The Role of Cell Cycle Regulators p53 and p16\textsuperscript{INK4a} in the Pathogenesis of Primary Brain Tumours

by

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A Thesis submitted in conformity with the requirements for the degree of Master's of Science
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University of Toronto

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ABSTRACT

Both p53 and p16\textsuperscript{INK4a} inhibit progression through G1/S and are altered in a wide variety of malignancies including glial cell tumors. In this study, the role of p53 and p16\textsuperscript{INK4a} in the pathogenesis of primary brain tumors is examined. Thirty-six frozen tissue samples were obtained from adult glial cell tumors and both genomic DNA and total RNA were extracted simultaneously using a CsCl technique. Single strand conformation polymorphism (SSCP) analysis followed by DNA sequencing was performed to characterize specific gene mutations for both p53 and p16\textsuperscript{INK4a}. In addition, Southern blot analysis and multiplex-PCR were used to identify large deletions of the p16\textsuperscript{INK4a} gene. Quantitative reverse transcriptase-PCR was performed to analyze p53 mRNA levels. Immunohistochemistry was done using monoclonal antibodies specific for the amino terminus of p53 (D0-7 and 1801). Six of the tumors examined harboured mutations involving the p53 gene. No mutations of the p16\textsuperscript{INK4a} gene were detected using sequencing techniques, however analysis of the same tumors using multiplex-PCR showed evidence of deletions in 14 samples. A positive correlation was found between the presence of p53 mutations and altered forms of the p53 protein as detected by immunohistochemistry. There was no evidence of any correlation between p53 mRNA expression levels and p53 mutations or p53 protein abnormalities. Overall 18 tumours harboured alterations of either p16\textsuperscript{INK4a} or p53 and only two tumours contained mutations/deletions of both of these products. These findings suggest that using sequencing techniques to investigate primary brain tumours will miss deletions of the p16\textsuperscript{INK4a} gene. Mutations/deletions of the p53 and p16\textsuperscript{INK4a} genes in brain tumours are often reciprocal in nature, and deletion of p16\textsuperscript{INK4a} may offer an alternative mechanism of tumor pathogenesis in the presence of wild-type p53.
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**LIST of ABBREVIATIONS**

AA anaplastic astrocytoma
bcl-2 B cell leukemia/lymphoma 2
CAT chloramphenicol acetyltransferase
CDI cyclin dependent kinase inhibitor
CDK cyclin dependent kinase
cyt cytoplasm
Dx diagnosis
GADD growth arrest and DNA damage
GBM glioblastoma multiforme
GGM ganglioglioma
INK inhibitor kinase
LGA low grade astrocytoma
LGO low grade oligodendroglioma
MDM2 murine double minute 2
MO malignant oligodendroglioma
NBM neuroblastoma
NCM neurocytoma
PCNA proliferating cell nuclear antigen
PCR polymerase chain reaction
PNET primitive neuroectodermal tumour
Rb retinoblastoma
RT-PCR reverse transcriptase PCR
<table>
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<tr>
<th>SSCP</th>
<th>single strand conformation polymorphism</th>
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<td>TGF</td>
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INTRODUCTION

Brain Tumours as a Health Care Burden

Primary brain tumours account for 2-3% of the cancer burden in North America and are the sixth most common cause of cancer mortality in the population over the age of twenty years (Cutler and Young, 1975; Mao et al., 1991). Gliomas are tumours derived from one of the various types of cells that form the interstitial tissue of the brain or spinal cord. Approximately 40% of all central nervous system tumours are gliomas (Russel and Rubenstein, 1989). Unfortunately, with the exception of some pediatric astrocytomas, gliomas are generally not curable by surgery or other forms of therapy currently available. In order to discover more effective therapies for combating brain tumours new avenues of attack must be explored.

Pathology of astrocytomas and oligodendrogliomas

Tumours derived from astrocytic lineage are a large and diverse group. Over the years, various grading systems have been designed to categorize the spectrum of astrocytic tumours from the "benign" to more malignant variety. The most recent classification scheme was presented by the World Health Organization in 1993 (Kleihues et al., 1993). This system is based on the presence or absence of four histologic features which include: nuclear atypia, mitotic figures, microvascular proliferation, and necrosis. Tumours with one of these features present are called astrocytomas, those with two features are referred to as anaplastic astrocytomas, and those with three or four of the histologic features are called glioblastoma multiforme (GBM) (Fuller and Burger, 1996).

Based on clinical observations of GBMs, it has been hypothesized that there are two different pathways that can lead to their formation. In one pathway the tumour
begins as an astrocytoma and progressively develops more malignant features until it becomes a GBM. In the second pathway the GBM is speculated to arise "de novo" (Collins et al., 1993; Louis et al., 1995; Kleihues and Ohgaki, 1997). Oligodendrogliomas are tumours derived from oligodendrocytes, which are cells that produce myelin in the brain. Oligodendrogliomas are usually classified into two categories, either low grade or high grade. The high grade oligodendrogliomas possess the same histological features as the more malignant astrocytic tumours including: mitotic activity, microvascular proliferation, and necrosis. Despite these histologic similarities, high grade oligodendrogliomas generally have a better prognosis than GBMs (Fuller and Burger, 1996).

**Review of the cell cycle**

_i) Overview_

The capacity for unchecked growth is one of the unique qualities that distinguishes cancerous cells from normal cells. Despite this difference, in order to divide, both normal dividing cells, as well as malignant cells, pass through the same orderly sequence of events referred to as the cell cycle. This cycle is divided into four phases: G₁, during which time the cell prepares to synthesize DNA; S phase, during which DNA is synthesized; G₂, a second "rest period" during which the cell prepares to divide, and M phase during which mitosis occurs. A phase called G₀ is also described and cells that are either terminally differentiated, or cells that are not actively dividing are "arrested" in this phase. Cells enter G₀ following successful division, and under special circumstances are induced to exit this phase and reenter the cell cycle at the G₁ phase.
ii) Cyclins and cyclin dependent kinases

Coordination of the multiple interactions that must take place during the cell cycle is orchestrated by a class of molecules called cyclins. Four major groups of cyclins have been identified (cyclins D, E, A, B) with the quantity of each individual cyclin varying during specific stages of the cell cycle. There is evidence that alterations of at least some of these cyclins may be involved in the development of cancer. For example, amplification of cyclin D1 has been found to occur in a variety of malignancies including carcinomas of breast (Gillett, et al., 1994) or head and neck (Bartkova, et al., 1995). In order for these cyclins to be active they must combine with a second group of molecules called cyclin dependent kinases (CDKs). At least seven different CDKs, (CDKs 1 through 7), all of which are homologous serine/threonine kinases, have been described (Hirama and Koeffler, 1995). In addition, there are several families of cyclin dependent kinase inhibitors (CDIs) that have been discovered. These molecules can control the activities of different cyclin/CDK complexes in response to specific external signals. They also play a role in the functioning of internal feedback loops that control the progression of the cell cycle (Grana and Reddy, 1995). It is only through the tightly controlled activation and inactivation of specific CDKs, and the transcription of cyclin genes and degradation of cyclin proteins, at precise time points during the cell cycle, that cell division can successfully occur (Hartwell and Kastan, 1994; Hirama and Koeffler, 1995).
iii) The G1/S checkpoint

There are various points during the cell cycle, referred to as "checkpoints", where progression through the cycle may be halted. A checkpoint can be formally defined as a biochemical pathway that ensures dependence of one process upon another process that is otherwise biochemically unrelated (Elledge, 1996). Many checkpoint pathways have been identified, including checkpoints that coordinate cell size and cell cycle progression, that inhibit mitosis while in G1, that restrict DNA replication to once per cell cycle, and that make DNA synthesis dependent upon G1 cyclins (Elledge, 1996).

However, the signaling pathways that regulate cell cycle progression seem to be primarily associated with the G1 phase of the cell cycle (Grana and Reddy, 1995). Furthermore, it appears that all eukaryotic cells are subject to extracellular signals until a specific point late in G1 called the restriction or "R" point is reached (Pardee, 1974). The existence of this "restriction point" was first proposed after it was observed that BHK cells derived from hamsters were all blocked at the same place in the G1 phase of the cell cycle after being exposed to a wide variety of adverse conditions such as nutritional deprivation or increased cellular density (Pardee, 1974; Sherr, 1995).

Of the various checkpoints identified, the DNA damage checkpoint in late G1 is understood in the most detail (Elledge, 1996). Furthermore, the effectiveness of both p53 and p16^{INK4a} as cell cycle regulators are thought to be greatest at this stage of the cell cycle. Key regulators of G1 progression in mammalian cells include three D-type cyclins (D1, D2, and D3) which combine with either CDK4 or CDK6, and cyclin E which associates later in G1 with CDK2 (reviewed by Sherr, 1993). Evidence to support the importance of the D-type cyclins at this stage of the cell cycle include the observations that peak levels of these cyclins are attained near the G1/S transition (though cyclin D is present throughout the cell cycle) (Sherr, 1994), that cells fail to enter S phase if these cyclins are absent, and their degradation later in the cell cycle is without effect (Baldin,
et al., 1993). Similar evidence exists for the role of cyclin E whose levels peak just before the G1/S transition and which is degraded once the cell enters S phase (Koff et al., 1992). Both the cyclin D/CDK4 complex and the cyclin E/CDK2 complex are activated by the phosphorylation of a single threonine residue (Sherr, 1994).

There are likely several downstream targets for the cyclin D catalytic complexes (Resnitzky and Reed, 1995), the best characterized and most understood one being the retinoblastoma tumour suppressor protein (pRb) (Ewen, et al. 1993). This protein is phosphorylated in a cell cycle dependent manner, with the active form of the protein being its hypophosphorylated isoform (Matsushime, et al., 1994). pRb becomes phosphorylated during the middle to late G1 phase of the cell cycle. This phosphorylation reverses the growth suppressive effects of pRb by releasing the transcription factor E2F-1 (Nevins, 1992). Once pRb is inactivated, the cell undergoes an irreversible commitment to enter S phase (Sherr, 1996). Furthermore, it has been demonstrated that E2F-1 microinjection into quiescent cells can induce entry into S phase of the cell cycle (Johnson, 1993). The entire process, leading to the liberation of the E2F-1 transcription factor from its association with pRb, is initiated by the cyclin D/CDK4 complex and then accelerated by the cyclin E/CDK2 complex (Hinds et al., 1992; reviewed by Sherr, 1996). As would be expected, cells that do not contain functional pRb do not require the cyclin D/CDK4 complex to progress from G1 to S phase (Lukas et al., 1995).

The ability of the cyclin D and E complexes to inactivate pRb and thus drive the cell cycle is in part regulated by at least two different classes of CDIs. One group of CDIs, called the INK4 proteins, can directly block cyclin D dependent kinase activity and cause G1 phase arrest (Sherr, 1996). These proteins, which include p16^{INK4a}, p15^{INK4B}, p18^{INK4C}, and p19^{INK4D}, specifically bind and inhibit CDK4 and CDK6 before they have an opportunity to form a complex with the D cyclins (Hall et al., 1995; Sherr, 1996). It
has been determined that $p15^{INK4b}$ levels can be dramatically increased in human keratinocytes treated with transforming growth factor β (TGFβ) (Hannon and Beach, 1994). A second group of CDIs involved during the G₁ phase of the cell cycle includes at least three distinct proteins: $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$. This group of inhibitors is thought to act as broad spectrum regulators of CDK function by binding to cyclin/CDK complexes that have already associated, including cyclin D/CDK4, and cyclin E/CDK2 complexes, and inhibiting their ability to function (Hall et al., 1995; Hannon and Beach, 1996). The gene coding for the $p21^{CIP1}$ protein product is inducible by the p53 tumour suppressor protein (El-Deiry et al., 1993; Xiong et al., 1993), and this has been considered by some to be the “most remarkable” feature in the relationship between the cell cycle and cancer (Sherr, 1996).
**FIGURE 1: Cell Cycle Regulators at the G1/S Junction**

The cyclin/cyclin dependent kinase complexes cause the phosphorylation of pRb. This results in release of E2F from pRb which helps drive the cell cycle from G1 to S phase.

