expression levels, the CDK4 specific primers (T12GT, T12CC, KE5, KE5L) were used in DDPCRs with the cell line MCF7 (1x CDK4) and tumor samples 512 (2x CDK4), 45 (8x CDK4) and 105 (16x CDK4) (Figure 4-2). CDK4 bands were present in each experiment and were correspondingly more intense in the samples with higher CDK4 expression. Thus, the reactions displayed a semi-quantitative pattern for CDK4 in that even 2 fold differences in expression were detectable.

In addition to the ~420 bp CDK4 band, another band (approximately 160bp) was detected in the reaction using T12CC which presented an expression pattern identical to CDK4 (Figure 4-2). It was present with both KE5/T12CC and KE5L/T12CC but not in the KE5/T12GT and KE5L/T12GT reactions. Therefore it seemed possible that the band resulted from either another gene, perhaps on the same amplicon or degenerate priming of T12CC to CDK4. Examination of the published CDK4 sequence revealed an alternate binding site for T12CC with 4 mismatches, which Liang and Pardee had shown to be tolerable by DDPCR. Sequencing confirmed the identity of the band as a result of T12CC degenerately binding to CDK4. These data confirmed that DDPCR can correctly detect differences in expression when using sub-optimal PCR conditions.

ii. DDPCR Primer Pairs

The tumor samples available for analysis contained varying amounts of salts and other contaminating substances. Each DDPCR primer pair must therefore be resistant to changes in salt
Figure 4-2. Differential Display of Cdk4 Control Gene in Osteosarcoma Samples. DDPCR of 4 samples using DDPCR primers capable of amplifying cdk4. The smaller cdk4 band results from degenerate binding of the T12CC anchored primer. A=MCF7 breast carcinoma cell line, B=tumor 512, C=tumor 45, D=tumor 105. ‘+’ represents the presence of reverse transcriptase while ‘-’ means that there is no RT present and bands result from DNA contamination. The cdk4 mRNA level was independently determined using quantitative RTPCR.
concentration and be capable of producing a consistent pattern of PCR products. For each primer pair, I found it was necessary to test the PCR using a range of MgCl₂ concentrations to determine the optimal concentration and also the sensitivity of the primer pair to different MgCl₂ concentrations. To this end, all primer pairs were tested in PCR reactions with MgCl₂ concentrations ranging from 0.9 to 1.5 mM. This was determined to be the optimal MgCl₂ range for the majority of DDPCR type primers and, generally, if a primer pair failed to work well in that range it did not function well at any concentration of MgCl₂. Figure 4-3 shows an example of a series of primer pairs over a range of PCR cycles and MgCl₂ concentrations. As can be seen in Figure 4-3, the T₁₂AG/KE7 and T₁₂AG/KE8 primer pairs were not suitable for DDPCR, while the T₁₂AG/KE6 pair gave reproducible results. Seven T₁₂XY primers and 7 random 10mer primers were tested in combination. Of the 49 primer pairs, 34 were stable over a range of cycles and MgCl₂ concentrations. However, 5 primer pairs gave redundant banding patterns due to similarities in the T₁₂XY sequences (e.g. T₁₂GG and T₁₂CG) leaving 29 suitable primer pairs.

iii. RNA Samples - Quality and DNA Contamination

One shortcoming of DDPCR is the high frequency of false differentially displayed bands, some of which may be due to varying amounts of DNA contamination. This problem can be compounded by the use of previously extracted RNA from sarcoma tumor samples, which may have DNA contamination and too little RNA to treat with DNase. While it was possible to include a control reaction that lacked reverse transcriptase in order to identify bands that originated from DNA contamination, the use of tumor RNA for this purpose was impractical as the quantity of tumor RNA was severely limited. In addition, DNA may compete with the cDNA in the PCR amplification and thereby distort the pattern of DDPCR bands among samples with
DDPCR primer | KE6 | KE7 | KE8
---|---|---|---

| Mg | 0.9 | 1.2 | 1.5 | 0.9 | 1.2 | 1.5 | 0.9 | 1.2 | 1.5 |

**Figure 4-3.** Differential Display Condition Optimization. Differential display of three primer pairs at varying magnesium concentration. Each sample was done at two different cycles (33 and 36) using T12AG/KE6, T12AG/KE7, T12AG/KE8 primers. T12AG/KE7 and T12AG/KE8 had bands which were inconsistent over a range of MgCl₂ concentrations. Only T12AG/KE6 was stable over a range of magnesium concentration and was suitable for use in DDPCR reactions.
different amounts of DNA contamination. Therefore, the potential to use UV irradiation to eliminate this source of spurious bands was explored.

