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The Epidemiology, Biology and Genetics of Human Astrocytic Tumours

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Vi upptäckte mer och mer
och jorden blev större och större.
Upptäckte ändå mer
och jorden blev bara en prick,
en liten leksaksballong
i oändligheten.
Nils Ferlin

To my family

ABSTRACT

In Sweden >1000 primary central nervous system (CNS) tumours are diagnosed each year. The majority are malignant and in adults astrocytic gliomas dominate. These are classified into four malignancy grades according to WHO criteria, including Glioblastoma (GB), which is the most common and most malignant of all CNS tumours (malignancy grade IV), Anaplastic Astrocytoma (AA; malignancy grade III) and Astrocytoma (AII; malignancy grade II). Prognosis is generally poor but varies markedly between the malignancy grades and even between individuals with the same malignancy grade. While numerous studies have addressed the issue of genetic prognostic markers in astrocytic tumours, little of clinical value has been established despite all the genetic information accumulated during the last two decades.

From 1987 to 1997 we collected >700 primary CNS tumours for molecular genetic analysis and the patients were followed-up clinically regarding age, sex, date of death and therapy received. We also collected blood samples from the patients, making it possible to pair-wise compare tumour DNA with normal DNA. The number of astrocytic glioma patients included in **Paper I-III** was 246 (172 GB, 54 AA and 20 AII). The data was used for correlation analysis with the primary endpoint to identify associations between gene abnormalities and patient survival. We analyzed 129 GB and 37 AA for loss of six tumour suppressor genes (*PTEN*, *RB1*, *CDKN2A*, *CDKN2B*, $p14^{ARF}$ and *TP53*) and amplification of three proto-oncogenes (*CDK4*, *MDM2*, and *EGFR*). These are the most commonly altered genes in astrocytic tumours. We found that abnormalities in any of the four genes coding for components of the Rb1 pathway (*i.e. CDKN2A, CDKN2B, RB1* and *CDK4*), were associated with shorter survival (p=0.002) in GB. In combination with loss of wild-type *PTEN* the association was even stronger (p<0.001). The survival difference (median survival in the two groups was 166 days compared to 437 days) was statistically significant in bivariate analysis adjusting for age (p=0.012). In AA we found that defects in any of the four Rb1 pathway-related genes were significantly associated with short survival (median survival 1.7 years compared to 9.9 years) in univariate (p=0.009) and multivariate analysis (p=0.013).

The Epidermal Growth Factor Receptor (EGFR) gene is frequently amplified and/or rearranged in astrocytic tumours, particularly in "primary" GB. The reported results on the prognostic impact of this have so far been contradictory and few reports have correlated both amplification and rearrangements with survival. We analyzed 221 astrocytic tumours for the presence of *EGFR* amplification and expression of the aberrant EGFRvIII transcript. In 160 "primary" GB patients we found no significant associations between *EGFR* abnormalities and survival. In 41 AA we found a tendency towards shorter survival in patients with tumours expressing the EGFRvIII transcript (p=0.069). These patients were significantly older than those with no EGFRvIII present (p=0.023).

As the aetiology of benign and malignant CNS tumours is largely unknown we performed an epidemiological study exploring if an association between CNS tumours and parathyroid adenomas exists (**Paper IV**). Known hereditary factors explain only a few percent of the incidence, as do the best characterized exogenous factors. We used the Swedish Cancer Registry (SCR) to identify all individuals operated for parathyroid adenomas between 1958 and 1999 (n=12,468) and using the SCR they were followed-up for the subsequent development of CNS tumours. There were 70 such cases observed. Compared to the 35 expected, the standard incidence ratio (SIR) was 2.0. This increased risk was independent of duration of follow-up and was confined to meningiomas (SIR=2.4), the most common type of benign tumour of the CNS, and neurinomas (SIR=3.4). For astrocytic tumours, after excluding the first year, the observed number was close to the expected (SIR=0.8).

With the studies included in this thesis, and the conclusions drawn, I hope to add something to the understanding of what causes CNS tumours as well as what dictates their prognosis.

PUBLICATIONS AND MANUSCRIPTS

I. **L. Magnus Bäcklund**, Bo R. Nilsson, Helena M. Goike, Esther E. Schmidt, Lu Liu, Koichi Ichimura and V. Peter Collins.
Short post-operative survival for glioblastoma patients with a dysfunctional Rb1 pathway in combination with no wild-type *PTEN*
Clinical Cancer Research, (9) Sept 15, 2003, 4151-8

II. **L. Magnus Bäcklund**, Bo R. Nilsson, Lu Liu, Koichi Ichimura and V. Peter Collins.
Anaplastic astrocytoma patients with glioblastoma-like tumor genotype have a poor outcome
Submitted for publication

III. Lu Liu, **L. Magnus Bäcklund**, Bo R. Nilsson, D. Grandér, Koichi Ichimura and V. Peter Collins.
Incidence and clinical significance of *EGFR* amplification and the aberrant EGFRvIII transcript in conventionally treated astrocytic gliomas
Submitted for publication

IV. **L. Magnus Bäcklund**, Dan Grandér, Lena Brandt, Per Hall and Anders Ekbom.
Parathyroid adenoma and primary CNS tumors
International Journal of Cancer, 113, 2005, 866-9 (Published online Oct 28, 2004)

ABBREVIATIONS

APC Adenomatous Polyposis Coli
ARF Alternative Reading Frame
ATM Ataxia Telangiectasia Mutated
ATR Ataxia Telangiectasia and Rad3-related
bp base pairs
BBB Blood Brain Barrier
CDK Cyclin Dependent Kinase
cDNA complementary DNA
CNS Central Nervous System
CpG Cytosine-Guanine dinucleotide pair
CT Computer Tomography
DGGE Denaturing Gradient Gel Electrophoresis
DNA Deoxyribonucleic Acid
EGFR Epidermal Growth Factor Receptor
FAP Familial Adenomatosis Polyposis
GF Growth Factor
GFR Growth Factor Receptor
HDAC Histone Deacetylase
HPNCC Hereditary Non-Polyposis Colon Cancer
HRT Hormone Replacement Therapy
HPT-JT Hyperparathyroidism–Jaw Tumour
IGF-R Insulin-like Growth Factor Receptor
kb kilobase
kDa kiloDalton
LOH Loss of Heterozygousity
Mb Megabase
MEN Multiple Endocrine Neoplasia
MRI Magnetic Resonance Imaging
NF Neurofibromatosis
PA Parathyroid Adenoma
PCR Polymerase Chain Reaction
PCV Procarbazine, CCNU, Vincristine
PDGFR Platelet Derived Growth Factor Receptor
PI3-K Phosphatidyl Inositol 3'-Kinase
PTEN Phosphatase and Tensin
homolog deleted on chromosome Ten
PTH Parathyroid Hormone
PTH-rp Parathyroid Hormone-related protein
RNA Ribonucleic Acid
RNase Ribonuclease
RTK receptor tyrosine kinase
RT-PCR Reverse Transcription-PCR
SCR Swedish Cancer Registry
SIR Standard Incidence Ratio
SSCP Single Strand Conformational Polymorphism
TGFß-receptor Transforming Growth Factor beta receptor
Thr Threonine
TSG Tumour Suppressor Gene
VEGF Vascular Endothelial Growth Factor
WHO World Health Organization

TABLE OF CONTENTS

INTRODUCTION

Introduction to cancer

The multistep genetic basis of cancer

A cancer consists of malignant cells that are derived from normal somatic cells but have alterations of their genome. The hypothesis that the transformation from a normal cell to a malignant cell is a multistep process was presented already in the 1950's (Armitage & Doll, 1957; Nordling, 1953) and has become generally accepted. The transformation process is a consequence of the acquisition of genetic defects. Alterations of normal gene-related DNA, *i.e.* mutations, can be inherited or be induced by factors produced in the normal cellular metabolism, including oxygen radicals, or by environmental factors, such as radiation and genotoxic compounds (Hoeijmakers, 2001). However, single mutations often have little or no effect on a cell's behaviour. To contribute to the malignant transformation of a cell, significant mutations of critical genes must occur and be transmitted to the next cell generation.

In addition to acquiring mutations, gene expression in cancer cells can be aberrant as a result of epigenetic changes, such as altered methylation of CpG islands in promoter regions (Feinberg & Tycko, 2004). The minimum number of specific genetic events needed is debated but believed to be somewhere between 2 and 7, each leading to a selective advantage as regards cell survival and proliferation (Hahn & Weinberg, 2002b; Knudson, 1971). This has been best demonstrated in studies of the development of colon carcinomas from adenomas (Fearon & Vogelstein, 1990), but also in *in vitro* model systems, *e.g.* by Hahn and co-workers that elegantly showed that 3 "hits" were enough to create a human tumour cell (Hahn et al., 1999). Several cellular programs are disrupted by the genetic defects in the cancer cells. Deregulated programs that many cancers have in common permit the cells to become growth factor independent, genetically instable and lacking in normal cell cycle control mechanisms and the capacity to differentiate and to apoptose. In addition most malignant tumour cells acquire the abilities to recruit new blood vessels (*i.e.* angiogenesis), to invade surrounding tissues and to metastasize (Hanahan & Weinberg, 2000).