*Figure 1*: A simplified diagram of some of the regulators involved at the G1/S junction of the cell cycle. Arrows represent activation, blunt ends represent inhibition. The cyclin/cyclin dependent kinase complexes cause the phosphorylation of pRb. This results in release of E2F from pRb which helps drive the cell cycle from G1 to S phase. **TGFβ** Transforming Growth Factor β; **PCNA** proliferating cell nuclear antigen; **CDK** cyclin dependent kinase. See text for detail (Hunter and Pines, 1994; Kamb, 1995).
The p53 tumour suppressor gene

i) History of its discovery

Alterations of p53, a tumour suppressor involved in the regulation of cellular proliferation, are the most frequently observed genetic events in human cancer (Zambetti and Levine, 1993). p53 was discovered in 1979 as a 53 kilodalton (kD) nuclear phosphoprotein in extracts of transformed cells reacting with antiserum from animals with tumours induced by simian virus 40 (SV40) (Linzer and Levine, 1979; Lane and Crawford, 1979), and cloned in 1983 (Oren and Levine, 1983). As a result of its association with the large T antigen, an oncogenic product of SV40, p53 came to be classified as a tumour antigen (Levine et al., 1991). Subsequently, p53 was found in large amounts in tumour derived or transformed cell lines (Linzer and Levine, 1979; Lane and Crawford, 1979; Levine et al., 1991) and thus earned the distinction as an oncogene. However, it was soon realized that the increased quantities of p53 found in these cancerous cells was in fact a mutant form of p53 (Hinds and Finlay, 1989). Furthermore, evidence began accumulating in support of p53’s role as a tumour suppressor, such as its ability to suppress transformation of cells in culture by other oncogenes (Finlay and Hinds, 1989; Eliyahu et al., 1989). In addition, evidence that inactivation of p53 confers a strong selective advantage on erythroid cells during transfection with Friend leukemia virus also supported p53’s role as a tumour suppressor (Ben David, et al., 1988). Further support of p53’s ability to act as a tumour suppressor was obtained when it was discovered that a wide variety of tumours including brain, breast, lung, and colon harbour point mutations of the p53 gene (Nigro et al., 1989). Currently, p53 is accepted as a tumour suppressor and the multitude of mechanisms through which p53 performs this role continue to be discovered.
ii) Structure of the p53 gene and protein

The p53 gene is located on human chromosome 17p13.1 and is approximately 20 kb long, with eleven exons, the first of which does not code for the final 393 amino acid p53 protein product. The mRNA transcript is 2.6 kb in size and contains a large 3' noncoding region that is thought to be involved in stabilization of the molecule (Harlow et al., 1985). This untranslated region has been demonstrated to inhibit translation of a chimeric reporter mRNA and may explain a mechanism of p53 gene expression (Fu and Benchimol, 1997). There are five highly conserved domains in the p53 gene, 4 of them located within exons 5-8 (codons 126-306) in which most of the mutations associated with malignancy have been located (Nigro et al., 1989; Louis, 1994). Furthermore, within these conserved domains there are several sites called "hot spots" where mutations are found with a particularly high frequency (Prives, 1994; Zambetti and Levine, 1993). Apparently the location of the "hot spots" is at least in part dependent on the tumour tissue type and a hot spot common in brain tumours will not necessarily be common in hepatocellular carcinoma (Zambetti and Levine, 1993; Greenblatt et al., 1994). The tendency for the majority of p53 mutations to be located between exons 5-8 has led many investigators to examine only this region of the gene, and may account for an underestimation of the incidence of p53 mutations by as much as 20% (Greenblatt et al., 1994). The majority of mutations identified are in the form of point mutations, with G:C→A:T transitions accounting for 41% of 2567 tumours reported in the literature and deletions and insertions accounting for only 13% (Greenblatt et al., 1994).

The amino terminus of the p53 protein is highly acidic with a net charge similar to other known transcriptional transactivators, whereas the carboxy terminus is rich in basic amino acids similar to those found on DNA binding domains (Zambetti and Levine, 1993). In addition, there are three nuclear localization signals located in the carboxy terminus of the p53 protein (Shaulsky et al., 1990). Other functional domains that have
been identified include a sequence-specific DNA binding domain located in the middle of p53 (amino acid residues 100-300), an oligomerization domain allowing the p53 molecule to form stable tetramers (amino acid residues 320-360), and a nonspecific nucleic acid binding region (amino acid residues 330-393) (Prives, 1994).

Full length p53 exists as a tetramer in solution and p53 only functions effectively as a tumour suppressor when it is in this form (Pellegata et al., 1995). Evidence supporting the importance of this tetrameric structure includes the finding that the DNA binding site for p53 is made of two palindromic repeat elements that could accommodate a tetramer (El-Deiry et al, 1992; Pellegata et al., 1995). More recently it was found that oligomerization domain-deficient p53 proteins were unable to inhibit the growth of tumour derived cell lines, though it did maintain some of its ability for transcriptional activation (Pellegata et al., 1995). In addition to forming dimeric and tetrameric complexes with itself, p53 is also able to bind a wide variety of other molecules. Among viral proteins, p53 can bind to the SV40 large T antigen (Linzer and Levine, 1979), the human papilloma virus E6 protein (Sarnow et al., 1982), and the adenovirus E1b (Werness et al., 1990). It has been speculated that the ability of these viral proteins to bind and inactivate p53 may be a mechanism by which these viruses can transform cells (Lee et al., 1994). p53 can also bind to endogenous proteins including MDM2 and the heat shock protein hsp 70 (Pinhasi-Kimhi et al., 1986). Recently a new protein with an unidentified cellular role, called 53BP2 was identified to bind to p53. Interestingly when six different p53 molecules with common mutations were examined, none of them retained the ability to complex with this protein. The significance of this finding remains unclear (Gorina and Pavletich, 1996). Another protein called p300 has also been shown to form a complex with p53. This protein normally stimulates the transcriptional activity of p53, however a dominant negative form of p300 prevents transcriptional activation by p53 and counteracts p53 mediated G1 arrest and apoptosis (Avantaggiati et al., 1997).
A novel gene called p73 encoding a protein that shares considerable homology with p53 was recently discovered by Kaghad and his colleagues (Kaghad, et.al., 1997). This gene which is located on chromosome 1p36, a region found to be frequently deleted in neuroblastomas and other tumours encodes two distinct polypeptides (Kaghad, et.al., 1997). Based on its similar homology to p53, it has been suggested that p73 is a sequence specific transactivator which probably requires oligomerization and regulates genes that at least partially overlap those targeted by p53 (Oren, 1997). Similar to p53, p73 has been shown to activate p21, and induce apoptosis, however unlike p53, it is not stabilized nor activated by DNA damage (Kaghad, et. al., 1997, Jost et. al., 1997). Mutation rates of this gene in many tumours is yet to be determined (Oren, 1997).

**iii) Immunohistochemistry of p53**

Wild-type p53 protein has a half life of approximately thirty minutes (Gronostajski et al., 1984) and is normally not detectable with antibodies. However this short half-life is often significantly increased in the presence of mutations, or when p53 is bound to viral or cellular proteins. In addition the half-life of p53 can be prolonged in response to DNA damage within the cell. The increased stability of many of the mutant forms of p53 allow it to be detected by antibodies (Bruner et al., 1991). Many different antibodies that identify the p53 protein have been generated. Some are specific for mutant p53 and others recognize both wild-type and mutant forms of the protein. D0-7 is a murine monoclonal antibody that recognizes the amino terminus of both wild-type and mutant p53 (Novocastra) (Bartek et. al., 1993). A second commonly used antibody is PAb 1801 which also is a murine monoclonal antibody that is specific for human p53 and recognizes wild-type and most mutant forms of the protein (Novocastra) (Banks et. al., 1986). Investigation of p53 protein alterations using immunohistochemical techniques is fraught with difficulty. Many investigators have attempted to use p53 immunoreactivity
as a prognostic indicator in a wide variety of malignancies, however there have been large discrepancies between studies (Pietilainen et al., 1995; Horne et al., 1996; Lahoti et al., 1996). To date, there has not been any standardization of the methods or interpretation of p53 immunostaining and it has been shown that the method of fixation affects the stability of the p53 protein (Fisher et al., 1993). Furthermore, since many studies employ antibodies that recognize both wild-type and mutant forms of the protein, it is likely that some tissue specimens are being misinterpreted as mutant when they are actually wild-type. This may account, in part, for the lack of correlation between immunohistochemical studies and genetic studies examining for p53 protein and gene alterations respectively (Macgeoch et al., 1993).

iv) Function of p53

In order to investigate the function of p53 in vivo, mice have been created which are deficient for the p53 gene (Donehower et al., 1992). As well, transgenic mice with extra copies of mutated forms of the p53 gene have also been designed (Lavigneur and Bernstein, 1991). Both the transgenic and p53 deficient mice are fertile and demonstrate virtually normal development, however, they both develop a spectrum of malignancies at an early age. The type of tumours that occur and the timing of their appearance in part depends on the specific model, with the “knockout” mice developing primarily lymphomas and sarcomas at four months of age, and the transgenic mice developing lung adenocarcinomas, lymphomas, osteosarcomas, and other sarcomas and adenomas by 12 months of age (Lee et al., 1994). Possible explanations for the discrepancies seen between the two types of mice include differences in genetic background as well as the possibility that the presence of wild-type p53 in the transgenic mice may diminish the effect of the mutant p53 (Lee et al., 1994). As might be predicted, mice that are null for p53 have also been found to be more prone to develop tumours when exposed to
cancer than those that are wild-type for the gene (Harvey et al., 1993). A human syndrome, somewhat analogous to the mouse models is the Li-Fraumeni Syndrome which is a rare familial cancer syndrome that is characterized by a predisposition to early onset of a wide variety of tumours. Family members afflicted with this syndrome develop osteosarcomas, brain tumours, leukemias, adrenocortical carcinomas, soft tissue sarcomas and breast cancer (Li and Fraumeni, 1969; Li, et. al., 1988; Malkin, 1994). It has been determined that many of these families carry a germline mutation of p53 which is speculated to be responsible for the tendency of these family members to develop cancer (Malkin et al., 1990).

Wild-type p53 protein is normally found in the nucleus, which is assumed to be its primary site of function (Zambetti and Levine, 1993). Aside from the finding that p53 is the most commonly altered gene in human cancer (Zambetti and Levine, 1993), substantial functional evidence exists in support of p53’s ability to act as a tumor suppressor. Wild-type p53 has been shown to decrease the ability of the myc and ras oncogenes to cooperate in transforming cultured primary rat embryo fibroblasts (Finlay et al., 1989; Eliyahu et al., 1989). In addition, experiments using a temperature sensitive mutant demonstrated that at 32°C, when the wild-type p53 was expressed, cell growth arrest was induced. However, at 39°C, when the mutated form of the protein was present, growth arrest no longer occurred (Michalovitz et al., 1990). Furthermore, introduction of wild-type p53 into a human osteogenic cell line that does not normally express the p53 protein, reverses its malignant phenotype (Chen et al., 1990).