Initial attempts at simply treating the concentrated cell line RNA sample with UV using a UV light box resulted in a decrease in the consistency and intensities of bands from both cDNA and DNA template. In order to stabilize the RNA and have greater control over the UV exposure, the RNA was diluted in the cDNA reaction cocktail (dNTPs, reverse transcriptase and primers were added afterwards) and exposed to UV using the Stratagene Stratalinker (254 nm) for varying amounts of time (Figure 4-4). Under these conditions, the UV treatment was successful in reducing the amount of DNA contamination. As expected, longer UV exposure resulted in a greater loss of DNA.

RNA from tumor samples have varying amounts of DNA contamination, and therefore this DNA may have varying effects. To determine the amount of DNA contamination necessary for disruption of the RNA pattern and the effectiveness of UV treatment on the removal of DNA, varying amounts of DNA were added to DNA-free RNA and the effect of UV treatment was investigated (Figure 4-5). Up to 15 ng of DNA was added to DNase treated T47D cell line RNA, which was then treated with UV for 5 minutes. DNA bands began to be visible in the RNA lanes at 5ng DNA / 100ng RNA, with substantial contamination at 15ng DNA / 100ng RNA. UV treatment was successful in eliminating the contamination from 5ng DNA and significantly reducing the contamination from 15ng DNA. Comparison of the T47D RNA (without DNase treatment) against the samples with DNA added indicated that there was less than 0.5ng DNA / 100ng RNA in the cell line. In addition, other experiments which involved UV treatment showed that, with the exception of the removal of spurious DNA bands, treatment did not significantly affect the RNA pattern of the DDPCR (data not shown).
Figure 4-4. Differential Display of Cell Line RNA Treated With UV Radiation. DDPCR of T47D breast carcinoma cell line RNA treated with UV radiation for varying lengths of time. '+' represents the presence of reverse transcriptase while '-' means that there is no RT present and bands result from DNA contamination. Lane X and Y simply represent the use of longer primers (20mers as opposed to 10mers).
<table>
<thead>
<tr>
<th>Untreated RNA</th>
<th>0 ng DNA</th>
<th>0.5 ng DNA</th>
<th>2 ng DNA</th>
<th>5 ng DNA</th>
<th>15 ng DNA</th>
<th>control</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT +</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DNA band</td>
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<tr>
<td>RNA band</td>
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<tr>
<td>DNA band</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Figure 4-5. Differential Display of UV treated RNA with DNA added. DDPCR of T47D cell line RNA with up to 15 ng of DNA added (pre-UV treatment). ‘Normal RNA’ is untreated and contains DNA. ‘DNase, UV treated RNA’ was treated with DNase and UV to remove contaminating DNA and then varying amounts of DNA was added back. The lanes labeled ‘uv’ were exposed to UV radiation after the introduction of DNA. The minimum amount of contaminating DNA that has an effect in the RNA is 5 ng. The UV treatment is able to deal with a maximum of 15 ng contaminating DNA.
RNA from osteosarcoma tumor samples can contain significant amounts of DNA contamination (more than cell lines) and additional contaminants. Any method that lessens the effect of DNA contamination in cell lines must therefore be attempted in tumors to determine the true efficacy of the technique. Towards this end, DDPCR was performed on multiple tumor samples using T12GT, including the use of UV irradiation of RNA. The control cell line produced a regular pattern but the tumor samples were less consistent (Figure 4-6). Therefore, the use of UV irradiation to simplify the interpretation of DDPCR gels was not a practical method and reactions which lacked reverse transcriptase were used to provide a control for DNA contamination.

In order to have high quality RNA for use with DDPCR, it was necessary to extract fresh RNA from the tumor samples in order to obtain a large amount of RNA of similar quality, treat each with DNase and quantitate the concentration by both spectrophometry and RT-PCR. Twenty six osteosarcoma samples were extracted to yield high quality DNA-free RNA which generated consistent results in the subsequent DDPCR reactions.