Resting (or quiescent) cells will, if they have not undergone malignant transformation, not proliferate without being stimulated by growth promoting mitogens. Positive growth factor signalling is counteracted by anti-proliferative signals *e.g.* via the transmembranic transforming growth factor beta (TGFß) receptor (Figure1). An excess of growth factor signalling does not only drive resting cells towards entry of the cell cycle, but frequently also promotes survival by inhibiting initiation of the cell suicide program apoptosis. This program is, together with accurate cell cycle control, crucial for regulating the number of cells in a tissue or organ and thereby normally protects an organism against inadequate proliferation.

Developments in tumour biology have during the last 30 years or so not only increased our understanding of cancer but also of cell biology in general and how proliferation, differentiation and death of cells are normally regulated. When trying to define the molecular details of these fundamental cellular processes a number of molecular "pathways" have been identified. These pathways are often complex with examples of auto-regulation, negative feedback loops as well as cascade scenarios. Examples of pathways regularly altered in cancer cells are the p53 and Rb1 pathways. They are for simplicity often described as linear processes starting with a stimulus triggering a domino effect ending with a particular event. This complexity is likely to be much greater than we understand today, since we have only identified a minority of the thousands of proteins involved in even the most fundamental cellular processes and as yet know almost nothing about how these proteins are modified in time and space in living cells. Each pathway may be involved in several cellular programs and, in addition, there are clear evidence of interactions between the different pathways. It is perhaps not surprising to see that both the p53 and Rb1 pathways are

involved in cell cycle control, differentiation, apoptosis and senescence and the p53 pathway also in angiogenesis and DNA repair (Sherr & McCormick, 2002).

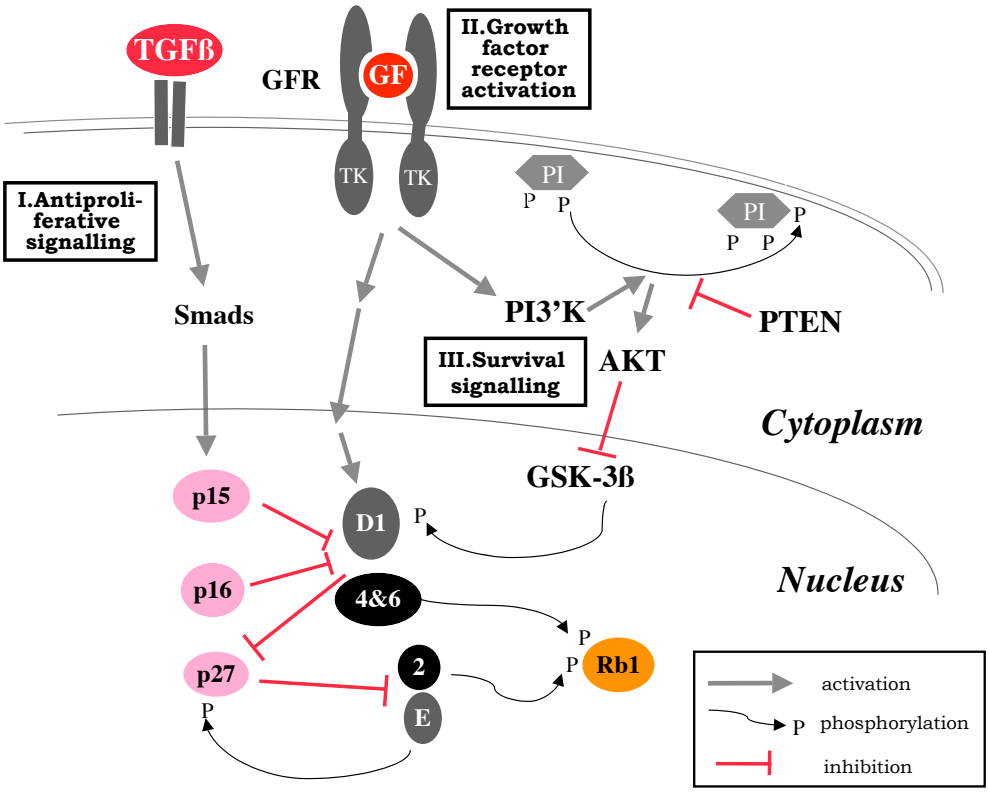

Figure 1. I. TGFß trigger the TGFß-receptor's anti-proliferative signals, which are transduced via the phosphorylation of members of the Smad family of proteins, leading to p15 upregulation. **II.** Wild-type GFR like EGFR form homo- or heterodimers when stimulated by a growth factor (GF) and becomes active, triggering a series of signals transduced to the nucleus. Via the Ras-MAPK pathway the synthesis of cyclin D1 is induced and forms active complexes with CDK4 and CDK6. These complexes bind and thereby inactivate p27 and phosphorylate Rb1. Cyclin E complexing with CDK2 also may phosphorylate Rb1, but the cyclin-CDK complexes can be inhibited by p15, p16 and p27, respectively. **III.** Survival signalling enhances PI3K-mediated phosphorylation of PI(4,5) diphosphate to PI (3,4,5) triphosphate, leading to activation of Akt. This is counteracted by PTEN. Akt can inhibit GSK-3B. Active GSK-3ß can mark cyclin D1 for degradation by phosphorylation. (D1=cyclin D1; E=cyclin E; 4&6=CDK4 and CDK6; GF=Growth Factor; GFR=Growth Factor Receptor)

Cancer genes

Mutations of even a few genes can dramatically alter many cellular functions. The majority of mutations causing cancer are so called "somatic", *i.e.* arising after conception (*in utero*, in childhood or later in life). However, the malignant transformation process is markedly speeded up if there is a significant inherited mutation, as this then will be present in all cells of an individual (Lindblom & Nordenskjold, 2000). The genes most often targeted in cancer cells are often roughly divided into oncogenes and tumour suppressor genes (TSGs). Both these classes of genes are involved in the regulation of fundamental aspects of the cell's daily life, such as decisions to proliferate or to rest or to enter the apoptosis program. A third category of cancer-related genes belongs to the DNA repair genes.

Oncogenes are activated variants of normal cellular genes called proto-oncogenes. Their activities promote cellular proliferation and survival. The activation is a consequence of either DNA sequence alterations, leading to the production of a protein with altered function, or of over-expression due to amplifications or translocations. The corresponding gene products are called onco-proteins. Important examples of onco-proteins are the transcription factor c-myc, the anti-apoptotic agent bcl-

2, growth factor receptors (*e.g.* EGFR, IGF-R and PDGF-R) and intracellular signalling molecules and kinases (*e.g.* ras and CDK4).

The role of TSGs is almost the opposite of that of oncogenes, as their protein products inhibit progression through the cell cycle or promote apoptosis. Examples of TSGs altered in high frequencies in many types of cancers are *TP53*, *CDKN2A*, *p14^{ARF}*, *RB1* and *PTEN*. While oncogenes are overactive in cancer cells, TSGs are frequently lost or inactivated, leading to a defective or absent protein. This generally requires bi-allelic inactivation, which may occur by a complete loss of both gene copies (*i.e.* homozygous deletion) or deletion of one allele and a point mutation of the other or even by loss, or mutation, of one copy and hypermethylation of the promoter region of the other, resulting in no expression of that otherwise normal allele. However, if the gene product functions as a di- or polymer the mutation of one allele may result in a non-functional protein complex. An example of this situation is when *TP53* mutations of one allele have a dominant negative effect or even lead to new functions of the p53 protein (Cadwell & Zambetti, 2001).

The third category of genes involved in the development and progression of cancer is the DNA repair genes. Examples of such are the mismatch repair genes *hMLH1* and *hMSH2* and the *BRCA1*, *BRCA2*, *ATM*, *ATR* and *DNA-PK* genes involved in detection and repair of DNA double-strand breaks (Hoeijmakers, 2001). Unlike oncogenes and TSGs, DNA repair genes usually do not control cell growth and death directly. Instead loss of these genes promote cancer development indirectly because DNA damage will to a lesser extent be recognized and repaired, thus elevating the probability of mutations of TSGs and oncogenes to be permanent.

Is there any clinical value in knowing about the status of cancer genes?

Our increased understanding of the molecular basis of cancer has many potential clinical implications. These include the areas of hereditary cancers, diagnosis, prognosis, identification of new therapeutic targets and prediction of treatment outcome. There are already quite a number of examples of this. In breast cancer amplification and consequent over-expression of erbB2, found in approximately 30% of breast cancers, is an established negative prognostic factor and a predictor of response to erbB2-targeting therapy (Sjogren et al., 1998). In acute lymphatic leukaemia (ALL) specific translocations are common events and t(9;22) is associated with poor outcome and has for many years been used in treatment decisions (Mrozek et al., 2004; Priest et al., 1980). In anaplastic oligodendroglioma there has been considerable interest in the association between allelic losses of 1p and 19q and response to the polychemotherapy combination PCV (Bauman et al., 2000; Cairncross et al., 1998).