\(v\) Mechanisms of p53 action

p53, which has been called the “guardian of the genome” (Lane, 1992), acts as a tumour suppressor via several mechanisms. Wild-type p53 protein is able to act as a cell cycle regulator by blocking cells in the G1 phase of the cycle. Accumulation of p53
protein in the nucleus has been demonstrated in response to ionizing radiation and this is associated with G1 arrest (Lu and Lane, 1993). Furthermore, cells which lack p53 are unable to arrest at G1 in response to radiation (Lee et al., 1994). In addition, it has been shown that cell lines from patients with ataxia-telangiectasia, a rare human autosomal recessive disorder characterized by cancer predisposition and hypersensitivity to ionizing radiation, are unable to respond to radiation exposure by accumulating p53 (Kastan et al., 1992; Khanna and Lavin, 1993; Canman et al., 1994). p53 is capable of inducing this cell growth arrest by transactivating p21(CIP1/WAF1), which is a cell cycle control protein involved in G1/S progression (El-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993). Another mechanism that p53 may use in causing cell cycle arrest at the G1/S phase is through its ability to down regulate transcription of the Rb gene (Shiio et al., 1992).

When a cell has been arrested in G1 in response to DNA damage, it can either repair its DNA or undergo apoptosis (programmed cell death). It is speculated that p53 plays a role in both of these processes. As with its role in preventing progression into S phase, p53’s ability to induce DNA repair is related to its ability to act as a transcription factor (Farmer et al., 1992). It has been shown that p53 can bind to a promoter sequence of the growth arrest and DNA damage-inducible (GADD) family of genes (Kastan et al., 1992). This family of genes, as its name implies, is thought to be involved in the initiation of repair to damaged DNA (Kastan et al., 1992). There is also evidence that p53 has intrinsic 3’-to-5’ exonuclease activity, located in its central DNA binding domain, which may also be involved in DNA repair, although the mechanism for this activity remains uncertain (Mummenbrauer et al., 1996).

When a cell in a multicellular organism has undergone extensive DNA damage, cellular apoptosis may be the most efficient solution for the organism to prevent perpetuation of the error. The pathways leading to apoptosis are complex and not yet
fully understood. p53 clearly plays a role in some but not all apoptotic pathways. Evidence exists that p53 is involved in the apoptotic processes of skin (Ziegler et al., 1994), and gastrointestinal tract (Clarke et al., 1994). However, in the case of thymocytes, apoptosis has been shown to have both a p53 dependent, as well as a p53 independent pathway (Clarke et al., 1993). Similarly, adenovirus E1A proteins have also been shown to induce apoptosis by both p53 dependent, and p53 independent mechanisms (Teodoro et al., 1995). The manner in which p53 induces apoptosis remains controversial. It has been speculated that p53 might activate the transcription of bax (a gene known to cause apoptosis), or repress the transcription of bcl-2 (a gene known to inhibit apoptosis) (White, 1996). Evidence in support of this hypothesis comes from experiments in which p53 has been found to induce bax expression in promoter-reporter assays in cultured cells (Miyashita and Reed, 1995). In addition, it has been found that cells from p53 deficient mice have decreased bax expression and increased bcl-2 expression (Miyashita et al., 1994).

In addition to its activity at the G1/S junction of the cell cycle, p53 is also capable of acting at the G2/M checkpoint. Evidence in support of this role is provided in experiments in which it was shown that fibroblasts which are null for p53 do not exhibit delay of entry into G2 after exposure to radiation (Paules et. al., 1995). Further evidence indicating that p53 has a role in progression at the G2/M includes the finding that p53 deficient murine fibroblasts exposed to spindle inhibitors formed tetraploid cells (Cross, et. al., 1995) and the demonstration that G2/M cyclin dependent kinases phosphorylate p53 and stimulate its binding to p21 and GADD45 (Wang and Prives, 1995).

vi) Mechanisms of p53 inactivation

Several mechanisms lead to the inactivation of p53 at either the gene or protein level. The most common form of inactivation that has been detected in a wide variety of
different malignancies is mutation of the gene (Zambetti and Levine, 1993; Louis, 1994; and Greenblatt et al., 1994). Most of the mutations are point mutations (transitions) and occur in one of five frequently identified “hotspots.” Codons 175, 245, 248, 249, and 273 are most frequently mutated. Nonsense mutations, insertions, and deletions have also been reported (Greenblatt et al., 1994). A mutation in one allele of the p53 gene is usually accompanied by loss of a portion of chromosome 17p that contains the second copy of the gene (Louis, 1994). Even if the second allele is not altered, the wild-type p53 protein that is produced may be inactivated by forming a complex with the altered version of the protein derived from the mutated allele in a process referred to as the “dominant negative” effect (Milner and Medcalf, 1991). As previously noted, several viral oncogenic proteins have the ability to bind to the p53 protein which results in its inactivation (Linzer and Levine, 1979; Sarnow et al., 1982; Werness et al., 1990). In addition, wild-type p53 function can be negatively regulated by the product of the murine double minute 2 (mdm-2) gene (Momand et al., 1992). It is now speculated that this gene can function as an oncogene by inhibiting the action of p53 (Momand et al., 1992; He et al., 1994). The ability of mdm-2 to bind and inactivate p53 is demonstrated in experiments in which expression of a p53 responsive chloramphenicol acetyltransferase (CAT) reporter gene, in cells known to contain wild-type p53, is inhibited by the transfection of a cosmid expressing mdm-2 (Momand et al., 1992). Observations suggestive of mdm-2’s ability to inactivate wild-type p53 was presented in a study examining 157 primary brain tumours where it was found that mdm-2 was overexpressed in 10% of the samples that were wild-type for the p53 gene (Reifenberger et al., 1993). Interestingly mice that are null for the mdm-2 gene are not viable unless they are also null for p53 (Montes de Oca Luna et al., 1995; Jones et al., 1995). A possible explanation for this finding is that p53 causes wide spread and uncontrolled apoptosis when the regulatory effect of mdm-2 on p53 is absent (Montes de Oca Luna et al., 1995; Jones et
Another mechanism for p53 inactivation is thought to be its exclusion from the nucleus where it normally functions in the cell. The mechanism for this exclusion remains unclear, however support of this theory is found in inflammatory breast carcinoma in which accumulation of wild-type p53 has been found in the cytoplasm (Moll et al., 1992).

**vii) p53 and primary brain tumours**

Brain tumours have been examined for p53 status by many different investigators. In a large review of the prevalence of p53 mutations in a wide variety of tumours, Greenblatt et al. found 456 brain tumours that had an overall 25% prevalence for these mutations, with the most common "hotspots" in this group being codons 178 and 248 (Greenblatt et al., 1994). Unfortunately the specific studies from which these data were generated, as well as the exact pathology of the tumours was not specified in this review. In another review, a cohort of 68 astrocytic brain tumours from seven different investigators were found to have p53 mutations, representing a prevalence of approximately 33% (Louis, 1994). Of these mutations, 80% were missense, 17% were nonsense or frameshift and 3% were intronic. Overall 90% of these mutations were point mutations, with 85% of these being transitions and 15% transversions (Louis, 1994). Considerably fewer studies have examined oligodendrogliomas for p53 mutations. One study using SSCP/sequencing analysis detected a mutation rate of 12% (2/17), interestingly both of these mutations were found in low grade varieties of this tumour (Ohgaki et al., 1991). Evidence suggests that mutations of p53 are an early event in the pathogenesis of GBMs. In one study genetic analysis of low grade astrocytomases revealed a subpopulation of cells that contained a p53 mutation, when the tumours recurred as GBMs they were reexamined and were found to have the same p53 mutation predominant in the malignant tumour (Sidransky et al., 1992). In another study Bogler
and his colleagues found that the loss of wild-type p53 gene gave a growth advantage and facilitated the transformation of primary cortical astrocytes (Bogler et al., 1995). Furthermore, others have found equal frequencies of p53 mutations in astrocytomas and GBM (Louis et al., 1993; Chung et al., 1991).

Analysis of p53 protein alterations in primary brain tumours using immunohistochemical techniques are much more difficult to interpret, and not surprisingly show a greater discrepancy in their results. Overall these studies, many of which were reviewed by Louis (Louis, 1994) show increasing p53 protein alterations as the grade of the tumour increases, with approximately 15-40% of astrocytomas, 35-60% of anaplastic astrocytomas, and 45-70% of GBMs harbouring alterations. One study examining p53 protein alterations in oligodendrogliomas using immunohistochemistry found that 75% of these tumours had evidence that at least some of the cells contained abnormal p53 protein (Kros et al., 1993). The results found in any one study are dependent on the type of antibody or antibodies used, the manner in which the samples were processed, and the methods of interpretation of staining patterns. Furthermore, in some studies that examined the relationship between p53 mutations and p53 protein alterations detected using immunohistochemistry no correlation between the two was found (Louis et al., 1993). Yet in other studies using similar techniques a correlation between p53 mutations and p53 protein alteration was found (van Meyel et al., 1994).

Whether p53 status at the gene or protein level is independently related to clinical outcome is another question that remains to be resolved. One study did not find p53 status to be of any prognostic significance (Rasheed et al., 1994), whereas another study found staining of more than 75% of cells, presumably indicating the presence of p53 protein alterations, was prognostic of a poorer outcome (Kros et al., 1993). An additional two unpublished studies cited by Louis also showed conflicting results with respect to the capability of immunohistochemical analysis of p53 to independently predict clinical
outcome (Louis, 1994). In yet another study, patients with p53 mutations or p53 protein alterations were found to survive almost twice as long as those without mutations (van Meyel et al., 1994).

**The p16\(^{INK4a}\) tumour suppressor gene**

*i) Location and structure*

p16\(^{INK4a}\), the first CDKI of its class discovered, was first observed as a CDK4 associated protein in human cells (Serrano et al., 1993). It was subsequently cloned and characterized as a specific inhibitor of the CDK4 and CDK6 molecules (Hannon and Beach, 1994; Serrano et al., 1996). The p16\(^{INK4a}\) protein has 156 amino acid residues and contains four ankyrin repeats of approximately 32 amino acids (Hannon and Beach, 1994; Okamoto et al., 1994). The gene coding for this protein is located at chromosome 9p21, and consists of three exons: exon 1 containing 126 base pairs (bp), exon 2 containing 307 bp, and exon 3 containing only 11 bp (Kamb et al., 1994). Interestingly, a second promoter exits for the p16\(^{INK4a}\) gene, located approximately 10-20 kilobases (kb) upstream of the originally identified exon 1, which enables an alternative first exon, called exon 1\(^{\beta}\), to be spliced into exon 2 of p16\(^{INK4a}\) (Mao et al., 1995; Stone et al., 1995).

A second, and closely related CDKI called p15\(^{INK4b}\) was identified by Hannon and Beach in 1994 (Hannon and Beach, 1994). This protein, comprised of 137 amino acids, is very similar to p16\(^{INK4a}\), with 44% homology in the first fifty amino acids and 97% homology in the subsequent 81 amino acid residues (Hannon and Beach, 1994). As is the case with p16\(^{INK4a}\), p15\(^{INK4b}\) contains four ankyrin repeats. Furthermore, it is located on the same locus as p16\(^{INK4a}\), approximately 30 kb upstream (Kamb et al., 1994). Though both p16\(^{INK4a}\) and p15\(^{INK4b}\) are able to bind CDK4 and CDK6 and inhibit its function, p15\(^{INK4b}\) is unique in that it can be induced up to thirty fold by TGF\(\beta\) (Hannon and Beach, 1994).
**ii) p16\[^{INK4a}\] as a tumour suppressor**

The 9p21 locus for the p16\[^{INK4a}\] and p15\[^{INK4b}\] genes is the site of chromosomal abnormalities found in a wide variety of malignancies including melanomas, gliomas, and leukemias (Kamb et al., 1994). For this reason, it has been speculated that this site likely contains at least one tumour suppressor gene (Shapiro and Rollins, 1996). The expectation that p16\[^{INK4a}\] is that gene was partially substantiated when it was found that cell lines derived from a large number of different tumours, including brain, bladder, lung and breast tumours harbour deletions of the gene in as many as 82% of the specimens examined (Kamb et al., 1994). Furthermore, germline mutations were found in exon 2 of the p16\[^{INK4a}\] gene in several families with a history of inherited familial melanoma (Hussussian et al., 1994; Liu et al., 1995; Grants et al., 1995). However, after an initial period of excitement, doubts were expressed about the significance of the high deletion rate of p16\[^{INK4a}\] in cell lines derived from tumours. The primary reason for concern involved the initial inability to find the same high mutation, or deletion rates in primary tumour specimens. This led to speculation that the high mutation rate observed in the cell culture samples may be the result of a laboratory artifact in producing these cell lines (Spruck et al., 1994; Cairns et al., 1994; Bonetta, 1994). Cell lines with alterations of the p16\[^{INK4a}\] gene would be easier to establish, and would have a growth advantage over those lines containing wild-type p16\[^{INK4a}\].