B. Differential Display of a Panel of Tumor Samples and Cell Lines

I examined 26 tumors of different stages and 9 cell culture lines using the differential display technique. The tumor samples consisted of 14 primary tumors of different grade and 12 metastatic samples (see Materials and Methods section). Included in these samples were five progression sets of tumors (samples taken from the biopsy and/or resection and metastasis from the same patient). One of the cell cultures was an osteoblast culture (second passage) used to represent "normal" cells.
Figure 4-6. Differential Display of UV Treated Tumor Samples. Differential display reaction of 4 osteosarcoma resection tumor samples which were treated with UV radiation in order to minimize DNA contamination. Each sample is represented by 2 lanes (33 and 36 PCR cycles). The DDPCR bands are inconsistent between PCR cycles and also from sample to sample.
Using 10 primer pairs, 64 bands were selected as potentially being differentially expressed. Forty nine were successfully reamplified and directly sequenced. A further round of selection was performed on these bands based on the following criteria: i) how significant is the range of expression of a band (based upon band intensity among samples); ii) is the band present in multiple samples; iii) if the expression pattern is consistently different among the different tumor stages and different tumor grades.; and iv) how potentially relevant to cancer a known gene is. Eleven genes including 7 known genes and 4 novel sequences were selected for further characterization on this basis (Tables 4-1 and Figure 4-7). One of these, the band representing von Willebrand factor (vWF), was initially chosen from the DD-PCR gel because it was overexpressed in 2 metastases compared to the paired primary tumors from the same patients (Figure 4-7). The differential expression of vWF was confirmed using quantitative RT-PCR (see Table 4-2 and Figures 4-7, 4-8, and 4-9). The expression pattern determined by RT-PCR corresponded well with the DDPCR gel results: vWF was expressed more highly in the metastatic samples in primary/metastatic pairs. In addition, the vWF expression was higher in unmatched metastatic samples as compared to high grade primary samples (p=0.05).
Table 4-1. Isolated Genes

<table>
<thead>
<tr>
<th>Known Genes</th>
<th>Chromosome location</th>
</tr>
</thead>
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<td>Collagen type I (preproalpha 2)</td>
<td>7q22.1</td>
</tr>
<tr>
<td>Collagen type III (alpha 1 precursor)</td>
<td>2q31</td>
</tr>
<tr>
<td>Collagen type XII (alpha 1 precursor)</td>
<td>6q12-q13</td>
</tr>
<tr>
<td>Cathepsin K</td>
<td>1q21</td>
</tr>
<tr>
<td>Lung resistance protein (LRP)</td>
<td>16p13.1-p11.2</td>
</tr>
<tr>
<td>von Willebrand factor (vWF)*</td>
<td>(Figure 4-7)</td>
</tr>
<tr>
<td>F1F0-ATP synthase complex, F0 domain g subunit</td>
<td>?</td>
</tr>
</tbody>
</table>

* Novel Genes

<table>
<thead>
<tr>
<th>Known Genes</th>
<th>Chromosome location</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>VB4</td>
<td></td>
</tr>
<tr>
<td>VC2</td>
<td></td>
</tr>
</tbody>
</table>

* Differential expression confirmed by RT-PCR (Figure 4-8).
Figure 4-7. Differential Display PCR with von Willebrand Factor (vWF) Band. A section of a differential display gel showing 5 progression sets consisting of 10 osteosarcoma samples from 5 patients exhibiting the vWF specific band. Each sample is represented by 2 lanes (36 and 39 PCR cycles). B represents a biopsy sample, R represents a resection sample and M represents a metastatic sample. The vWF mRNA relative expression level was determined by quantitative RTPCR (see Figure 4-8).
**Figure 4-8.** Quantitative Reverse Transcriptase/PCR of von Willebrand Factor in Tumor Samples.