Growth factor signalling

Cell growth is under normal conditions controlled by interactions between the cell and its environment. These interactions can trigger a cascade of intracellular molecular signals, leading to diverse effects on genes and proteins, which regulate the life and death of a cell. In the presence of relevant ligands, growth factor receptors (GFR) become activated and, in turn, either homo- or heterodimerize (with other members of their GFR family) and subsequently their intracellular tyrosine kinase domains are activated permitting the binding and phosphorylation of signal transduction molecules initiating a cascade (Schlessinger, 2000). In malignant cells, growth factors and GFRs can be either inadequately expressed or mutated, resulting in constitutive activation.

From a cancer perspective one group of GFRs are of particular interest: so called receptor tyrosine kinases (RTKs). One important and well-characterized sub-group of RTKs is the erbB family of receptors, with its four members erbB1, 2, 3 and 4. The first RTK discovered was erbB1, also called epidermal growth factor receptor (EGFR) or Her1. As *EGFR* gene status (and the aberrant transcript EGFRvIII) has been studied in **Paper I-III** of this thesis, and the EGFR constitutes a good principle example of a GFR, is it described more in detail here. Wild-type EGFR form homo- or heterodimers and at least 6 ligands are known to stimulate this formation (Yarden & Sliwkowski, 2001). When

activated, EGFR triggers a number of signalling cascades, via *e.g.* Ras-mitogen-activated protein kinase (Ras-MAPK) and phosphatidyl-inositol 3' kinase (PI3K)–Akt pathways (Schlessinger, 2000)(Figure 1). The consequence of an exaggerated growth signalling is increased proliferation and decreased apoptosis.

The normal *EGFR* gene is located on the short arm of chromosome 7 and has 28 exons that code for a 170 kDa transmembranic glycoprotein (Lin et al., 1984; Reiter et al., 2001). The wild-type EGFR is composed of an N-terminal extracellular ligand-binding domain, a hydrophobic transmembrane region, a cytoplasmic domain with intrinsic tyrosine kinase activity and a carboxy-terminal region with tyrosine residues and regulatory auto-phosphorylation sites (Schlessinger, 2000)(Figure 2). Aberrant expression of the EGFR in cancer is well documented, *e.g.* in lung and gastrointestinal cancers (Salomon et al., 1995). Its role in lung cancer has been of particular interest recently since novel therapies targeting EGFR have shown a dramatic positive effect in patients with specific mutations of the cytoplasmic tyrosine kinase domain (Paez et al., 2004).

EGFR over-expression is also found in primary central nervous system (CNS) tumours, both gliomas and meningiomas (Andersson et al., 2004; Ekstrand et al., 1991; Libermann et al., 1984), with the highest frequency reported, at about 50%, in Glioblastomas (GB). Up to 40% of GB carry *EGFR* amplification (Ekstrand et al., 1991; Ohgaki et al., 2004; Wong et al., 1987) and these amplified copies are typically present as double-minute extra-chromosomal elements. Additionally, the amplified *EGFR* copies frequently have DNA sequence alterations. A number of different rearrangements have been reported including the so called EGFRvIII (or ΔEGFR), where a deletion of exons 2–7 gives rise to an in-frame transcript lacking 801 bases that codes for a constitutively active membrane protein that can not bind a ligand (Ekstrand et al., 1994; Moscatello et al., 1996; Sugawa et al., 1990).

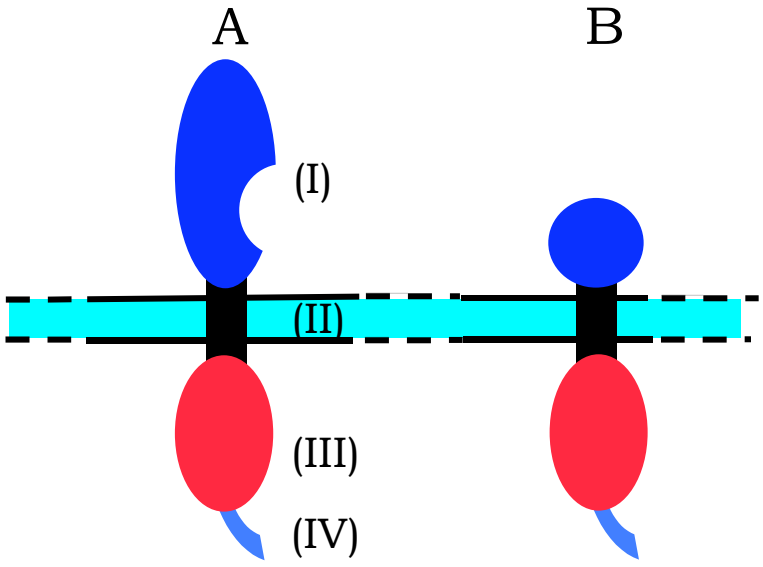

Figure 2A. Principle components of the wild-type EGFR. The N-terminal extracellular domain, including the ligand-binding part (I). The trans-membrane domain (II). The intracellular domains with the tyrosine kinase region (III) and the carboxy-terminal regulatory motifs (IV).
B. The truncated receptor variant coded by the EGFRvIII transcript. As shown here, the extracellular domain is lacking the ligand-binding region, which is a result of an in-frame deletion involving exons 2-7.

As aberrant EGFR signalling has been identified in several tumour forms, different EGFR-targeting therapies have developed, of which monoclonal antibodies and small molecule tyrosine kinase inhibitors are the most extensively tested (Baselga, 2001).

Another example of RTK signalling deregulated in many malignancies is that mediated by platelet-derived growth factors (PDGF) and their receptors. PDGF are homo- or heterodimeric ligands formed by 4 sub-units, PDGF-A, –B, -C and -D. Their corresponding receptors, the PDGF-receptor variants □ and □, also homo- or heterodimerize. The specificity of the 5 known ligand dimer complexes depends on which receptor dimer is present. Over-expression of both ligands and receptors in tumour cells forms the basis for autocrine stimulatory loops. Additionally, endothelial cells of tumour capillaries may have high levels of both PDGF-□ receptor and ligands, potentially enhancing angiogenesis as has been studied in sarcoma and GB (Nister et al., 1988; Ostman, 2004; Smits et al., 1992).

Growth factors and their receptors also mediate survival signals. This is mainly achieved via the PI3K–Akt pathway. PI3K phosphorylates PI(4,5) diphosphate to PI(3,4,5) triphosphate. The serine/threonine kinase Akt (=protein kinase B, PKB) is recognized by PI(3,4,5) triphosphate and aggregates at the inside of the cell membrane and subsequently becomes activated by phosphorylation on Thr308 by PDK-1 and another, yet unidentified, actor (Scheid & Woodgett, 2003). Akt has many targets, including several pro-apoptotic proteins that, when phosphorylated by Akt, become less likely to be activated (Di Cristofano & Pandolfi, 2000). Akt also phosphorylates and inactivates GSK-3ß (Cross et al., 1995). Inactivation of GSK-3ß, among other things, hampers its phosphorylation of cyclin D1 leading to the stabilization of cyclin D1 levels (Diehl et al., 1998). The dual phosphatase PTEN dephosphorylates PI(3,4,5) and is thus a suppressor of the PI3K–Akt pathway (Figure 1).

The *PTEN* (Phosphatase and Tensin homolog deleted on chromosome Ten) gene (also named *MMAC* or *TEP1*), located on 10q23, is a TSG that was cloned in 1997 (Li & Sun, 1997; Li et al., 1997; Steck et al., 1997) and its product have been found to have diverse functions. When PTEN is absent, the cell, in addition to lost inhibition of the PI3K–Akt pathway, increases its motility and invasiveness as well as its angiogenic capacity (Sansal & Sellers, 2004). Loss of wild-type *PTEN* (generally due to loss of one copy and mutation of the retained copy) is frequent in many cancers, *e.g.* in GB, breast and prostate cancers (Li et al., 1997). Promoter methylation, combined with hemizygous deletion, has also been observed in a high frequency in GB (Baeza et al., 2003). Inherited mutations of *PTEN* have been linked to the autosomal dominant syndromes Cowden disease, Lhermitte-Duclos disease and Bannayan-Zonana syndrome, which all are associated with multiple hamartomas (Sansal & Sellers, 2004).

Cell cycle regulation and the Rb1 pathway

Although an upregulated growth factor signalling is common in neoplastic cells, this alone does not cause a malignant phenotype. Normal cells are protected from uncontrolled proliferation by several mechanisms, including the thoroughly investigated cell cycle checkpoints. In the classical model of the cell cycle it is divided into four phases: the G1, S, G2 and M phases. During S phase (S for synthesis) the DNA is doubled to provide a complete genome for each of the two daughter cells in the end of the M phase (M for mitosis). The S and M phases are interspaced by the preparatory and strictly regulated G1 and G2 phases (G for gap). Non-proliferating cells are said to be in G0 (or quiescent). External growth factor stimulation is a necessity for a cell in G0 to re-enter the cell cycle.