There are compelling reasons to suggest that p16\[^{INK4a}\] is a tumour suppressor gene of significance in the pathogenesis of at least a subset of malignancies. More recently, a number of studies have found high levels of p16\[^{INK4a}\] alterations in a variety of selected primary tumours including gliomas (Moulton et al., 1995), leukemias (Ohnishi et al., 1995), and carcinomas of the pancreas (Caladas et al., 1994). In addition, the frequency of p16\[^{INK4a}\] loss of function in Rb positive tumours is much higher than the frequency of other genetic abnormalities in these tumours (Shapiro and Rollins, 1996).
iii) Mechanisms of action

Investigations of p16\textsuperscript{INK4a} function in the cell have also helped make this gene an attractive candidate as a cell cycle regulator involved in the development of cancer. Proof that p16\textsuperscript{INK4a} ability to inhibit CDK4 and CDK6 is functionally significant was obtained when co-injection of plasmids encoding CDK4 and CDK6 was able to prevent p16\textsuperscript{INK4a} mediated growth arrest (Koh et al., 1995). It is speculated that the mechanism of reversal of arrest is through binding and titration of the p16\textsuperscript{INK4a} protein by the large amount of CDK4 and CDK6 produced by the plasmids (Koh et al., 1995). Furthermore, evidence that p16\textsuperscript{INK4a} exerts its ability to inhibit cell cycle progression through the Rb pathway comes from experiments demonstrating p16\textsuperscript{INK4a} inability to cause cell cycle arrest in cells that do not contain the Rb protein (Lukas et al., 1995; Medema et al., 1995). Consistent with this finding is the inverse correlation observed between the expression of wild-type pRb and p16\textsuperscript{INK4a} in many human tumour cell lines (Okamoto et al., 1994; Tam et al., 1994; Medema et al., 1995). Recently, mice that are homozygously null for the p16\textsuperscript{INK4a} gene were generated (Serrano et al., 1996). Although these mice are viable, they develop spontaneous tumours at an early age, and are much more sensitive to carcinogenic treatments than p16\textsuperscript{INK4a} wild-type controls (Serrano et al., 1996).
iv) \( p16^{INK4a} \) in primary brain tumours

The reported incidence of \( p16^{INK4a} \) alterations in primary brain tumours varies depending on which group of tumours is examined and the techniques employed to detect the alterations. Moulton et al. used Southern blot analysis to examine 27 glioblastomas and found a 33% occurrence of homozygous deletions, in the same study they were unable to detect any deletions in the 12 low grade cerebral gliomas (Moulton et al., 1995). Jen et al. used PCR analysis on brain tumour xenografts established in nude mice and found that 26/38 glioblastomas had deletions of the \( p16^{INK4a} \) gene, however they were unable to find any point mutations involving this gene in their samples (Jen et al., 1994). Ueki and his team found deletions in 24/48 glioblastomas, and 2/8 anaplastic astrocytomas using a comparative multiplex PCR technique (Ueki et al., 1996). Li et al. examined 46 gliomas for mutations of both the \( p16^{INK4a} \) and \( p15^{INK4B} \) genes using a direct sequencing technique and found only one mutation of the \( p16^{INK4a} \) gene (Li et al., 1995). In an interesting study by Schmidt et al., 41% of glioblastomas examined using Southern analysis were found to have deletions of the \( p16^{INK4a} \) gene (Schmidt et al., 1994). This group then examined the same cohort of tumours and found that 50% of the tumours with normal \( p16^{INK4a} \) had amplification of the gene coding for CDK4. In a similar study examining glioma cell lines it was concluded by He and his colleagues that CDK4 amplification is an alternate mechanism to \( p16^{INK4a} \) gene deletion (He et al., 1994). It has been suggested that abnormal hypermethylation of the 5' end of the \( p16^{INK4a} \) gene may be an alternative mechanism to cause the functional inactivation of this gene in gliomas with \( p16^{INK4a} \) genes present (Fueyo et al., 1996).

Further evidence of the functional role of \( p16^{INK4a} \) as a critical factor in the development of primary brain tumours is provided by experiments in which this gene was introduced into malignant glioma cell lines using a viral vector. It was found that transfection of this gene into malignant cell lines which were known to have deletions of
the \(p16^{\text{INK4a}}\) gene caused marked growth inhibition of these cells. However, when the same vector containing the \(p16^{\text{INK4a}}\) gene was transfected into malignant cells that already had wild-type \(p16^{\text{INK4a}}\) no change in cellular morphology was observed (Arap, 1995; Fueyo et al., 1996).
**Hypothesis and experimental purpose**

The central hypothesis of this presentation is that the cell cycle regulators p53 and p16\[^{INK4a}\] play an instrumental role in the pathogenesis of primary brain tumours. If this hypothesis were correct, it would be expected that a large percentage of these tumours would exhibit alterations of at least one of these genes. Furthermore, it is hypothesized that alteration of either one of these genes is sufficient to tip the balance in favour of uncontrolled cellular division and the development of malignancy. Therefore it would be expected that many tumours would contain alterations of either one or the other of these genes, with a smaller number of tumours harbouring alterations of both p53 and p16\[^{INK4a}\].

It is hoped that by examining genomic DNA for p53 mutations, total RNA for p53 expression levels, and proteins for altered forms of p53 (using immunochemistry) from the same tumour samples, a better understanding of the mechanisms controlling the production of p53 protein will be obtained. In addition, by studying the p53 and p16\[^{INK4a}\] mutation rates in the same cohort of tumours an improved appreciation of how these two genes interact will be acquired.
METHODS

Extraction of genomic DNA and total RNA from frozen tissue samples

Thirty-six primary brain tumours (snap frozen in liquid nitrogen at the time of surgery) were obtained from the brain tumour bank of Toronto located at the Toronto Hospital, Western Division. The specimens, which varied in size from 20 mg to 400 mg, were individually taken from liquid nitrogen and placed into Guanidinium thiocyanate (GTC) homogenization buffer (4.0 M guanidinium thiocyanate, 0.1 M Tris pH 7.5, 1% β-mercaptoethanol, 0.5% sodium lauryl sarcosinate) and homogenized using a Kinematica AG polytron 1200. 3.5 ml of the homogenized solution was carefully layered onto a 1.3 ml cushion of 5.7 M CsCl, 0.01 M EDTA. The specimens were placed into a SW55 swinging-bucket rotor and centrifuged for 14 hours in a Beckman L8-55 ultracentrifuge at a speed of 35,000 RPM.

Following centrifugation the upper layers of the GTC buffer solution were discarded and the remaining GTC buffer, down to the last 250 μl, was carefully removed and mixed with an equal volume of ddH₂O and six times the volume of ethanol. At this point the DNA precipitates out as a white fibrous aggregate and was removed with a clean pipette, placed in an Eppendorf tube and redisolved at room temperature in TE solution (10 mM Tris, 1 mM EDTA) pH 8.0. The amount of TE used was dependent on the approximate yield of DNA.

The small amount of residual GTC buffer remaining in the centrifuge tube was discarded with caution to avoid dislodging the RNA pellet which was washed with 70% ethanol and allowed to dry at room temperature. The dried RNA pellet was resuspended in water treated with diethyl pyrocarbonate (DEPC) and stored at -70°C.

The DNA and RNA samples extracted from each specimen were analyzed using optical densitometry to determine yield. The quality of these samples was determined by
calculating the 260 nm/280 nm ratio. Each sample was run on an a 1% agarose gel (the RNA was run on a formaldehyde agarose gel).

**Polymerase chain reaction amplification (PCR) of DNA samples for single strand conformation polymorphism (SSCP) analysis**

PCR amplification of exons 2 and 4-11 of the p53 gene was done using primers and conditions already well established for this purpose (Mashiyama S et al., 1991; Borresen al. et al., 1991). The sequence of the primer pairs used is as follows:

<table>
<thead>
<tr>
<th>EXON</th>
<th>CODONS AMPLIFIED</th>
<th>LENGTH (bp)</th>
<th>PRIMER SEQUENCE</th>
</tr>
</thead>
</table>
| exon 2 | 1-25             | 133         | 5'-CTTTTCCTCTTGAGCAGCC-3'  
|       |                   |             | 5'-CAATGGATCCACTCAGTTT-3' |
| exon 4 | 33-125           | 293         | 5'-ATCTACAGTCCCCCTGCGC-3'  
|       |                   |             | 5'-GCAACTGACGGTGCAAGTCA-3' |
| exon 5 | 126-187          | 239         | 5'-TTCCCTCTCTCAGTACCT-3'   
|       |                   |             | 5'-GCAACAGCGGCTGTCGCTC-3' |
| exon 6 | 187-225          | 236         | 5'-ACCATGAGGCCTGACAGAT-3'  
|       |                   |             | 5'-AGTTGCCAACAGACCTCAG-3' |
| exon 7 | 226-261          | 139         | 5'-GTTTTGCTCTCTAGGTTGCC-3'  
|       |                   |             | 5'-CAAGTGGCTCCTGACCTGGA-3' |
| exon 8 | 262-307          | 212         | 5'-CTATCCTGAGTGTGGTAA-3'   
|       |                   |             | 5'-TGAATCTCTGAGCATAACTGC-3' |
| exon 9 | 308-331          | 135         | 5'-TTATGCTCATAGATTCCTTT-3'  
|       |                   |             | 5'-CCAAGACTTATGTAAGTGAAG-3' |
| exon 10 | 332-367         | 218         | 5'-ACTTCTCCCCCTCTCGTT-3'   
|       |                   |             | 5'-AAGGCAAGTGGAAATGGGA-3' |
| exon 11 | 368-393         | 218         | 5'-TCTCTCATAGCCACCTGAAG-3'  
|       |                   |             | 5'-CTGACGCCACCTATTGCCA-3' |

Genomic DNA from the tumour specimens was diluted to a concentration of 50 ng/µl; 2.5 µl was added to the PCR reaction mixture which consisted of: 2.5 µl 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3), 4 µl dNTP (10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP), 0.5 µl of each primer for any given exon (250 ng/µl), 0.25 µl
AmpliTaq DNA polymerase (Perkin Elmer Cetus, 5 U/µl), 0.05 µl 32P-dCTP (3000 Ci/mmol), 1.5 µl MgCl2 (25 mM for final concentration of 1.5 mM), and 13.5 µl ddH2O for a final volume of 25 µl. PCR conditions were the same for all exons amplified and were as follows: 85°C for 15', denature at 94°C for 6', then cycle 32 times at 94°C for 45", 55°C for 45", 72°C for 45". The 32 cycles were followed by a final extension at 72°C for 7', then denaturation at 94°C for 3'. 2.5 µl of SSCP loading buffer (42.5 ml formamide, 5 ml of 0.5% bromophenol blue and xylene cyanol in formamide, 2 ml 0.5 M ethylenediamine tetraacetic acid (EDTA), and 0.5 ml H2O) were added to the final PCR product which was kept stored at 4°C.