RT-PCR of 6 osteosarcoma tumor samples. B represents a biopsy sample, R represents a resection sample and M represents a metastatic sample. Sample 511 (resection) and 670 (metastasis) came from the same patient. In addition, sample 1092 (biopsy) and sample 1349 (resection) were from the same patient. AS represents asparagine synthetase, a housekeeping gene used as a control.
Figure 4-9. vWF mRNA Expression in Osteosarcoma Tumor Samples. Expression was determined by quantitative RT-PCR and normalized versus osteoblast culture expression level (Figure 4-8).
<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Sample</th>
<th>Relative vWF mRNA expression</th>
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</thead>
<tbody>
<tr>
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</tr>
<tr>
<td></td>
<td>MCF7</td>
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</tr>
<tr>
<td></td>
<td>MDA231</td>
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</tr>
<tr>
<td></td>
<td>KHOS</td>
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</tr>
<tr>
<td></td>
<td>SAOS2</td>
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</tr>
<tr>
<td>Biopsy</td>
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<tr>
<td>Metastasis</td>
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<td>Resection</td>
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<td>Metastasis</td>
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<td>Biopsy</td>
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<tr>
<td>Resection</td>
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<td>Metastasis</td>
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<td>16.7</td>
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<tr>
<td>No Metastases</td>
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<td>High Grade Biopsy</td>
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<tr>
<td>Low Grade</td>
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<td>Metastatic Samples</td>
<td>835</td>
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<tr>
<td></td>
<td>1116</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>1432</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>1257</td>
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<tr>
<td></td>
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<td></td>
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<td></td>
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<td>2.3</td>
</tr>
<tr>
<td></td>
<td>1273</td>
<td>7.7</td>
</tr>
</tbody>
</table>

vWF RNA expression was determined by quantitative RT-PCR (Figure 4-8).
II. Discussion

A. Differential Display Optimization

i. Differential Display Reaction Conditions

It was necessary to optimize general DDPCR conditions due to the condition of the tumor RNA samples to be analyzed. The RNA from each sample can contain slight differences in the concentration of salts (especially magnesium), the amount of contaminating DNA and the amount of RNA degradation (if any). It was therefore necessary to develop DDPCR conditions that were robust and capable of producing consistent results in each sample. The annealing temperature of 37°C was found to give the best results for most DDPCR primer pairs. The primer pairs used for DDPCR were tested to see if they generated consistent PCR products over a range of Mg concentrations. If a DDPCR reaction was reproducible over a range of Mg concentrations from 0.9 to 1.5 mM, those primers were suitable for DDPCR. If not, the primers were too sensitive and generally would not be sufficiently stable over any range of MgCl₂ concentration and were therefore not suitable for DDPCR.

ii. CDK4 Control

Due to the high rate of false positives/background for DDPCR as stated in the literature, it was necessary to ensure that the optimized DDPCR conditions were capable of detecting genes that really are differentially expressed. Towards this end, the CDK4 gene was used as a positive control for the experiments. CDK4 had previously been shown to be expressed at different levels in the osteosarcoma tumour samples being used in this DDPCR analysis. The level of CDK4
expression in the tumour samples varied from 0.5x to 16x the MCF7 cell line CDK4 expression level. CDK4 fulfills the criteria of the type of gene I am attempting to isolate: a putative oncogene (or tumour suppressor gene) which maps to a chromosomal region commonly altered in sarcomas and expresses its mRNA in a corresponding manner (differing levels in different samples). It therefore is an ideal control gene for the DDPCR reaction.

In an attempt to isolate a CDK4 specific band, primers capable of amplifying CDK4 (along with other genes) were used in DDPCR reactions of tumour samples known to represent a range of CDK4 expression levels. Although various bands of approximately the correct size were visible, none of them varied in intensity. This raised the possibility that CDK4 was being amplified but not in a quantitative manner, i.e. the CDK4 product may be saturating at the high cycles used for DDPCR.

An experiment was therefore performed that involved a DDPCR (using the CDK4 capable primers pairs) in which the PCR was sampled over a range of cycles. This revealed that CDK4 was amplified semi-quantitatively at lower cycles and the PCR reaction was not amplifying the CDK4 product efficiently at the higher cycles due to the accumulation of a large amount of PCR product. When the experiment was repeated with a number of tumour samples (with different levels of CDK4 expression), a similar quantitative result was obtained, indicating that the DDPCR reaction is capable of detecting small differences in expression in osteosarcoma tumour samples under the correct conditions. It was, therefore, determined that DDPCR must be performed over a range of lower cycles (30-35) before the PCR products can saturate the reaction.

It was necessary to confirm that the DDPCR conditions, as worked out for CDK4, are capable of functioning under suboptimal PCR conditions, namely, the degenerate binding of primers that produces a substantial portion of the PCR products in a DDPCR. This concern was
addressed by the identification of a band of approximately 160 basepairs in the CDK4 DDPCR experiments. This band had a similar expression pattern to the expected CDK4 band. Sequence analysis confirmed this band was CDK4, a result of degenerate priming of the T12CC primer (4 mismatches). These DDPCR conditions are therefore capable of semi-quantitative amplification of genes even under suboptimal conditions.

iii. RNA Quality

DDPCR can have a high rate of false positives in the screening step. This is further compounded by the high amount of DNA contamination in the tumour RNA samples, possibly resulting in additional spurious DNA bands. In addition, the presence of the DNA raises the potential that the banding pattern produced by the RNA may be affected by the presence of competing DNA, of which there is variable amounts in the tumour RNA samples. The DNA could have various effects: DNA specific bands could mask RNA specific bands in the DDPCR, and the DNA PCR products could affect the kinetics of amplification of RNA specific products. Since there is a variable amount of DNA in the tumour samples, this effect would be variable from sample to sample and therefore compensation would be extremely difficult.