A critical point in late G1 is called the Restriction point, after which cells independently of growth factor stimuli will enter S phase and progress through the cell cycle to mitosis (Pardee, 1974). The Restriction point is strictly regulated in normal cells and occurs approximately 4 hours before entry into S phase. This transition from G1 to S phase is aberrant in the majority of, if not all of, malignant cells that have been studied thoroughly. Many cancer cells are tetraploid or aneuploid, which might be explained by the fact that even G2/M transition may be deregulated in cancer cells. So far, only a few genes regulating the G2/M checkpoint have been found mutated in cancer cells. One example is the *hBUB* gene, which is found altered in colorectal cancer, lymphoma and leukaemia. Mutations in *hBUB* may lead to a deregulated mitotic checkpoint, giving rise to a

situation of genome instability associated with an increased mutation rate in affected cells (Cahill et al., 1998; Ru et al., 2002).

Cyclins and CDKs

Central players in the fine-tuned cell cycle control are cyclins and cyclin-dependent kinases (CDKs). Cyclins have a 100 amino acid long region in common, the "cyclin box", which bind to and activate CDKs. Persistent mitogenic stimulation by growth factors leads to the sequential accumulation of specific cyclin-CDK complexes that drive the cell cycle (Murray, 2004). Some cyclins and CDKs are coded by proto-oncogenes and have been intensively studied in cancer cells. Cyclin D1, coded by *CCND1* (or *BCL1*), is over-expressed in as diverse tumour types as parathyroid adenoma, lymphomas and breast cancer (Malumbres & Barbacid, 2001; Motokura et al., 1991). Cyclin E over-expression in breast cancer has been repeatedly reported and associations with poor prognosis have been suggested (Nielsen et al., 1996). CDK4 is amplified and over-expressed in a considerable fraction of GBs, malignant melanomas and breast cancers (An et al., 1999; Reifenberger et al., 1994; Tam et al., 1994), while CDK6 over-expression is more rare in epithelial tumours but is seen in some lymphomas and sarcomas (Corcoran et al., 1999; Dei Tos et al., 1997). Also point mutations of CDK4 and CDK6 that block the binding of specific inhibitors have been found in some human cancers (Easton et al., 1998; Wolfel et al., 1995).

Assembly of particular cyclins and CDKs into holoenzymes are more or less restricted to different parts of the cell cycle (Figure 3). In early G1 phase D-type cyclins associate with CDK4 and CDK6, respectively. The six possible cyclin D1, 2 and 3 and CDK 4 and 6 combinations exhibit similar functions, phosphorylating Rb1 and promoting the release of the sequestered E2F transcription factors. Cyclin D expression, its synthesis and assembly with CDK4, and the stability and activation of the complex, is strictly dependent on growth factor signalling, but is also influenced by TGFß-receptor mediated anti-proliferative signalling and survival signalling (Figure 1). Later in G1, CDK2 forms complexes with cyclin E and one of its main activities is to phosphorylate the Rb1 family of proteins. Cyclin A and D and their kinases maintain the inactivating phosphorylation of Rb1 during G2 and further on into M phase (Ludlow et al., 1993; Ludlow et al., 1990).

Levels of mitotic cyclins increase as cells progress through S- and G2 phases and peak at mitosis. The primary mitotic cyclin is the B-type that associates with CDK1 (=$p34^{cdc2}$). Cyclin A also associates with CDK1, which in mammalian cells is required for the initiation of mitosis. Both cyclin A and B are degraded during the progression through M phase. DNA damage can, just like in G1, lead to cell cycle arrest in G2 (O'Farrell, 2001).

Inhibitors of cyclins and CDKs

Cyclin D- and E-dependent kinases (CDK2, 4 and 6) are regulated by 2 groups of inhibitors: "Cip/Kip" proteins and "INK4" proteins. The "Cip/Kip" family includes $p27^{Kip1}$, $p21^{Cip1}$ and $p57^{Kip2}$ (from hereon and in the tables, only called p27, p21 and p57). Both p27 and p21 are potent inhibitors of cyclin E-bound CDK2, but not of cyclin D-CDK4 activity (Blain et al., 1997; Soos et al., 1996)(Figure 3).

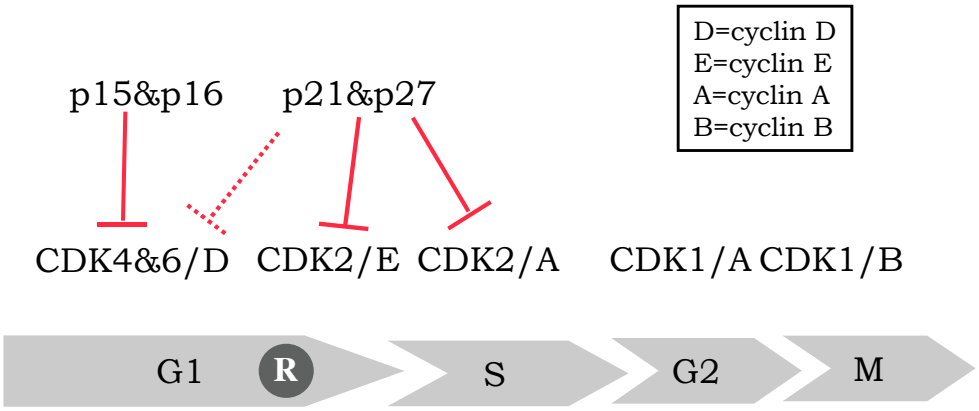

Figure 3. The cell-division cycle is usually divided into the G1, S, G2 and M phases. Growth factors (both positive and negative) exert their effect during G1 until the cycling cell has passed the restriction (R) point. Mitogens promote the synthesis of cyclin D1, which form active complexes with CDK4 and CDK6 in early G1, and these complexes phosphorylate Rb1. This leads to induction of cyclin E, followed by induction of cyclin A. p27 and p21 are potent inhibitors of cyclin E-bound CDK2, but not of cyclin D-CDK4. In the later phases, the cell cycle progression is instead driven by cyclin A and B, complexing with CDK1.

A high level of p27 is a characteristic of quiescent cells. With low levels of cyclin D1, cyclin D1-CDK4 complexes cannot form and cyclin E-CDK2 activity is suppressed by p27. Mitogen stimulation leads to induction of cyclin D1, enhancing its assembly with CDK4. Cyclin D1-CDK4 complexes sequester p27, decreasing the amount of p27 that may inhibit cyclin E-CDK2. The pool of p27 remains bound to cyclin D1-CDK4, whereas the remainder is phosphorylated by cyclin E-CDK2 triggering its ubiquitination and proteasomal degradation (Sheaff et al., 1997; Vlach et al., 1997)(Figure 1).

Members of the "INK4" proteins include $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$ (in mice) or $p14^{ARF}$ (in humans). The proteins $p16^{INK4a}$ and $p15^{INK4b}$ (from here on called just p16 and p15) are encoded by the genes *CDKN2A* and *CDKN2B*, respectively. p16 and p15 bind to CDK4 and CDK6 and block the binding of these CDKs to cyclins and thus prevent the phosphorylation of the Rb1 family of proteins (Ortega et al., 2002; Roussel, 1999). Mice lacking p16 develop normally but are prone to a wide range of tumours, particularly after exposure to chemical carcinogens or radiation (Krimpenfort et al., 2001; Sharpless et al., 2001). A progressive increase in p16 levels after each cell division argues for a role for p16 in cellular senescence (Serrano et al., 1997; Zindy et al., 1997). *CDKN2B* is located close to *CDKN2A* on 9p21. The two genes have DNA sequence similarities and are found homo- or hemizygously co-deleted in many tumour types. While the upstream events regulating p16 activities are fairly unknown, p15 is well known to be upregulated by signals mediated by the TGFß-receptor, via phosphorylation of the Smad family of proteins (Hannon & Beach, 1994; Seoane et al., 2001)(Figure 1).

It is notable that mutations affecting the Rb1 pathway genes are almost mutually exclusive, so that one "hit" (*e.g.* loss of wild-type *CDKN2A*) is unaccompanied by others. The frequency of particular genetic defects varies between tumour types but disruption of the Rb1 pathway is overall very common (Hahn & Weinberg, 2002a; Ichimura et al., 2000).

Pocket proteins

A major group of substrates for cyclin-CDK complexes is the "Rb family" of pocket proteins Rb1 (or pRb or p110), p130 and p107. They have considerable amino acid sequence homology, including multiple sites for phosphorylation by CDKs. The name "pocket proteins" comes from their evolutionary well-conserved pocket structure (Classon & Dyson, 2001). They physically interact with many proteins but their binding of the transactivation domain of members of the E2F family of transcription factors to the pocket domain is fundamental (Nevins, 2001).