All three of the exons making up the p16 gene were amplified by PCR using a modification of primers and conditions previously established (Okamoto et al., 1994). The sequence of the primer pairs used was as follows:

<table>
<thead>
<tr>
<th>EXON</th>
<th>CODONS AMPLIFIED</th>
<th>LENGTH (bp)</th>
<th>PRIMER SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>exon 1</td>
<td>1-50</td>
<td>249</td>
<td>5'-CGGAGAGGGGAGAGCAG-3' &lt;br/&gt; 5'-AACTTCGTCTCCAGAGTCG-3'</td>
</tr>
<tr>
<td>exon 2</td>
<td>51-151</td>
<td>461</td>
<td>5'-CTCTACCACAGCTTCTTCC-3' &lt;br/&gt; 5'-GGGCTGAACCTTCTGCTGG-3'</td>
</tr>
<tr>
<td>exon 3</td>
<td>152-155</td>
<td>136</td>
<td>5'-CCGGTAGGACGGCAAGAGA-3' &lt;br/&gt; 5'-TCCCGAGTTTCTCAGAGCC-3'</td>
</tr>
</tbody>
</table>

The PCR reaction mixture for p16 was as described above for p53, except that 10.4 µl of 12% dimethyl sulfoxide (DMSO) was substituted for ddH2O (resulting in a final concentration of 5% DMSO).

Several different PCR cycling conditions were attempted for the each of the three p16 exons. The following conditions were found to yield the best results. To amplify exon 1, denaturation at 94°C for 7', then 35 cycles starting with 94°C for 30", then annealing at 63°C for 30", and extension at 72°C for 1'. A final extension was permitted
to occur at 72°C for 7'. The conditions were the same for exons 2 and 3 except that the annealing temperatures were 55°C and 60°C, respectively.

As indicated above, the PCR product for exon 2 is 461 bp long. This is relatively long for the SSCP and sequencing techniques employed to analyze the specimens. Therefore, the PCR product of exon 2 was digested with the restriction endonuclease Smal, yielding two fragments of sizes 179 bp and 282 bp. This digestion was achieved using 20 units of Smal per PCR reaction tube and took place over 14 hours at 37°C. Following completion of the PCR, and digestion with Smal (for exon 2 only), 2.5 μl of SSCP loading buffer was added to the PCR product which was then stored at 4°C.

**Single strand conformation polymorphism analysis of DNA samples**

Each sample was examined using SSCP analysis under at least two different gel conditions. A standard 50 ml polyacrylamide gel, with a final concentration of 10% glycerol and 6% acrylamide, was made with 7.5 ml of 40% acrylamide (1:20 bis:acrylamide), 10 ml 50% glycerol, 10 ml 5X TBE (0.045 M Tris-borate, 0.001 M EDTA) and 22.5 ml ddH2O. 300 μl of 10% ammonium persulfate (APS) and 30 μl of Temed were then added to expedite polymerization. By varying the concentrations of glycerol and acrylamide, gels containing combinations between 2%-10% of glycerol and 4.5%-9% of acrylamide were made. All of the gels were run at room temperature (24°C), and the running conditions varied from 80 to 400 Watt hours depending on the gel conditions chosen and the size of the PCR product being analyzed. The buffer used to run the gels was 1X TBE. Prior to loading, each sample was heated to 85°C for 15 minutes, and 7 μl of each sample were run per lane. A wild-type control as well as a blank control (containing the PCR mixture but no DNA) was run on every gel. In addition, whenever available, a mutant control for the particular exon being examined was also run. The same technique was used for samples being screened for mutations of
the p53 as well as p16 gene. When the gel had completed its run, it was removed, picked up on 3MM Whatman paper and dried at 80°C for one hour under vacuum. An autoradiograph was made of each gel, the exposure time varied from 4 hours to one week depending on the intensity of the label. The autoradiographs were reviewed by at least two members of the laboratory familiar with the SSCP analysis technique to determine whether or not any band shifts were present.

**Sequencing of selected DNA samples**

If a sample were found to have a band shift on any of the gel conditions using SSCP analysis it was reamplified using PCR. The PCR reaction mixture consisted of 10 μl 10X PCR buffer, 20 μl dNTP mix, 2 μl of each primer of the set (same set of primers as described above), 1.0 μl of AmpliTaq, 55 μl ddH2O. 10 μl of the DNA from the sample of interest was added to this mixture to yield a final volume of 100 μl. The PCR cycling conditions were as follows: 85°C for 15' denaturing, then 35 cycles of 94°C for 1', 55°C for 1', 72°C for 2', followed by a final extension at 72°C for 7'. Once the PCR was completed 12 μl of 10X TAE loading buffer (10X TAE, 0.05% bromophenol blue, 0.05% xylene cyanol, 50% glycerol) was added to the product which was then stored at 4°C.

Purification of the PCR product was achieved by loading the entire sample into a 1% agarose gel containing approximately 0.2 μg/μl ethidium bromide and running it for 30 minutes at 100 V. A 100 bp ladder (Pharmacia Biotech) was also run simultaneously on the same gel. A photograph was taken of the gel and the band of appropriate size for the amplified exon was excised. The DNA was extracted from the gel using a Qiaex II extraction kit (Qiagen catalog #20021). Briefly, the excised piece of gel was solubilized and the DNA was adsorbed to silica-gel particles in the presence of high salt. The silica-gel particles were then washed with a high salt buffer as well as with a buffer containing
ethanol to remove all contaminants left by the agarose gel. Elution of the DNA was accomplished (using a basic solution, with a low salt concentration) in 10 mM Tris-HCl, pH 8.5.

Ligation of the purified DNA fragment into vector derived from pBluescript SK phagemid was carried out overnight at 12°C. This was done using 1 µl T4 DNA ligase (Pharmacia Biotech), 1 µl 10X One-Phor-All buffer (Pharmacia Biotech) (100 mM Tris-acetate, 100 mM magnesium acetate and 500 mM potassium acetate) 2 µl T-tailed vector prepared according to a protocol described by Collins (Marchuk, et al., 1991), 1 µl of Qiaex purified DNA, and 5 µl ddH2O. The final product was stored at -20°C until required for transformation.

Transformation of ligated vector was done into E. coli cell line XL-1 Blue. These cells were rendered competent according to the protocol described in Maniatis (protocol II p.1.79) (Sambrook et al., 1989) and stored frozen at -70°C. Transformation was accomplished by thawing a 100 µl aliquot of competent cells slowly on ice and then adding 1.7 µl of 1.4 M β-mercaptoethanol. Subsequently 2 µl of the ligated DNA product was added to the competent cells, the solution was gently shaken and left on ice for 30 minutes. Following this 30 minute period the cells were heat shocked for 45 seconds at 42°C, then rapidly transferred to ice for 2 minutes. 900 µl of SOC medium (2% w/v bacto-tryptone, 0.5% bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl), and were spread with x-gal and isopropylthio-b-D galactoside (IPTG). The agar plates were incubated overnight at 37°C.

After the incubation period the agar plates were examined for colonies. Those colonies that were white in colour, indicating that they contained a clone of bacteria containing a vector that had been successfully ligated were selected and transferred into a Falcon 2059 tube containing 4 ml of Luria-Bertani (LB) medium containing the same antibiotics that were included in the agar plates (one colony per tube). Between 9 and 18
colonies were selected for every sample that was to be sequenced. The cultures were incubated overnight at 37°C while shaking at 325 rpm.

The following day the cultures were centrifuged for 15 minutes at 5000 rpm at 4°C. Processing through the “mini prep” protocol as previously described (Sambrook, et al.; 1989), continued as follows: The supernatent was aspirated and the pellet was resuspended in 600 μl of STE (0.1 M NaCl, 10 mM Tris HCl pH 8.0, 1 mM EDTA pH 8.0) and transferred to a 1.5 ml microfuge tube. The sample was centrifuged at 14,000 rpm at 4°C for 10 minutes and the supernatant was aspirated. The pellet was resuspended in 150 μl of "Solution I" (50 mM glucose, 25 mM Tris HCl pH 8.0, 10 mM EDTA pH 8.0). 300 μl of "Solution II" (0.2 N NaOH, 1% SDS) was added to the mixture containing "Solution I" and the contents were mixed by inverting the microfuge tube and then left on ice for 5 minutes. 150 μl of "Solution III" (3 M Potassium, 5 M acetate) was then added to neutralize the mixture which was left for an additional 5 minutes on ice before being centrifuged for 10 minutes at 14,000 rpm and at 4°C. The supernatant was transferred to a clean 1.5 ml microfuge tube and 1 ml of isopropanol was added. The tube was stored at -20°C for at least 30 minutes to precipitate the plasmid DNA. Following precipitation with isopropanol, the microfuge tube was centrifuged at 14,000 rpm for 20 minutes and at 4°C, the supernatant was aspirated, and the pellet was washed with 500 μl of 70% ethanol. The microfuge tube was centrifuged at 14,000 rpm for 10 minutes at 4°C and the supernatant was aspirated. The pellet was air dried for 10 minutes and resuspended in 125 μl of TE (pH 8.0), with RNAse A added to yield a final concentration of 20 μg/μl.

To ensure that the miniprep plasmid contained the appropriate DNA insert, a 5 μl aliquote of miniprep DNA was digested using the restriction enzymes EcoRI and Xhol and the buffer solution was One-Phor-All. The digestion was allowed to occur at 37°C over one hour, and the product was then run on a 1% agarose gel containing ethidium
bromide alongside a 100 base-pair ladder. A photograph of the gel was taken and only minipreps from those clones containing an insert of the appropriate size were sequenced.

Sequencing was done using the chain-termination technique with the Sequenase Version 2.0 DNA Sequencing Kit from United States Biochemicals (USB). Briefly, the selected miniprep DNA was denatured using NaOH and then neutralized with sodium acetate. The DNA was then precipitated using 100% ethanol at -80°C and the pellet was washed with 70% ethanol and then air dried. Annealing of primer to template was done by adding 7 μl of ddH2O to the dried DNA pellet as well as 1 μl of either "universal" primer M13 (from USB sequence 5'-GTAAAACGACGGCCAGT-3') or "reverse" primer M13 (from USB sequence 5'-dTTCACACAGAACAG-3') and 2 μl of 5X sequenase buffer (200 mM Tris HCl pH 7.5, 100 mM MgCl2, 250 mM NaCl). The annealing process took place at 37°C over 30 minutes. Labeling reaction involved adding 1 μl 0.1 mM DTT, 2 μl "labeling mix" (7.5 mM dGTP, 7.5 mM dCTP, 7.5 mM dTTP) diluted 1:5, 1μl [α-35S]dATP, and 2 μl sequenase dilution (1:8 Sequenase Version 2.0 T7 DNA Polymerase, 1:16 inorganic pyrophosphatase, in enzyme dilution buffer consisting of 10 mM Tris HCl pH 7.5, 5 mM DTT, 0.5 mg/ml BSA). The labeling reaction took place over 5 minutes at 19°C. Following this 5 minute labeling incubation, termination was induced by transferring 3.5 μl of the labeling reaction to each of 4 different 0.5 ml tubes containing 2.5 μl of one of the four termination solutions. These termination solutions each consist of all 4 nucleoside triphosphates plus one of 4 particular dideoxy-nucleoside. The termination reaction took place over 5 minutes at 42°C, after which 4 μl of SSCP loading buffer was added to the solution. The solution was then stored at 4°C until it was loaded onto a sequencing gel.