Although it is possible to treat the previously extracted RNA samples with DNase, it is an inefficient method due to the small amount of the tumour RNA: too much RNA is lost or degraded during the DNase treatment. An attempt was therefore made to develop conditions for the use of UV radiation to treat the samples. The UV light causes thymidine dimers in DNA, thus preventing the DNA from being used as a template for PCR.

Cell line RNA was successfully treated with 254nm UV radiation, i.e. the banding pattern was not significantly affected by the treatment but the DNA contamination was reduced.
Unfortunately, when the technique was applied to osteosarcoma RNA samples, the effect was less satisfactory in that the RNA pattern was affected by the treatment. The low quality of the tumor RNA was not suited to the UV treatment and resulted in less reproducible DDPCR patterns. Hence, the use of UV treatment for the elimination of DNA effects in tumour samples was not a practical technique.

Previously extracted RNA is sufficient for more typical methodologies such as northern blots and quantitative RT-PCR. However, inconsistent factors such as DNA contamination, RNA degradation, and salt contamination have a negative impact upon the quality of DDPCR reactions and therefore these RNA samples were not optimal. This was partially due to the samples being extracted using three different methods and partially due to the nature of the tumor samples: they contained large amounts of bone, cartilage and other solid material that complicated the extractions. It was therefore necessary to produce RNA samples to be used in DDPCR by extracting fresh RNA from tumor samples, selecting the RNA samples with minimal degradation and then treating with DNase. Although this resulted in the loss of RNA (even to the point of rendering some samples as unusable for DDPCR) there were many benefits over the use of previously extracted RNA. Namely, this resulted in tumor RNA samples with similar amounts of salt contamination and very minimal degradation; the RNA was accurately quantitated (by UV quantitation and RT-PCR) and sufficient amounts of RNA were obtained, allowing for a large number of DDPCR reactions using the same tumor panel. In addition, it was necessary to include a lane in each DDPCR that was a sample with no reverse transcriptase added, producing bands using DNA contamination as the template. The bands that resulted from DNA contamination could then be accounted for in the RNA specific lanes. By standardising the RNA (accurate
quantitation, no DNA contamination and minimal degradation), the quality of the DDPCR was greatly increased over reactions using archival RNA.

B. DDPCR Bands

Using 10 primer pairs to examine 26 tumor and 9 cell cultures, 64 bands were isolated that were of potential interest. To minimise the likelihood of isolating false positives or genes that are modified due to random genetic changes, the following selection criteria were applied: (i) bands must be present in multiple samples, (ii) bands should display a consistent change in expression during tumor progression (e.g. increasing expression form low to high grade or primary tumor to metastasis) in multiple sample sets (i.e. progression sets or sets of tumors of the same stage), and (iii) the band should display a significant range of expression. In each DDPCR there are many bands which do not vary in intensity. Presumably, these are housekeeping genes and can be used as internal controls for comparing differentially expressed bands. In addition, a known gene whose function is potentially involved in the pathogenesis of OSA may be ranked more highly (e.g. a gene whose function is implicated in metastasis may be deserving of subsequent examination even though it does not present with very wide range of expression on the DDPCR gel). This selection resulted in 11 genes worthy of further investigation: 4 novel genes and 7 known genes (Table 4-1). Known genes identified by DDPCR include collagen types I, III, XII, cathepsin K and von Willebrand factor.

i. von Willebrand Factor

Von Willebrand Factor (vWF), a large glycoprotein expressed by endothelial cells and megakaryocytes, is involved in the platelet adhesion, aggregation and interaction with the
subendothelial matrix during coagulation (through the interaction of vWF and platelet specific vWF receptors) (199-201). It is present in the blood, platelet granules and subendothelial connective matrix as a multimeric protein (199-201).

vWF was originally isolated from the DDPCR gel as a differentially expressed band because it was expressed higher in metastasis from two patients compared to the paired primary tumor samples. The band was cut out and directly sequenced, identifying it as vWF. The possible role of vWF in metastasis (discussed below) and the pattern of expression seen on the DDPCR gel resulted in vWF being highly ranked as a candidate for further study.