Families with a high incidence of retinoblastoma, an otherwise rare disease, were described as having a dominant inheritance for the disease in early epidemiological reports (Knudson, 1971). When the targeted gene was later cloned (Friend et al., 1986; Fung et al., 1987; Lee et al., 1987) it was named *RB1* and it has become the classical example of a TSG. Individuals carrying a *RB1* germ-line mutation have close to 100% risk of getting retinoblastoma and often develop more than one primary retinoblastoma. In follow-up studies an over-risk for osteosarcoma and malignant melanoma have been found which might, at least partially, be explained by the radiotherapy given (Draper et al., 1986; Moll et al., 1996).

The human *RB1* gene, located at 13q14, is a large gene containing 27 exons that produces a 4.7 kb mRNA, translated into a 928 amino acid protein (Goodrich & Lee, 1993). *RB1* nullizygous mice suffer from early embryonic lethality (Lee et al., 1992). Many sporadic cancers show loss of wild-type *RB1*. Mutations of the other members of this family, p130 and p107, are less frequent. Mutations of the *RB2* gene, coding for p130, has however been found in lung cancer cells (Claudio et al., 2000).

Hypo-phosphorylated Rb1 binds and sequesters proteins of the E2F family, blocking their transcriptional activities (Chellappan et al., 1991). Rb1 also actively represses gene expression by recruiting histone deacetylases (HDACs) and other chromatin remodelling factors to E2F-responsive promoters (Siddiqui et al., 2003). When Rb1 becomes increasingly phosphorylated during progression through G1 its HDAC activities are repressed enabling increased expression of many genes, including E2Fs, A- and E-type cyclins and CDK2. As levels of cyclin E-CDK2 complexes then increases, Rb1 is further phosphorylated. Hyper-phosphorylated Rb1 cannot bind E2F and the cell progresses into S phase (Harbour & Dean, 2000).

The p53 pathway

Having a key role in the cellular responses to many forms of stress, p53 serves as a major obstacle that has to be overcome in order to allow tumour formation. *TP53* gene mutations are believed to be the most common genetic aberration in human cancer. Virtually all tumour-derived mutants show defects in their ability to specifically bind DNA, implying that there is a strong selective pressure to disable this p53 property during tumour development. Approximately half of all human cancers have *TP53* gene mutations and in the most of the other half is p53 activity inhibited by alternative mechanisms (Vogelstein et al., 2000).

In the late 1970's p53 was found over-expressed in various tumours and was therefore for several years suspected to be an onco-protein. Not until 10 years later was wild-type p53 documented to suppress cell transformation (Finlay et al., 1989). In 1989 came also the first report on mutations of *TP53* in human colorectal carcinomas (Baker et al., 1989). Later that year it was confirmed that *TP53* mutations are frequent in several other human tumour types (Nigro et al., 1989). Analyzing tumour DNA revealed that the most common type of *TP53* abnormality is deletion of one allele and a point mutation of the retained copy. Missense mutations are most frequent, of which >90% occur in the highly conserved sequences coding for the DNA binding domain (corresponding to amino acids 125-325) (Levine, 1997).

The *TP53* gene is located at 17q13 and spans over 20 kb and has 11 exons. The normal human p53 transcript is 2.8 kb long and is translated to a protein of 393 amino acids. Expression of the p53 protein is mainly regulated post-transcriptionally and maintained at very low levels due to a short half-life in normal, unstressed cells. In general, acetylation or phosphorylation on specific sites leads to increased p53 stability (Bode & Dong, 2004). On the basis of its different functions, the protein can be described as having different regions: an N-terminal transcription-activation domain, a highly conserved DNA-binding domain and a C-terminal region responsible for the oligomerization into a tetramer. In the tetrameric state the DNA binding conditions are optimal (Jeffrey et al., 1995). Due to this dependence on oligomerization, point mutations of one allele of *TP53* (with retention of the other allele) may have a dominant negative effect on p53 function (Willis et al., 2004). Additionally there

have been suggestions of ”gain-of-function” mutations. One example is the evidence that tumour-derived mutant p53, in contrast to wild-type p53, has the ability to transactivate promoters of various oncogenes, *e.g.* those coding for c-myc, E2F-5 and EGFR, as well as other cancer-related genes such as *MDR1* and *hMLH1* (Chin et al., 1992; Deb et al., 1994; Frazier et al., 1998; Scian et al., 2004). The different functions of the p53 protein and its regulators together form what is commonly called the p53 pathway.

Signals activating or de-activating p53

p53 is a key regulator of a number of cell cycle checkpoints. It is still not entirely clear why activation of p53 in some cell types, and under certain conditions, results in cell cycle arrest and under others induces apoptosis. Intrinsic pro-apoptosis signals, collectively described as situations of “cellular stress” (*e.g.* DNA damage, hypoxia and oncogene activation), increase the levels of p53. DNA damage is sensed by ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3-related) protein kinases (Hoeijmakers, 2001; Khanna et al., 1998). High expression of specific oncogenes, *e.g.* myc and E2F, up-regulates $p14^{ARF}$ (Bates et al., 1998; Zindy et al., 1998). The signals are transduced via various phosphorylations and acetylations of specific sites on p53 (Gu & Roeder, 1997; Sakaguchi et al., 1998).

The control of p53 protein levels is orchestrated by multiple factors with the E3 ubiquitin ligase MDM2 considered to be one of the major regulators. MDM2 binds to p53 and inhibits its function by concealing the activation domain of p53 (Momand et al., 1992) and transfers it to the cytosol for degradation. Under otherwise normal conditions DNA damage leads to phosphorylation of the MDM2 binding site of p53 inhibiting MDM2 binding and p53 rapidly accumulates (Prives & Hall, 1999). MDM2 is coded by a proto-oncogene situated 4 Megabases from the *CDK4* gene on chromosome 12q13-14. Under normal conditions MDM2 is upregulated by p53 via transcriptional activation, creating a negative feedback loop for p53 levels (Piette et al., 1997).

Another important regulator of the p53 pathway is $p14^{ARF}$, first described in 1997 (Kamijo et al., 1997). The $p14^{ARF}$ protein is encoded using a unique promoter and first exon, followed by an alternative reading frame of exon 2 and 3 of the *CDKN2A* gene on 9p21 (Quelle et al., 1995). The *p14ARF* gene includes the unique exon 1β located between exon 1α of *CDKN2A* and exon 2 of *CDKN2B*. The $p14^{ARF}$ protein can physically interact with both p53 and MDM2 and thereby inhibits MDM2-mediated degradation of p53 (Kamijo et al., 1998; Zhang et al., 1998).

The ATM and ATR proteins are activated in response to DNA damage, but it is not entirely clear how. ATM plays an important role in initiating DNA repair checkpoint by the phosphorylation of several proteins such as p53, MDM2, BRCA1 and Chk2. ATR shares a number of substrates with ATM but its function is less well studied (Giaccia & Kastan, 1998; Iliakis et al., 2003).

Cellular effects of p53

Binding to DNA in a sequence-specific manner, p53 functions as a transcription activator of several genes. One primary target for cell cycle control by p53 is through transcriptional induction of the *p21* gene. p21 can bind and inhibit cyclin–CDK complexes and PCNA (proliferating cell nuclear antigen) protein, inhibiting DNA replication (Prives & Hall, 1999). p53 also transactivates the *GADD45* (growth arrest and DNA damage inducible) gene, whose product binds directly to PCNA (Azam et al., 2001).

Apoptosis can be induced in physiological or pathological processes, resulting in the elimination of individual cells. Cell death during apoptosis results from an energy-dependent, endogenous cellular process, which in normal cells is initiated either via an extrinsic, receptor-mediated pathway or via an intrinsic pathway. Important regulators of apoptosis are the Bcl-2 family of proteins. Members of this family all have amino acid sequence similarities, but some members are anti-apoptotic, including Bcl-2, while others, *e.g.* Bax, Noxa and Puma are pro-apoptotic (Cory et al., 2003).

Pro-apoptotic signals activate a family of cystein proteases called caspases, which can be divided into initiator and effector caspases. They are first synthesized as inert zymogens known as pro-caspases. When cleaved and activated caspases oligomerize and can, in turn, cleave and activate a number of different proteins, including caspase family members, such as pro-caspase 3 and 7, thus causing an irreversible cascade effect. Eventually fundamental cell structures, including the DNA, are degraded. The remaining polypeptide and nucleic acid fragments are captured in liposomes and subsequently phagocytosed and endocytosed by surrounding cells and re-cycled (Lowe et al., 2004).

Bax gene transactivation is an important part of p53-induced apoptosis and over-expression of Bax is sufficient to induce cell death (Miyashita & Reed, 1995). The pro-apoptotic bcl-2 family member Bax forms homodimers causing cytochrome c release from the mitochondrial intermembrane space. Cytochrome c then interacts with Apaf-1 and caspase 9 forming the active caspase complex called the apoptosome, that subsequently activates effector caspases such as caspase 3 (Lowe et al., 2004). Other proteins of the intrinsic apoptotic pathway induced by p53 are the pro-apoptotic Noxa and Puma (Oda et al., 2000; Yu et al., 2001). Wild-type p53 is also known to be a transcriptional repressor of *e.g.* Bcl-2, that is located at the outer mitochondrial membrane (Miyashita et al., 1994), and which gene first was found to be involved in a t(14:18) translocation in malignant B-cells (Tsujimoto et al., 1984). In addition, p53 transactivates pro-apoptotic genes of the extrinsic pathway coding for so called death receptors of the tumour necrosis factor (TNF) receptor family (Owen-Schaub et al., 1995; Wu et al., 1997).