The urea containing gels for sequencing consisted of 6% final concentration of polyacrylamide, and 21 g of urea in a total volume of 50 ml of 1X TBE. Polymerization of the gels was catalyzed with Temed and ammonium persulfate. Prior to loading the gels
they were heated by running at 60 watts for 1/2 hour. The gels were run in 1X TBE buffer. 3.5 µl from each of the sequencing reactions was loaded on each lane of the gel and the gel was run at 60 W for between 2 and 5 hours depending on the size of the insert that was being sequenced. The gels were dried and film exposed and developed in the same manner as for SSCP gels.

Every tumour sample that was sequenced had to have at least six different clones sequenced successfully, and was considered to harbour a mutation only if three or more of these clones harboured the same base pair changes. If there were any uncertainty about reading a sequence, the same sample was resequenced using "reverse" primer.

**Quantitative reverse transcriptase PCR analysis of RNA samples**

Quantitative reverse transcriptase PCR was used to determine the RNA expression levels for the p53 gene. This technique, which involves making a cDNA copy from an RNA template and then amplifying a specific portion of this cDNA, requires significantly less RNA than Northern blot analysis (Ozcelik, et al., 1995) and was thus preferred given the small quantity of the specimens available.

The cDNA template was made by adding 100 ng of total RNA (1.45 µl of total RNA of 69 ng/µl concentration) to 0.8 µl 5X MMLV (Moloney Murine Leukemia Virus) reverse transcriptase buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl₂, Gibco BRL cat. No. 18057-018), 0.4 ml 0.1M DTT, 0.8 ml 10 mM dNTP, 0.4 ml pd(N)₆ a hexanucleotide "random" primer (50 ng/µl, Boehringer Mannhem cat. No. 1 034 731), as well as 0.05 µl RNasin ribonuclease inhibitor (40 u/µl, Promega), and 0.1 µl MMLV reverse transcriptase (200 u/µl, Gibco BRL cat. No. 28025-013). This solution was incubated at 37°C for one hour after which the DNA/RNA complex was denatured at 94°C for 5 minutes. The newly made cDNA was stored at -20°C until required for PCR amplification.
In quantitative reverse transcriptase PCR each sample of cDNA is amplified for both the gene of interest as well as for a second "housekeeping" gene which acts as an internal control. In addition each tumour specimen was amplified three times under the same conditions, but for varying number of cycles. Theoretically, the amount of product should double with every increase of two in the number of cycles. The primers used in quantitative reverse transcriptase PCR should ideally amplify a product that spans an intron to insure that only cDNA is being amplified and not any residual contaminant DNA. The primer set used to detect p53 had one primer located on exon 10 (5'-AACTCAAGGATGCCCAGGCT-3') and the other on exon 11 (5'-GTCTGAGTCAGGCCCTTCTG-3') and was expected to yield a product of 135 base-pairs in length. The primers used for phosphoglycerate kinase (PGK, 5'-CAGTTTGAGCTCCTGGAAG-3' and 5'-TGCAAAATCCAGGCTGCAGTG-3'), the "housekeeping" gene, was expected to yield a product 247 base-pairs in size.

PCR amplification of cDNA was accomplished by adding 1.2 μl 10X PCR buffer, 0.54 μl 10 mM dNTP, 0.5 μl of each primer (30 mM) for both p53 and PGK, 2 μl 1.4 mM MgCl₂, 0.2 ml Taq Polymerase (Cetus), and 2.06 μl ddH₂O to 4 μl of cDNA made as described above. The PCR solution was heated to 95°C for 4 minutes and then cycled at 94°C for 30", 55°C for 30", and 72°C for 60". Separate samples derived from each tumour were cycled for 24, 26, 28, and sometimes 30 cycles while maintaining all the other PCR parameters constant. Following PCR amplification the samples were stored at 4°C until they were loaded on an acrylamide gel prepared by combining 12.5 ml of 40% polyacrylamide (29:1 acrylamide:bis acrylamide), 10 ml of 5X TBE, and 27.5 ml ddH₂O as well as 300 μl APS and 30 μl temed. 2 μl of SSCP loading buffer was added to each PCR product to make a final volume of 14 μl and 12 μl from each sample was loaded in each lane of the vertical gel. Samples derived from the same tumour but cycled for different number of cycles were run next to one another for easy comparison. Positive
controls obtained from total RNA derived from brain tumour cell line U87 known to be wild-type p53 were included in each group of samples analyzed. In addition, negative controls containing all of the PCR components except for the cDNA, as well as a second negative control in which no RNA, but all of the other components of the reverse transcriptase reaction along with the PCR components were run. The 50 ml acrylamide gel was 1mm thick and was run on a vertical gel kit using 1X TBE as running buffer. The gel was run for approximately 15 hours at 30 V.

When the gel had completed its run it was stained with ethidium bromide and then photographed using Polaroid type 55 positive/negative film. Optical densitometry was used to analyze the negative. A ratio of the band representing p53 over the intensity of the band representing the housekeeping gene was taken for each sample.

**Multiplex-PCR**

Multiplex-PCR involves the simultaneous PCR amplification of two or more different DNA fragments. Aside from the exclusion of radioactive isotope, the PCR reaction mixture used in this technique contains the same reagents at the same concentrations as that described above for PCR amplification for p16. However, two sets of primers are used instead of using one set as is done with standard PCR. The primer sets used were for exon 4 of p53 and for exon 2 of p16, the sequences of which are detailed above. The PCR cycling conditions were as follows: denature at 94°C for 9 minutes; then 23 or 25 cycles at 94°C for 30", 59°C for 30" and 72°C for 1 minute; followed by an extension at 72°C for 7 minutes. Each specimen had two reaction mixtures prepared, one was cycled for 23 cycles and the other for 25 cycles. Each set of reactions was done with a control using wild-type genomic DNA. In addition, a set of controls using various dilutions of genomic DNA (extracted from MCF-10F cell line derived from breast tissue) known to contain deletion of the p16 gene (Brenner and
Aldaz, 1995) with wild-type genomic gene were also amplified. Subsequent to PCR amplification, the samples were run on a 1% agarose gel containing ethidium bromide for approximately 30 minutes at 100 volts, and then photographed. The intensity of the amplified products was quantified using optical densitometry.

**Immunohistochemical analysis of samples**

Specimens from each tumour investigated were fixed in 10% formalin, embedded in paraffin, and sectioned. Multiple sections were made from each specimen and immunostaining for p53 was done with an indirect immunoperoxidase method and enhanced by an antigen retrieval technique (Lahoti, 1996). Two monoclonal antibodies (D0-7 and 1801) which recognize amino-terminal epitopes of both wild-type and mutant p53 were used. Briefly, the sections were microwaved in 0.01M sodium citrate pH 6.0. Subsequently, the specimens were incubated for 30 minutes at room temperature in 0.3% H2O2 in order to block endogenous peroxidase. 1% normal goat serum was then used to block the sections, after which one of the p53 antibodies (D0-7 1:100, or 1801 1:60 both from Novocastra Laboratories) was applied. The specimens were incubated overnight at 4°C with the antibody. The following day, the slides were washed 3 times using buffer solution (30mM Tris HCl pH 7.5, 150mM NaCl, 1% bovine serum albumin, 0.05% Triron X-100) and then incubated for 1 hour at room temperature with biotinylated goat anti-mouse IgG (Molecular Probes, Eugene OR) diluted 1:250 in antibody diluting buffer (Dimenshions Lab). The slides were washed 3 times in buffer solution and incubated with avidin peroxidase (Vector Laboratories, Burlingame, CA) diluted 1:1000 in diluting buffer for 1 hour at room temperature. This incubation was followed by one wash in buffer solution and then 3 washes in Tris-buffered saline. The bound antibody complex was visualized by incubating with 3,3'-diaminobenzidine (Vector) at room temperature for 15 minutes, and counterstaining was done with Mayer's hematoxylin. Both a positive
control known to stain strongly for the p53 antibodies as well as a negative control in which the primary antibody was not applied were also processed.
RESULTS

Overview

A total of 36 tumours were examined. Twenty-one of these were of astrocytic origin and of these four were low grade astrocytomas, one was an anaplastic astrocytoma and the remaining 16 were diagnosed as glioblastoma multiforme. Ten of the tumours were derived from oligodendrocytes, with four of these being classified as low grade oligodendrogliomas, and the remaining six as malignant oligodendrogliomas. In addition five other rarer tumours were examined, including two neurocytomas, a neuroblastoma, a primitive neuroectodermal tumour and a ganglioglioma. The quantity and quality of the various tumour specimens available for analysis varied considerably and as a result not all of the tumours were successfully analyzed using all of the techniques described above.

p53 and p16\textsuperscript{INK4a} mutation analysis

All of the tumours were screened for p53 mutations with SSCP analysis (Figure 2), and of these, 14 were found to have a band shift indicating the possible presence of a mutation. DNA from these tumours was subsequently sequenced (Figure 3). Of these only two of the glioblastomas were found to harbour mutations (HIS193ARG and ARG273CYS). However, examination of the four low grade astrocytic tumours revealed that three contained a mutation (two had ARG273CYS mutations and one a PHE270LEU mutation). SSCP and sequencing analysis of the same group of tumours for mutations of the p16\textsuperscript{INK4a} gene did not reveal any evidence of mutations, despite the observation of band-shifts in six samples when they were analyzed with SSCP. Possible explanations for this discrepancy include interaction of the PCR products giving rise to additional bands on SSCP analysis, as well as PCR errors.
Analysis of the ten oligodendrogliomas revealed that one of the malignant oligodendrogliomas harboured a p53 mutation (PRO191del). None of the five rarer tumours analyzed harboured any p53 mutations.

Multiplex PCR was successfully completed on 24 samples (Figure 5). In addition, controls containing DNA known to harbour a complete deletion of p16\textsuperscript{INK4a}, diluted with varying amounts of DNA known to be wild-type for p16\textsuperscript{INK4a} were also analyzed. In a sample with a heterozygous deletion of the p16\textsuperscript{INK4a} gene half of the DNA present will be wild-type, and in the presence of contamination from normal tissue the proportion of wild-type DNA in that sample will be even higher. The control that contained an equal mixture of DNA with a p16\textsuperscript{INK4a} deletion and wild-type DNA, had a value of 0.7, thus indicating that any sample with a value of less than 0.7 has at least a heterozygous deletion. Using this criterion 14/24 samples harbour at least heterozygous deletions of the p16\textsuperscript{INK4a} gene. Six of these had values of less than 0.5, the value obtained with 75% DNA with a p16 deletion and 25% wild-type DNA, and thus likely harbour homozygous deletions of this gene.
Figure 2a: SSCP screening analysis for exon 6 of p53. Note the band shift present in sample 620. Subsequent sequencing analysis (see Figure 3) revealed a mutation in this sample.

Figure 2b: SSCP screening analysis for exon 2 of p16\textsuperscript{INK4a}. Note the presence of band shifts in multiple samples. All of these were sequenced but none were found to harbour mutations (see Figure 3).
**FIGURE 3: Sequencing analysis for p53 and p16<sup>INK4a</sup>**

**Figure 3a:** Sequencing analysis for exon 6 of p53 for sample 620. Note that there is a three base-pair deletion of codon 191. Compare to wild-type sequence (on left).