RT-PCR conditions were established and confirmed the original differential expression pattern seen in the matched samples (increased expression in the metastatic sample vs the primary tumor sample). In addition, comparison of unmatched high grade primary and metastatic tumors revealed higher expression in metastases (p=0.05) (Figure 4-9). This demonstrated that the DDPCR analysis of gene expression in osteosarcoma tumor samples is an effective method for the isolation of differentially expressed genes potentially involved in cancer.

The expression of vWF in osteosarcoma tumor samples may result from two sources: the tumor cells themselves and the endothelial cells present in the vasculature of the tumor. The amount of vasculature, referred to as vascular index or microvessel density, has been to shown to be of prognostic value in cancers such as breast, melanoma, prostate, ovarian, gastric, and colon carcinoma (202,203). vWF staining of endothelial cells is commonly used as a marker for vascularity in tumor sections. Thus the differential expression of vWF may be partially due to differences of microvessel density in the tumor samples. In support of this, vWF expression by tumor cells originating from cells which do not express vWF has not been previously reported. However, I found that the osteosarcoma cell line SAOS2 expressed vWF. This indicated that
osteosarcoma tumor cells may themselves express vWF and contribute to the differential expression seen in the tumor samples assayed.

The vWF expression by tumor cells may play a role in the aggressive metastatic spread of osteosarcoma. Platelet aggregation and adhesion has been shown to be involved in hematogenous tumor cell metastasis(204-212). The aggregation of platelets and tumor cells may result in metastasis by producing a mixed platelet-tumor cell thrombus which can then easily lodge in a capillary or arteriole. Gasic et al found that tumors capable of forming platelet aggregates usually metastasize to the lung, the first sub-vasculature a metastatic cell would encounter in the bloodstream, while those lacking this ability gave a more wide spread occurrence in mice (210). This tumor cell-platelet mechanism may be partially responsible for metastasis of osteosarcoma (which tend to metastasize to the lung). In support of this, Clezardin et al (1993) and Chiang et al (1995) reported that the osteosarcoma cell lines MG63, HOS and SAOS2 induced platelet aggregation (213,214).

vWF expressed by osteosarcoma tumor cells may assist in tumor cell-platelet aggregation as well as tumor-sub endothelium adhesion, increasing the likelihood of successful blood-borne metastasis to the lung. Pre-treatment of either tumor cells or platelets with an antibody or peptide which neutralizes vWF or blocks vWF receptor binding has been shown to inhibit tumor cell-platelet interaction in vitro and tumor cell metastases in vivo (in mice) for both colon carcinoma and melanoma cell lines(207,208). As well, a platelet receptor that binds vWF (GPIIb/IIIa) has been found on many types of human tumor cell lines including melanoma, head and neck, colon, prostate, liver and breast carcinomas (215-217). The melanoma cell line M3Dau has been shown to directly interact with human platelets and induce aggregation, mediated by tumor cell GPIIb/IIIa (218). In addition, GPIIb/IIIa has been shown to be involved in osteosarcoma platelet
aggregation. Chiang et al reported that SAOS2 platelet aggregation can be prevented by treatment with rhodostomin, an inhibitor of GPIIb/IIIa, while Clezardin et al found platelet GPIIb/IIIa mediates the platelet aggregation induced by MG63 and HOS (213,214).

Although vWF may not provide a growth advantage to osteosarcoma cells in the primary tumor and therefore may not be uniformly expressed, the metastatic cells may be derived from the clonal expansion of a single primary vWF expressing cell, resulting in a pure population of cells that express vWF. Hence, the metastatic advantage provided by vWF expression may result in a net increase of vWF expression between the primary tumor and the metastatic sample.