The p53 pathway and cancer

Disruption of the p53 pathway, and/or other defects in the regulation of apoptosis, is shared by most malignant cells. The consequence of this is not only an unregulated proliferation, but also that cells may become protected from therapy-induced cell death (Johnstone et al., 2002; Lewanski & Gullick, 2001). As in the case of Rb1 pathway abnormalities, are genes associated with the p53 pathway commonly altered and each occurs in an almost mutually exclusive fashion. Common alternative p53 pathway disrupting gene alterations such as *MDM2* amplification, which is seen in the highest frequencies in soft tissue sarcomas, or loss of wild-type $p14^{ARF}$, is preferably found in tumours harbouring wild-type *TP53* (Ichimura et al., 2000; Momand et al., 1998; Reifenberger et al., 1993; Sherr, 2001). Apart from its association with the cancer syndrome ataxia telangiectasia, are somatic mutations in *ATM* found in haematological malignancies while ATR mutations have not been associated with human disease (Gronbaek et al., 2002; Iliakis et al., 2003). Mutations of the *bax* gene are observed in *e.g.* colon cancer (Rampino et al., 1997).

Connections between Rb1 and p53 pathways in cancer

As mentioned, the regulation of each pathway is complex. There are also examples of "cross-talk" between them. That the Rb1 and p53 pathways are connected can be exemplified with the ability of E2F to induce $p14^{ARF}$ transcription (Bates et al., 1998; DeGregori et al., 1997). Another connection is encountered by the geographical proximity of the *CDK4* and *MDM2* genes on chromosome 12. When co-amplified, *e.g.* in GB (Reifenberger et al., 1994)**,** both pathways are immediately disrupted. Another variant of hitting both pathways with one single genetic event is mutating the 9p21 locus targeting the *CDKN2A* and *p14ARF* (and sometimes also *CDKN2B*) genes, *e.g.* seen in GB, malignant melanoma and leukaemia (Calero Moreno et al., 2002; Ichimura et al., 2000; Krug et al., 2002; Sharpless & Chin, 2003). It has also been reported that MDM2 can physically interact with and inhibit Rb1 (Xiao et al., 1995) and, as mentioned, the transcriptional activation of p21 by p53 inhibits cyclin/CDK-mediated phosphorylation of Rb1 (Prives & Hall, 1999).

Introduction to primary CNS tumours

Types

In Sweden about 1100 primary central nervous system (CNS) tumours are diagnosed each year (according to Swedish Cancer Registry data) (Swedish National Board of Health and Welfare, 2004 online). The majority are malignant. They are classified by histopathological evaluation on empirically derived criteria. Because of the many different tumour types with a wide range of biological aggressiveness, it is highly important to carefully consider the diagnosis, both in patient management and in clinical research. A major group of tumours of the neuro-epithelial tissues are called gliomas, which is the most common type in adults. Among gliomas astrocytic tumours are most frequent, followed by oligoastrocytic and oligodendrocytic gliomas (Kleihues & Cavanee, 2000). They are composed of cells resembling the phenotype of normal astrocytes. The most common benign CNS tumour is meningioma, which as the name indicates, is a tumour of the meninges and it accounts for about 1/3 of all primary CNS tumours (Swedish National Board of Health and Welfare, 2004 online).

Astrocytic tumours are subdivided on the basis of histological grading criteria into grades I-IV. Astrocytomas of grade I, also called Pilocytic Astrocytomas, are rare in adults and have a favourable prognosis if optimally treated by surgery. These tumours will not be considered further in this thesis. Astrocytomas of grade II-IV occur most commonly in the cerebral hemispheres and are more frequent in males than in females. Astrocytoma grade II (AII) has a peak incidence in young adults. Histologically the tumours are made up of fairly well differentiated tumour cells with a moderate increase in cellularity and few, if any, mitoses. Radiologically AII can generally be differentiated from other grades of astrocytic tumour by the absence of contrast enhancement. Astrocytoma grade III, or Anaplastic Astrocytoma (AA), are more cellular, show greater levels of dysplasia and mitoses are relatively common. The peak incidence is in early middle age.

Astrocytoma grade IV, or Glioblastoma (GB), is the most malignant among astrocytic tumours and has the poorest prognosis of all primary tumours of the CNS. Although the peak incidence comes is in late middle age they can occur at any age, with even case reports of *in utero* diagnosed GB published (Doren et al., 1998; Sylvestre & Sherer, 1998). GBs are on a clinical basis frequently divided into “primary” and ”secondary” GBs. These terms were introduced by H.J. Scherer already in 1940 (Scherer, 1940). By definition ”primary” (or ”*de novo*”) GBs are diagnosed when there is no evidence of a previous astrocytic tumour of lower grade. ”Secondary” GBs occur in patients who have had a diagnosis of an astrocytic tumour of lower grade. Histologically the two groups are indistinguishable.

GB histology is characterized by high cellularity, nuclear pleomorphism and prominent microvascular proliferation. Areas of necrosis are commonly seen. However, within the same tumour there are often different areas with varying histological appearance. There may, for instance, be a mix of areas with high and low proliferation rate as well as varying expression of astrocyte lineage markers, such as the intermediate filament protein GFAP (glial fibrillary acidic protein). These examples of intra-tumoural variations underscores the diagnostic limitations of a small biopsy as compared to a well sampled operation specimen.

Epidemiology and aetiology

An increase in primary CNS tumour incidence during the last few decades has been observed, which has created a concern about some modern environmental and life-style factors. However, the general view to day is that the increase, mainly seen in around 1980 in many countries, is explained by improved diagnostic procedures with the introduction of CT and MRI and the development of the stereotactic biopsy technique, increasing our ability to make an accurate diagnosis both in paediatric patients and in the elderly (Gurney & Kadan-Lottick, 2001; Krieger et al., 1998; Legler et al., 1999; Lonn et al., 2004b; Smith et al., 1998).

With the exception of radiation there are no well-documented factors that are known to increase the incidence of CNS tumours. In the groups exposed to therapeutic radiation it is the incidence of meningioma that from the literature appears to be most markedly increased. Several cancer syndromes are associated with an excess risk of specific types of primary CNS tumours, but these can only help to explain a small fraction of all cases. Thus, the vast majority of primary CNS tumours are considered sporadic and their aetiology is largely unknown (Inskip et al., 1995; Marsh & Zori, 2002). However, some epidemiological reports indicate that as yet unidentified hereditary components may be important (Grossman et al., 1999; Malmer et al., 2003). There may exist more complex genetic factors than those that explain the monogenic cancer predisposition syndromes that have been relatively well characterized so far. However, the reported associations may also be proven to be chance findings or explained by environmental factors shared between relatives.

Environmental factors

Finding out what causes a disease is fundamental for both understanding the biology of the disease and to identify suggestive preventive measures. So far, ionizing radiation is believed to be the most important external factor increasing the risk of developing CNS tumours. This applies to both the malignant tumours, such as the gliomas, and the benign tumours, such as meningiomas, and possibly neurinomas. An excessive risk is found at as low therapeutic doses as 1 Gy (Ron et al., 1988). Atomic bomb survivors have had an excess risk of developing nervous system tumours, and the risk was found related to the exposure dose. The highest risk estimates are seen for neurinomas (Preston et al., 2002). The data in support of diagnostic X-rays increasing the incidence of CNS tumours is much more weak and contradictory, as is the evidence for an association between exposure to electromagnetic fields and brain tumours (Inskip et al., 1995; Kleinerman et al., 2005). Some occupations, *e.g.* physicians, nurses, dentists and other medical workers, have been reported to have an elevated risk (McLaughlin et al., 1987). These results were however not confirmed in a large recent study of occupation and glioma (Schlehofer et al., 2005). The use of cellular phones has increased dramatically in some countries, including Sweden. One recent Swedish study reported an elevation of the relative risk of developing a vestibular neurinoma after the use of mobile phones for >10 years to 1.9 and when restricting to tumours on the same side of the head as the phone was normally used, the relative risk was 3.9 (Lonn et al., 2004a).

Hereditary factors

Hereditary cancer syndromes are often, but not always, associated with germ-line mutations of tumour suppressor genes. For some familial aggregations including primary brain tumours a monogenetic background has been suggested: The LiFraumeni syndrome includes early occurrence of soft tissue sarcomas, breast cancer, astrocytic tumours, medulloblastoma, meningioma and neurinoma and is most often explained by germ-line mutations of *TP53* (Li & Fraumeni, 1969; Malkin et al., 1990; Srivastava et al., 1990). Later studies have revealed that a similar familial clustering of tumours could instead be due to inherited mutations in *hCHK2*, which codes for a DNA-damage activated protein that normally phosphorylates and stabilizes p53 (Bell et al., 1999).