**Figure 3b:** Sequencing analysis for exon 2 of p16<sup>INK4a</sup> for sample 612. Note that all the clones have wild-type p16<sup>INK4a</sup> with no evidence of point mutations or deletions.
Figure 4: Multiplex-PCR analysis to detect deletions of p16^{INK4a}. Each sample was amplified for 23 and 25 cycles. The lack of amplifications of p16^{INK4a} in sample 612 is striking and this likely represents a homozygous deletion of the p16^{INK4a} gene. Note that exon of p53 was used as an internal control (see next for more details).
**p53 message analysis**

RT-PCR was used to determine the expression level of p53 mRNA (Figure 6). 24/36 of the tumours were successfully analyzed using this technique. In addition some brain tissue obtained from a trauma victim with no known malignancy was also analyzed. The values obtained for the RT-PCR analysis were determined by obtaining the ratio of p53 to PGK and then dividing that value by the value of the same ratio obtained for the positive control that was run on each gel. The values obtained from this analysis ranged from 0.34 to 2.53 with a mean of 1.28, the value for traumatized brain being 0.74.
Figure 5: Reverse Transcriptase-PRC analysis for p53. Note that each sample was amplified for 24, 26, 28 and 30 cycles (going from right to left for each individual sample). The upper band represents the housekeeping gene PGK and the lower band p53. U 87, a glioblastoma multiforme cell line known to be wild-type for p53 was used as a positive control (see next for more details).
**Immunohistochemical analysis**

Immunohistochemical analysis (Figure 7) was successfully performed on 35/36 and 31/36 specimens for the D0-7 and 1801 monoclonal antibodies, respectively. Each of these was independently graded by two people (myself and Dr. John Provias, a neuropathologist) and in the case of any discrepancy the slide was reviewed by both graders. As there is no consensus on how to quantify immunohistochemical staining, with many papers not even detailing how they graded the presence of staining, a scale was devised to grade these preparations as follows:

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In addition, those tumours that showed prominent staining in the cytoplasm were also noted. Interestingly, four of the samples with no nuclear staining were found to have cytoplasmic staining present when the D0-7 antibody was used and seven samples had cytoplasmic staining in the absence of nuclear staining when the 1801 antibody was used. Overall 9/35 of the tumours examined had greater than 25% positive nuclear staining when the D0-7 antibody was used and 8/31 had positive staining when the 1801 antibody was employed.

All of the results are summarized on the following pages on Tables 1 and 2.
FIGURE 6a: Immunohistochemical analysis

Figure 6a: Immunohistochemical analysis using p53 specific monoclonal antibody DO-7. Note the presence of diffuse and intense nuclear staining.
Figure 6b: Immunohistochemical analysis using p53 specific monoclonal antibody DO-7. Note the purity of nuclear staining and compare with figure 6a.
FIGURE 6c: Immunohistochemical analysis

Figure 6c: Immunohistochemical analysis using p53 specific monoclonal antibody DO-7. Note the impressive cytoplasmic staining despite the paucity of nuclear staining.
Table 1: Comparison of p53 gene mutations, p53 mRNA expression levels and p53 immunohistochemical staining

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</table>

**Table 3: Controls for Multiplex-PCR analysis**

<table>
<thead>
<tr>
<th>sample</th>
<th>p16 MPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>0.0</td>
</tr>
<tr>
<td>25%</td>
<td>0.5</td>
</tr>
<tr>
<td>50%</td>
<td>0.7</td>
</tr>
<tr>
<td>75%</td>
<td>0.8</td>
</tr>
<tr>
<td>100%</td>
<td>1.0</td>
</tr>
</tbody>
</table>

0%-- control using only DNA known to harbour p16\(^{\text{INK4a}}\) deletion

25%-- control dilution containing 3/4 DNA known to harbour p16\(^{\text{INK4a}}\) deletion and 1/4 DNA known to be wild-type for p16\(^{\text{INK4a}}\)

50%-- control dilution containing 1/2 DNA known to harbour p16\(^{\text{INK4a}}\) deletion and 1/2 DNA known to be wild-type for p16\(^{\text{INK4a}}\)

75%-- control dilution using 1/4 DNA known to harbour p16\(^{\text{INK4a}}\) deletion and 3/4 DNA known to be wild-type for p16\(^{\text{INK4a}}\)

100%-- control using only DNA known to be wild-type for p16\(^{\text{INK4a}}\)
**Statistical analysis of results**

Multivariate analysis of the data reveals that the results of the immunohistochemistry using the two antibodies 1801 and D0-7 correlate highly (p=0.0001). Furthermore, with the scale used to grade the degree of staining it was found that with the 1801 antibody the mean grade was 1.2 when there was no p53 mutation detected and the mean increased to 3.4 in the presence of a p53 mutation. Using a Student's T test this difference was found to be significant (p=0.0081). When the D0-7 antibody was used the mean grade was 1.5 in the absence of p53 mutations and increased to 3.4 in the presence of p53 mutations. This difference was also found to be significant using a T test (p=0.024).

Analysis of the relationship between the presence of p53 mutations and the expression levels of p53 mRNA using partial correlation coefficients did not reveal any correlation between these two variables. Furthermore when the same methods were used to determine the relationship between mRNA expression levels and the degree of staining by immunochernistry, there was no correlation between these two variables regardless of which antibody was used.

Attempts to statistically analyze whether p53 or p16\(^{\text{INK4a}}\) mutations occur predominantly in low or high grade tumours could not be done because of the small sample size, particularly of the lower grade tumours. In addition, statistical analysis to determine the relationship of p53 and p16\(^{\text{INK4a}}\) with one another did not reveal any correlation, however the small sample size and low p53 mutation rate made it unlikely that a relationship would be detected.
DISCUSSION

The role of p53 and p16\(^{INK4a}\) in the pathogenesis of primary brain tumours

The p53 mutation rate of the 17 malignant astrocytic tumours examined in this study is considerably lower than that reported previously (Wu, et al., 1993; Louis, 1994; Louis et al., 1993). It is unlikely that this discrepancy is a result of the techniques employed in this investigation. In fact, it was anticipated that the mutation rate in the cohort of tumours studied might be higher than those previously published. All of the samples were screened with SSCP using at least two different gel conditions, decreasing the chances that a mutation would be missed as a result of unfavourable gel conditions, or subtle band-shifts. Furthermore, unlike many other studies which only examine the "hotspots" located between exons 5-8 (Greenblatt, et al., 1994), this study examined all of the exons of p53, including the intron-exon splice sites, except for exon 3 which is very small and has never been found to harbour a mutation, and the non coding exon 1. It is thus unlikely that any undetected mutations were present in the group of tumours investigated.

There was nothing unusual about the pathology of the astrocytic tumours examined and the patient population from which they were derived was also unremarkable (none were from pediatric patients). It is now generally accepted that glioblastoma multiforme can evolve via two distinct pathways and that certain genetic features of these tumours are characteristic of the pathway of their origin. In the "de novo" pathway which is believed to be more common, p53 mutations are a more rare event, whereas in the "progressive" pathway p53 mutations occur more frequently (von Deimling, von Ammon, 1993; von Deimling, 1995). Although there was nothing atypical about the cohort of patients from which the tumours were derived, given the relatively small number of glioblastomas examined, it is possible that a large majority of the
tumours investigated in this study were coincidentally "de novo" glioblastomas and this may account for the low p53 mutation rate.

Whether p53 mutations are an early or late event in the pathogenesis of astrocytomas has been a matter of debate. In this study, 3/4 low grade astrocytomas were found to harbour p53 mutations as compared to only 2/16 glioblastomas. Although the sample size is too small for any meaningful statistical analysis, these results would seem to support the notion that when mutations of the p53 gene are involved in the pathogenesis of astrocytic tumours they tend to occur as an early event. This is supported by the finding that loss of chromosome 17p occurs with equal incidence in low grade and malignant gliomas (El-Azouzi et al., 1989; Fults et al., 1989; and James et al., 1989), as well as by Sidransky et al. who demonstrated that clonal expansion of a subset of cells containing a p53 mutation occurs as a low grade astrocytoma evolves into a glioblastoma (Sidransky et al., 1992).

Analysis of the non astrocytic tumours for p53 mutations did not yield any surprising results. Of the ten oligodendrogliomas, only one was found to have a p53 mutation in keeping with results previously published (Ohgaki et al., 1991). It has been found that patients with oligodendrogliomas respond well to chemotherapeutic agents that are generally ineffective in the treatment of astrocytic tumours (Peterson et al., 1996; and Cairncross and Macdonald, 1988). Although there are many possible explanations for this difference, it is interesting to speculate that one of the reasons may be that the presence of wild-type p53 in the majority of oligodendrogliomas allows for the induction of apoptosis in the presence of chemotherapeutic agents. None of the remaining and less common tumours examined had p53 mutations in keeping with other reports in the literature (Ohgaki et al., 1991; Wu et al., 1993). Given the small sample size it is not possible to make any generalized conclusions based on this finding.
Immunohistochemical analysis of the same cohort of tumours using the antigen retrieval technique was performed using the monoclonal antibodies D0-7 and 1801. The decision to use this combination was based on a report suggesting that together these two are most effective in detecting the presence of altered forms of the p53 protein (Horne et al., 1995). Unfortunately there is no consensus on how to quantify the staining results using these antibodies. The grading scale used in this study was developed empirically. The difference between grade 0 (no nuclear staining) and grade 1 (0-5% staining) may seem trivial, but theoretically even one cell with an altered form of the p53 protein can clonally expand; thus, the presence of a very few scattered positively staining nuclei may be of biological and clinical significance. Using the same logic one can speculate that the difference between grade 4 (51-75% staining) and grade 5 (76-100% staining) is perhaps not of very great significance.

Since the antibodies used in this study recognize both altered as well as wild-type forms of the p53 protein the possibility remains that the staining represents an accumulation of wild-type p53 that has become post-translationally stabilized. Alternatively, if there were a nonsense mutation in the upstream part of the gene, or if a mutation occurred somewhere in the gene that changed the conformation of the epitope recognized by the antibodies, it is possible that the altered protein would not be stained with the technique used. Despite these caveats, unlike some other studies (Louis et al., 1993; Lang et al., 1994), but similar to another (van Meyel et al., 1994), a positive relationship was found between positive staining and the actual presence of p53 mutations. Furthermore, the degree of immunohistochemical staining with each of the two antibodies employed correlated very highly, indicating that in future studies staining with only one of these antibodies may be adequate.

p53 is normally located in the nucleus and the significance of specific cytoplasmic staining in the absence of any nuclear staining is unclear. Moll and her
colleagues examined 27 breast tumours using the 1801 antibody concluded that nuclear exclusion is a mechanism of p53 inactivation in these tumours (Moll et al., 1992). It is very unlikely that the p53 protein, even if it were wild-type, would be able to function effectively as a cell cycle regulator or in the induction of apoptosis, if it were excluded from the nuclear compartment. This has been supported in experiments in which p53 was no longer able to inhibit cellular proliferation in culture when it was excluded from the nucleus (Shaulsky et al., 1991). Possible explanations for the cytoplasmic location of the protein include its binding to cytoplasmic proteins, or a mutation in its nuclear localization signal domain. In this study, there does not appear to be any relationship between the presence of cytoplasmic p53 and the presence of p53 mutation, or the grade of tumour. Interestingly, both neurocytomas exhibited very strong cytoplasmic staining, with DO-7 and 1801 antibodies, in the absence of any nuclear staining or p53 DNA mutations. This observation suggests that p53 may be inactivated by a unique mechanism in this subset of tumours, but a larger number of these tumours would have to be examined to verify this finding.

The reverse transcriptase-PCR technique was used because it requires less RNA than needed for northern blot analysis. Unfortunately, even given these more modest requirements for RNA, many of the samples did not yield sufficient quantities or adequate quality RNA, to be analyzed. The finding that there is no correlation between p53 mRNA message levels and p53 mutations is not surprising given the fact that there are likely many factors involved in the regulation of p53 transcription, including regulation that has recently been found to occur in the promoter region of the p53 gene (Roy and Reisman, 1996; Desaintes et al., 1997). It remains unclear whether the presence of a mutation in the p53 gene has any effect on the manner in which transcription occurs. However, the results of this study as well as an appreciation for the complexities involved in the regulation of transcription indicate that if there is a
relationship between p53 mutations and p53 mRNA expression levels, it is not a simple linear one.