C. Future Studies

Currently, our knowledge of the genes involved in producing the OSA phenotype is severely limited. Only a few genes are known to be involved in OSA pathogenesis and the number of potential candidates for further study is also limited. The use of DDPCR to analyse multiple tumor samples can highlight a substantial number of areas worthy of further investigation. For example, vWF and the receptors involved in vWF binding may be involved in OSA metastasis and may explain the high rate of metastasis of OSA and its tendency of metastasizing to the lung. Further analysis of tumor samples with DDPCR and the investigation of the isolated genes may provide a greater global understanding of OSA development and highlight candidates for further study, thereby providing many potential prognostic markers and therapeutic targets.

i. Further DDPCR Experiments

Additional DDPCR experiments should be performed to isolate more bands for analysis.
Nineteen additional DDPCR primer pairs have been optimized and will be used to examine the 26 osteosarcoma tumor samples. This will likely lead to the isolation of approximately 20 more bands that fulfil the selection criteria's discussed above. Further studies must be done to prioritize the bands, including those already isolated and any future bands, to select for a small number of genes to be the subject of subsequent comprehensive investigations into the biology of each gene and what role it may play in neoplasia. These analyses of isolated bands, including both novel and known genes, involve the confirmation of the differential expression, analysis of novel sequences, mapping genes, mutational analysis and amplification studies, and functional studies. In addition, taking advantage of the established biology of the known genes, specific studies will be performed to establish if they play a role in neoplasia.

ii. von Willebrand Factor

The higher expression of vWF seen in metastatic samples may result from two factors: a greater amount of endothelial cells in the metastatic samples (more vasculature) and expression by the osteosarcoma tumor cells themselves. The next step involves the use of antibodies to confirm the major site(s) of vWF production (most likely tumor cells and tumor endothelium). Following that, there are three further studies: (i) expand the study of vWF expression to confirm the pattern seen in the 26 tumor samples already examined (ii) investigate the other genes involved in the vWF pathway (e.g. vWF receptors in tumor cells) and vascularity (e.g. angiogenesis) and (iii) studies of metastasis of OSA in mice (e.g. will differing levels of vWF expression affect frequency of metastasis?).
iii. Novel Sequences

Once a novel gene has been identified as a potential proto-oncogene or tumor suppressor gene (based upon the expression pattern in the tumor samples), and its differential expression has been confirmed, full length coding sequence must be obtained. Further sequence information can be obtained by examining expressed sequence tag (EST) databases. An EST may overlap a DDPCR fragment or be larger than the short fragments typically isolated in DDPCR. Furthermore, the Institute for Genomic Research (TIGR) has compiled tentative human consensus sequences (THCs) which are created by assembling ESTs into virtual transcripts. These THCs can provide substantial amounts of sequence data (i.e. potentially full length). Full length sequence can also be obtained through screening libraries or by RACE analysis. The coding region of the gene must then be examined for the presence of common motifs (such as leucine zippers or SH2 domains) or for homology with known genes, which may furnish a hint towards a possible function.

iv. Mapping of Genes

The localization of a gene to a region commonly altered in OSA indicates a potential tumor suppressor gene or oncogene. The chromosomal location for many genes is known. In other cases, such as novel sequences, mapping information may also be available from many STS (sequence tagged sites) and ESTs that have already been mapped. The remaining genes will be mapped in collaboration with Dr. S. Scherer and Dr. L.C. Tsui at The Hospital for Sick Children.
v. Mutational Analysis and Amplification Studies

Once a gene has been shown to be a potential tumor suppressor gene through differential gene expression, further studies must be done to confirm its status. Point mutations are a common way to inactivate the second copy. Mutational analysis of a gene may, therefore, provide strong genetic evidence that the gene is a tumor suppressor gene. One mechanism for the increased expression of an oncogene is amplification of its DNA, thereby deregulating mRNA expression. Examination of a potential oncogene for DNA amplification can supply further evidence of an oncogenic role for a gene.

vi. Functional Studies

In addition, functional studies of the isolated genes will provide a wealth of information about the role of these genes in neoplasia. In vitro studies can provide powerful proof that a gene is a tumor suppressor gene or oncogene. In the case of a potential tumor suppressor gene, it is possible to transfec it into a cell line that lacks the gene and then see a change in phenotype (i.e. a decrease in malignant behavior). Tests include loss of growth in soft agar, increased contact inhibition in culture, and decreased development of tumours in nude mice. For example, a tumor cell line (which lacks a wild type copy of the gene) that can no longer form tumours in nude mice after the introduction of a wild type copy of the gene would support the hypothesis that it is a tumor suppressor gene. Oncogenes can be studied in a similar manner. The gene would be transfected into a cell line in order to over-express it. If the cell line gains the ability to form foci with loss of contact inhibition, grow tumours in mice, or the ability to grow in soft agar, it would provide support for the idea that the gene is an oncogene.
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