Multiple endocrine neoplasia (MEN) type 1 is characterized by the occurrence of endocrine tumours and hyperparathyroidism in young adulthood. Meningioma is also over-represented and there are case reports on spinal ependymoma (Zarnegar et al., 2002). Neurofibromatosis type 1 (NF1) patients have germ-line mutations in *NF1*, a large gene on 17q12 carrying 3 other genes in one of its introns, and develop neurofibromas, sarcomas, phaeochromocytomas and optic nerve Pilocytic Astrocytomas (Lakkis & Tennekoon, 2000). The hallmark of neurofibromatosis, type 2 (NF2), is the development of bilateral vestibular neurinoma, but meningioma and low-grade gliomas are also overrepresented (Evans et al., 2000).

Familial clustering of primary CNS tumours and colorectal carcinomas define Turcot's syndrome. Epidemiologically and genetically this syndrome has been split into at least 2 variants; one with familial adenomatosis polyposis (FAP), associated with inborn *APC* mutations, colorectal polyposis

and medulloblastoma and one with mismatch repair gene mutations causing non-polyposis colorectal carcinoma (HNPCC) and GB (Hamilton et al., 1995).

Cowden's disease is a syndrome characterized by hamartomas at multiple sites, including in the CNS, and cerebellar dysplastic gangliocytomas. The cloning of *PTEN* in 1997 was quickly followed by the discovery of *PTEN* germ-line mutations in 80% of these families (Liaw et al., 1997; Zhou et al., 2003).

Clinical aspects

The metastasis process explains most cancer-related deaths. However, with the exception of the phenomenon of seeding, primary CNS tumours rarely metastasize. Particularly when intra-cranially located, it is usually instead uncontrolled growth on the primary site and/or local recurrences that, due to local mass effect and increased intracranial pressure, is lethal. The limitation in space for both intracranial and spinal tumours can cause morbidity and mortality even if histologically benign.

The most common presenting symptoms are headache, typically in the morning and sometimes combined with nausea and even cascade vomiting, seizures, focal neurological deficits and mental deterioration. When the clinical history and physical examination leads to a suspicion of a CNS tumour the patient is generally investigated with computer tomography (CT) or magnetic resonance imaging (MRI) with and without contrast added. The result of such radiological examinations will decide the further management of the patient. In order to determine the diagnosis accurately, access to the affected tissue for microscopic examination is usually necessary. This is, when possible, done performing a biopsy or an operation.

Surgical treatment

The number one treatment choice in most newly diagnosed primary CNS tumours is surgery. For benign CNS tumours this is often the only therapy needed, but benign and malignant tumours may be located in such critical regions that an extensive operation is hazardous. In high-grade CNS tumours the ability to define the tumour margins can be difficult despite the advances in microscopic surgery and radiological techniques. CT and MRI provide limited information regarding tumour extent and contrast enhancement only highlights the tumour where there is leakage of contrast through a damaged blood-brain barrier. With a heterogeneous tumour, as is often the case in GB, the tumour size may be underestimated. Additionally, GB cells may invade far from the primary site, as has been revealed by autopsy studies (Halperin et al., 1988). The radiologically defined tumour size can also markedly change due to variations in the amount of tumour necrosis and surrounding oedema. Steroid treatment may also affect the appearance of the tumour by reducing the oedema. These difficulties are also a concern when trying to evaluate treatment response, as well as early detection of a local recurrence, during follow-up (Kaplan, 1998).

Even with these limitations and the lack of curative treatment for GB patients, surgery can meaningfully reduce the mass effect and thus decrease the symptoms and the dose levels of steroids needed. Surgery may therefore be an important part of the individualized patient management also at recurrence or progression (Kreth et al., 1999).

Oncological treatment for astrocytic tumours

Unfortunately, most brain tumours are relatively insensitive to the standard oncological treatments radiotherapy (RT) and chemotherapy. As in the case of surgery there are a number of reasons for this. Gliomas, and in particular GBs, are considered to be rather radioresistant (Schultz & Geard, 1990), while the maximum tolerated total dose to the normal brain tissue when the radiation is administered is considered to be between 60 and 70 Gy. At these and higher doses there is a steep escalation in the probability of radionecrosis of the normal tissue (Emami et al., 1991). Radionecrosis is a feared "late" side-effect of CNS irradiation. It may occur months or years later and

is the major reason why conventional radiotherapy cannot be repeated at recurrence. The "early" side-effects are mainly referred to an increased oedema in the tumour area, which may require steroid treatment. Thus, there are difficulties in finding a "therapeutic window" for GB patients. Nevertheless, adjuvant post-operative RT is used regularly in treating both AA and GB patients, based on empirical data showing a survival benefit up to a total dose of 60 Gy (Kristiansen et al., 1981; Walker et al., 1979). Doses are usually administered in 1.8 or 2.0 Gy fractions daily 5 days per week to a total dose of 50-60 Gy. It has been debated if such RT, stretching over 5 to 6 weeks, is beneficial to older patients as well as other patients who have a low performance status (as measured for instance by the Karnofsky index), and thus have a very bad prognosis. To shorten the treatment time for these groups hypofractionated schedules (*i.e.* using fewer fractions and higher dose per fraction) have been tested and in some clinical trials seem to be as effective as the standard schedules (Laperriere et al., 2002). Hypofractionation is though likely to increase the risk of severe "late" side-effects and should therefore be restricted to poor-prognosis patients. Another area of debate is whether RT should be given to patients with low-grade gliomas. While there is consensus on benefits of RT, the timing, either post-operatively or at the time of recurrence or progression, is still debated (Kortmann, 2003; Mirimanoff & Stupp, 2003; Stupp & Baumert, 2003).

Due to the very small fraction of long-time GB survivors following standard therapy, surgery and RT, many attempts to improve patient survival using antitumoural agents have been carried out. When chemotherapy is used systemically it is generally administered orally or intravenously. This concept has its limitations in achieving an appropriate drug concentration in the tumour cells due to the blood-brain barrier (BBB), at least if the BBB is not completely disrupted. In addition, there is an upregulation of the P450 enzyme in the many patients treated with anti-convulsants to prevent seizures, which will have an impact on the efficacy of the chemotherapy (Vecht et al., 2003). Some large studies conclude that there is a slight survival advantage when using chemotherapy in the adjuvant setting, given after surgery and RT (Fine et al., 1993; Stewart, 2002). The drugs most commonly used are carmustine (BCNU) and lomustine (CCNU). These alkylating agents have been used for decades as monotherapy, both inside and outside clinical trials, mainly at the time of recurrence or progression. More recently has the alkylating drug temozolamide been introduced. The most commonly used polychemotherapy regimen has been PCV (procarbazine, CCNU and vincristine) and in an early trial it was found superior to BCNU alone in AA patients (Levin et al., 1990). Later studies have instead found PCV not to be superior to single agents in neither AA nor GB patients (MRCT, 2001; Prados et al., 1999).

Prognostic factors

As mentioned, GB is the CNS tumour subtype associated with the worst prognosis. In most studies median survival is shorter than 1 year (Barker et al., 1996b; Ohgaki et al., 2004) and only about 2% are alive at 5 years (McLendon & Halperin, 2003). AII patients have a comparably favourable prognosis (median survival approximately 7 years) while the AA group have a more variable survival, with a median survival intermediate to that of AII and GB (Herfarth et al., 2001; Kleihues & Cavanee, 2000; Lin et al., 2003). Thus, the grading system of adult astrocytic gliomas (according to WHO) provides important prognostic information. Since GB, AA and AII also differ in the age distribution, proliferation rates, frequencies of specific mutations and incidence rates, attempts to correlate clinical, histological or molecular findings with survival will be biased if histological grading is not considered. Within each grade young age is generally accepted as a positive prognostic factor (Burger & Green, 1987; Lote et al., 1997; Perry et al., 1999; Salminen et al., 1996) as is a high performance status score at the time of diagnosis (Curran et al., 1993; Leighton et al., 1997; Lote et al., 1997; Salminen et al., 1996).

"Secondary" GBs are generally considered to have a better prognosis than "primary" GBs (Kleihues & Cavanee, 2000). This may be explained by the fact that "secondary" GB occur at younger age, but there are also some indications that "primary" and "secondary" GB have different biological characteristics and show different responses to therapy (Kleihues & Ohgaki, 1999). A similar

difference in age distribution and prognosis has been suggested for anaplastic gliomas that have developed from lower grade as compared those that have arisen "de novo" (Winger et al., 1989). This was not confirmed in a case-control study (Dropcho & Soong, 1996).