Comparison of RT-PCR values and values obtained from p53 immunohistochemistry also did not show any correlation. It has been demonstrated that p53 can be phosphorylated at multiple serine and threonine residues in its amino and carboxy regions (Ko and Prives, 1996). This phosphorylation may increase the steady state level of p53 protein in the cell but at the same time may decrease its ability to function as a transcriptional activator (Zhang, et al., 1994). Furthermore, experiments done using growth-arrested mouse fibroblasts suggest that translation of p53 mRNA is negatively autoregulated by wild-type p53 protein (Mosner, et al., 1995). In addition, the p53 protein can bind to a wide variety of other cellular and viral proteins (Ko and Prives, 1996). Given this complex translational regulation, as well as these post-translational modifications, it is not too surprising that no correlation was found between p53 mRNA expression levels and p53 protein alterations detected using immunohistochemistry. Furthermore, uncertainty as to antibody specificity for wild-type versus altered forms of the p53 protein confounds any conclusions that can be derived from this analysis.

Initial screening analysis, employing SSCP and subsequent sequencing when indicated, for mutations or deletions involving p16\textsuperscript{\textit{NK4a}} in the cohort of samples examined did not reveal evidence of any abnormality involving this gene. However, this is not surprising given the infiltrative pathologic nature of these tumours, as well as evidence that the vast preponderance of mutations involving the p16\textsuperscript{\textit{NK4a}} gene are in the form of fairly large deletions. It is unclear how much non tumourigenic material was included in the samples analyzed but it is certain that there was enough DNA present from these tissues to be amplified by the PCR technique. Furthermore, DNA derived from tumour cells that have deletions of the p16\textsuperscript{\textit{NK4a}} gene will not be amplified by the PCR technique if the sequence recognized by the primers, located on either side of exon
2, is also involved in the deletion, which is likely to be the case when there is a deletion present. Thus the final PCR product will only contain the amplified DNA derived from the non-tumourigenic material and analysis of this product will appear completely normal. If the mutation of the p16^{NK4a} gene were in the form of a point mutation, or a deletion that did not involve the sequence that the primers recognize, then SSCP screening and subsequent sequencing would be very effective in detecting alterations of this gene, as is the case for p53. As a result, it is now apparent that in the case of p16^{NK4a}, where mutations are most commonly in the form of large deletions, this is a poor technique to use.

The results obtained from multiplex-PCR analysis verify that there are deletions present in at least some of the tumours examined. The multiplex-PCR technique was used because it avoids many of the problems encountered using the Southern blotting technique. There are fewer steps involved in the multiplex-PCR technique and the bands on the agarose gel are sharp and well defined making quantification by optical densitometry more reliable. In addition, only a small amount of DNA is required for this procedure, allowing samples to be run multiple times. Furthermore, DNA with a known deletion of p16^{NK4a} can be used to complement the internal control that was used for each sample. As a result, the values obtained could be more easily interpreted. Based on the controls which contained various dilutions of DNA known to contain a deletion of the p16^{NK4a} gene with DNA known to be wild-type for p16^{NK4a}, it appears that any value less than 0.7 obtained for a sample from the multiplex-PCR method represents at least a heterozygous deletion of the p16^{NK4a} gene (Table 3). This is a conservative estimate given that all the samples contain some contamination with non-tumourigenic material.

The 9/11 glioblastoma multiforme samples determined to have deletions involving the p16^{NK4a} gene is somewhat higher than that published previously (Schmidt et al., 1994; Moulton et al., 1995; Kyritsis et al., 1996). However, the literature
published to date with respect to the deletion rate of $p16^{\text{NK4a}}$ in brain tumours is quite confusing, with different groups employing different laboratory techniques and obtaining varying rates of $p16^{\text{NK4a}}$ deletions in these tumours. It is encouraging that Ueki and his collaborators also using the multiplex-PCR technique found a similar rate of mutations as that presented in this study, with evidence of $p16^{\text{NK4a}}$ deletions in 25/42 glioblastoma multiforme tumours (Ueki et al., 1996). The absence of any intragenic mutations involving $p16^{\text{NK4a}}$ in the cohort of tumours in this study, as determined by SSCP and sequencing, is in keeping with findings described by other groups (Giani and Finocchiaro, 1994; Jen et al., 1994; Moulton et al., 1995). Unfortunately, since only one of the low grade astrocytomas was analyzed and found not to harbour any $p16^{\text{NK4a}}$ deletion, no conclusion as to whether $p16^{\text{NK4a}}$ mutations are an early or late event in the pathogenesis of brain tumours can be made from that study. Schmidt and her colleagues did manage to investigate a cohort of tumours that included 46 glioblastomas and 11 anaplastic astrocytomas and found a deletion rate involving $p16^{\text{NK4a}}$ of 41% and 27% respectively; none of 8 astrocytomas investigated in the same study had any alterations of the $p16^{\text{NK4a}}$ gene (Schmidt et al., 1994). This finding would suggest that deletions of $p16^{\text{NK4a}}$ might be a later event in the pathogenesis of astrocytic brain tumours, but clearly more data is necessary before this trend can be verified.

The finding that 3/5 malignant oligodendrogliomas and 1/2 low grade oligodendrogliomas studied contained deletions of the $p16^{\text{NK4a}}$ gene represents a higher rate than that found by Sato et al. who did not find any $p16^{\text{NK4a}}$ deletions in 17 oligodendrogliomas investigated (Sato et al., 1996). Of the rarer tumours examined, only one, a neuroblastoma, had evidence of a $p16^{\text{NK4a}}$ deletion and this likely is a heterozygous deletion. One study found evidence of $p16^{\text{NK4a}}$ deletions in 32% of neuroblastomas (Takita et al., 1997), yet two other studies did not find any abnormalities of the $p16^{\text{NK4a}}$ gene in any of the neuroblastomas examined (Beltinger et al., 1995;
Kawamata et al., 1996). Examination of primitive neuroectodermal tumours by Raffel et al. did not reveal any $p16^{\text{NK4a}}$ deletions in either four established cell lines, or in 18 surgical specimens (Raffel et al., 1995). None of the other rarer tumours examined in this study have been examined elsewhere for $p16^{\text{NK4a}}$ deletions and it remains to be determined what role if any $p16^{\text{NK4a}}$ plays in their pathogenesis.

Analysis of the cohort of tumours for deletions of the $p16^{\text{NK4a}}$ gene and mutations of the p53 gene reveal a very interesting pattern. It appears that many of the tumours that are wild-type for p53 harbour deletions of the $p16^{\text{NK4a}}$ gene. Only two tumours were found to have an alteration involving both genes and 14 tumours had an alteration of one or the other gene. This suggests that a mutation in either one of these cell cycle regulators can have profound significance in the process of tumourigenesis regardless of whether or not the other gene is wild-type. Thus, both p53 and $p16^{\text{NK4a}}$ are critical for a cell to maintain a fine balance between division as required for the organism to function and uncontrolled proliferation that leads to malignancy. Both p53 and $p16^{\text{NK4a}}$ share a common downstream target, pRb and it appears that one cannot compensate when the other is unable to function properly. Furthermore, it is clear that p53 acts via several different mechanisms in its capacity to act as a tumour suppressor gene, including its ability to induce apoptosis. A recent study supporting the synergistic activity of p53 and $p16^{\text{NK4a}}$, found that overexpression of p53 and $p16^{\text{NK4a}}$, but not p53 on its own, induces apoptotic death. In the same study it was also determined that simultaneous adenoviral transfer of $p16^{\text{NK4a}}$ and p53 genes in nude mice caused the inhibition of tumour growth (Sandig et al., 1997). Furthermore, it has been found that $p16^{\text{NK4a}}$ has an alternative exon 1 which when spliced into exon 2 changes the reading frame of the gene thus ultimately producing an entirely different protein product (Mao et al., 1995; Stone et al., 1995). It is speculated that the different forms of $p16^{\text{NK4a}}$ (which have been alternately called $p16\alpha$ and $p16\beta$, or $p16^{\text{NK4a}}$ and $p19^{\text{ARF}}$) have different downstream targets (Quelle
et al., 1995) and that one form (likely p16β) can function effectively as a cell cycle regulator independently of pRb. The multiplex-PCR technique that was used in this study targeted exon 2 and thus would not be able to distinguish between these two forms of p16INK4a.

Recently, a novel tumour suppressor gene candidate called PTEN (phosphatase and tensin homolog deleted on chromosome 10) was isolated (Li et al., 1997; Steck, et al., 1997). This gene which is localized on chromosome 10q23.3, a region known to be deleted in many glioblastomas, is speculated to be involved in the progression from low grade astrocytomas to glioblastoma multiforme (Wayne et al., 1997; Rasheed et al., 1997). The mechanism through which PTEN acts as a tumour suppressor is thought to be, at least in part, via its intrinsic phosphatase activity (Li and Sun, 1997). Much remains to be determined about this gene, and it will be interesting to discover whether PTEN's ability to act as a tumour suppressor is related to either p53 or p16INK4a.

Conclusions

This study has examined the cycle regulators p53 and p16INK4a in the pathogenesis of primary brain tumours. Clearly the products of these genes are critical factors in the development of at least a subset of these tumours. The establishment of a positive relationship between p53 mutations and p53 protein alterations as detected by immunochemistry is encouraging. It indicates that examining specific tumours using less tedious immunochemical techniques can yield valuable information which reflects on the p53 mutational status of the tumours. The lack of correlation between p53 mRNA levels and either p53 mutations, or p53 protein alterations as detected using immunochemistry is likely explained by post transcriptional as well as post-translational changes.

SSCP analysis is a poor method of screening for p16INK4a mutations which are almost always in the form of deletions in primary brain tumours. Multiplex-PCR
analysis, or Southern analysis if sufficient DNA is available, are far superior techniques for detecting the presence of $p16^{INK4a}$ deletions in these tumours. In this study, $p16^{INK4a}$ deletions were found to be present in 82% of glioblastomas, suggesting that deletion of this gene is a common event in these tumours. Furthermore, the tendency for these deletions to occur in the presence of wild-type p53 indicates that alterations of either p53 or $p16^{INK4a}$ is a strong factor favouring the development of a malignancy, regardless of the ability for other cell cycle regulators to function normally.

**Future Directions:**

The establishment that cell cycle regulators play a role in the pathogenesis of primary brain tumours presents the opportunity for the development of new strategies and therapies in the management of these tumours. Use of radiation therapy “up front” in the treatment of low grade gliomas remains controversial and perhaps guidelines as to when to use this modality of therapy can be formulated depending upon the p53 status of the tumour. Exposing a low grade glioma to radiation therapy may not only be ineffective given the requirement of p53 for the induction of certain apoptotic pathways, but may actually be detrimental by inducing the accumulation of other mutations that would normally be repaired if wild-type p53 were present. Prospective clinical trials examining the outcome of patients with low grade gliomas based on their p53 status as well as whether or not they received radiation therapy would be useful. Other experiments in which gene therapy is used to introduce wild-type copies of the defective p53 or $p16^{INK4a}$ gene back into the tumour would also be of interest, as *in vitro* experiments already demonstrate that reintroduction of these genes is capable of transforming a malignant phenotype back to a more benign phenotype in cell lines which are null for p53 or $p16^{INK4a}$ (Van Meir *et. al.*, 1994; Fueyo *et. al.*, 1996). Using a different strategy, it has recently been shown that an adenovirus with a deleted E1B gene, which normally
inactivates the p53 gene, causes tumour-specific cytolysis particularly in cell lines that have mutated p53 (Bischoff et al., 1996; Heise et al., 1997). This attenuated adenovirus called ONYX-015 is currently being tested in phase I clinical trials in patients with cancer (Heise et al., 1997). Lastly experiments investigating the different roles that the two forms of p16\textsuperscript{INK4a} play in the development of brain tumours with respect to each other as well as with respect to p53 would be of interest and of possible clinical relevance.
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