The proliferation rate of a tumour is a potential indicator of prognosis. With tumours showing marked heterogeneity and a mix of areas with high and low proliferation rates, which is often the case in AAs and GBs, reliable proliferation indices and relevant cut-off levels can be hard to determine. Studies using immunohistochemistry, identifying the proliferation marker Ki-67 and/or assessing a mitotic index (number of cells in mitosis divided by the total number of cells) have shown varying results. One study of grade III gliomas identified Ki-67 as an independent negative prognostic factor (Tortosa et al., 2003). While in this study the majority of patients were AA, survival analysis was performed mixing AA patients with Anaplastic Oligoastrocytoma and Anaplastic Oligodendroglioma patients. In a study of only AA, presence of >1 mitotic cell per specimen examined was found to be a negative predictor of outcome in univariate analysis (Korshunov et al., 2002), while another large study of AAs was inconclusive (Perry et al., 1999). In a study of only GBs the fraction of GBs with no histological or radiological signs of tumour necrosis at diagnosis was found to have a favourable prognosis (Barker et al., 1996a).

Other factors that may contribute to the prognosis are treatment related. As mentioned post-operative RT is generally believed to improve survival for AA and GB patients in total doses up to 60 Gy (Walker et al., 1979). Studies on the extent of surgery show that surgery is superior to biopsy alone, while the extent of surgery tends to be of less importance (Kowalczuk et al., 1997; Lacroix et al., 2001; Simpson et al., 1993). Studies on RT and surgery should be performed as randomized trials, or otherwise there exists the potential bias that the patients with the youngest age and highest performance status, *i.e.* the patients with the best prognosis, are more likely to receive extensive surgery and full-dose RT.

Nevertheless, more refined prognostic and treatment predictive tools would be of value. With the enormous amount of molecular and genetic data on astrocytic tumours that has accumulated during the last 20 years it has been quite natural that attempts have been made to see whether this information is clinically useful or not.

Genes and prognosis

More than a dozen genes have been identified as involved in the development of human astrocytic gliomas. Loss of both wild-type copies of the TSGs *CDKN2A*, *CDKN2B*, *p14^{ARF}* and *PTEN* are found in relatively high frequencies in GB (He, 1995 #228;Ichimura, 2000 #115;Rasheed, 1994 #167;Ueki, 1996 #172;Reifenberger, 1993 #252;Schmidt, 1999 #169]. The losses are regularly due to homozygous deletions or deletion of one allele and point mutations or small deletions of the other allele. Such losses of these TSGs are to some extent found also in AA but rarely in AII (Ichimura et al., 2000; Kraus et al., 2000; Schmidt et al., 1999; Ueki et al., 1996). Hemizygous deletions of *RB1* are relatively frequent in GBs and AAs, while losses of both wild-type copies are only found in GBs (Ichimura et al., 2000; Ueki et al., 1996). *TP53* mutations are instead more common in AII and AA than in GB (Ichimura et al., 2000; Rasheed et al., 1994). Amplification of proto-oncogenes is almost restricted to GBs (Ichimura et al., 2000; Smith et al., 2001). The most frequently amplified proto-oncogenes in GBs are *EGFR* followed by *CDK4* and *MDM2* (Ekstrand et al., 1991; Ohgaki et al., 2004; Olson et al., 1998; Reifenberger et al., 1993), while amplifications of *MDM4, CDK6, CCND1, CCND3* and *PDGFA-R* only have been reported in a small number of cases (Buschges et al., 1999; Costello et al., 1997; Galanis et al., 1998; Riemenschneider et al., 1999; Smith et al., 2000).

Several studies have attempted to correlate the molecular genetic data with astrocytic glioma patient outcome. Since GB is both the most common type and the one best genetically characterized, correlation studies on GB patients are most prevalent, but have to-date not provided any convincing results (Galanis et al., 1998; James et al., 1999; Kraus et al., 2000; Ohgaki et al., 2004; Olson et al.,

1998; Rasheed et al., 2002; Simmons et al., 2001). Many studies have mixed tumour types and/or malignancy grades in their analyses, and used various definitions of anomalies of tumour suppressor genes and of amplification of oncogenes. A study of paediatric astrocytic tumours showed an association between loss of wild-type *PTEN* and poor outcome when AA and GB patients were analyzed together (Raffel et al., 1999). One study of 63 AA (including tumours only biopsied) analyzed alterations of the *EGFR*, *PTEN* and *TP53* genes and showed a negative association between loss of a functional *PTEN* and survival and a positive association between *TP53* mutations and survival (Smith et al., 2001). In AII, since there are few known targeted genes other than *TP53* mutations, most studies of AII patients have focused on p53 mutations and/or expression levels and survival, but have failed to find any clear associations (Kraus et al., 1994; Shinoda et al., 1998; Watanabe et al., 1996).

Thus, seemingly little of clinical value has come out of previous studies, considering the large amount of genetic data available.

AIMS OF THE THESIS

The overall aim of the thesis was to increase our understanding of the aetiology, molecular biology and prognosis of human CNS tumours. More specifically the aims were as follows:

• The primary aim of **Paper I** and **II** was to identify genetic prognostic markers in human Glioblastoma and Anaplastic Astrocytoma by analyzing the 9 cancer genes that are most commonly altered and believed to be important in the development of astrocytic tumours.

• The primary aim of **Paper III** was to characterize a large series of astrocytic tumours of various grades regarding amplification and rearrangement of the epidermal growth factor receptor (EGFR) gene and to correlate the findings to patient survival.

• The primary aim of **Paper IV** was to explore if there exists an association between the occurrence of CNS tumours and parathyroid adenomas.

MATERIALS AND METHODS

Patients and tumour tissues (Paper I-III)

During 1987-1997 the clinical group at the Ludwig Institute for Cancer Research in Stockholm collected, with detailed patient informed consent and Ethical committee approvals, >700 primary CNS tumours. Blood samples were collected from the majority of patients, making it possible to pair-wise compare tumour DNA with the same individuals' white blood cell DNA. This large series of tumours, collected from both paediatric and adult patients, include a number of different histological diagnoses. In some cases tumour tissue was obtained only from a second or third operation, while for a small number of patients tumour tissue from more than one operation is included in the series. Tissue was only allocated for research when the amount of material from an operation exceeded that needed for clinical and histopathological investigation.

The objective was to learn more about the genetic abnormalities of tumour cells and their biology, eventually developing information that could be of clinical value and lead to new innovative and successful therapies. The initial goal was to molecularly and genetically characterize different common types of primary CNS tumours. The results of the work has been published in several scientific articles, of which some include tumours studied for **Paper I-III**, *e.g.* (Ichimura et al., 2000; Ichimura et al., 1996; Ichimura et al., 1998; Liu et al., 1998; Liu et al., 2000; Miyakawa et al., 2000; Reifenberger et al., 1993; Reifenberger et al., 1996; Schmidt et al., 1999; Schmidt et al., 1994). Each tumour was given a publication number, which is constant in all publications.

Since astrocytic tumours are the most common type of malignant brain tumours in adults, many of the studies have been on these tumours. We reviewed the Swedish Cancer Registry (SCR) and found that about 20% of newly diagnosed GBs in Stockholm and Gothenburg areas are included in our tissue bank. The patient material was found to be representative regarding age and sex, although the mean age was slightly lower than the mean for all cases in the SCR, probably due to the fact that tumour resection was a prerequisite for inclusion in our studies.

Over the years the amount of detailed genetic data on the individual tumours accumulated and the ambition to also collect clinical data, and then try to correlate the data with patient survival, emerged.

The results presented in **Paper I** are based on detailed studies of 9 genes in a total of 130 Glioblastoma (GB) patients and the results in **Paper II** are based on detailed studies of the same 9 genes in 37 Anaplastic Astrocytoma (AA) patients, all from the period 1989-1994. During this time period the WHO histopathological criteria from 1979 were modified, why all cases were re-evaluated according to the WHO classifications of 1993 and 2000 (Kleihues, 1993; Kleihues & Cavanee, 2000). Initially 140 GB and 42 AA were included for mutation analysis and clinical follow-up. One GB and 4 AA were excluded after histopathological re-evaluation, because the diagnoses had to be modified according to the new criteria or there were significant diagnostic doubts. One AA patient from abroad could not be followed-up and had to be excluded. For the GB study (**Paper I**) 10 of the 140 GBs were from patients operated for a GB at least once before. Since we had not access to tumour tissues for molecular genetic analysis from their first GB operation, they were excluded from the correlation analyses of genetic abnormalities and survival. One of these recurrent GB patients was, however, included in the descriptive part of mutation rates in "secondary" GBs since that patient also fulfilled the criteria of a "secondary" GB (see below).

For later projects, including the study in **Paper III**, the series was expanded with additional cases from Karolinska Hospital and Sahlgrenska University Hospital (from 1988 to 1997), including Astrocytoma grade II patients. When necessary, the diagnoses were revised according to the most recent WHO classification (Kleihues & Cavanee, 2000). In total 263 patients were first included for

EGFR analysis and collection of clinical data. Of the 23 AII originally included, we had only access to tissue from the second operation from 2 of the patients and one cerebellar tumour was re-diagnosed as a PA. Of the 59 AAs originally included, one cerebellar tumour was re-diagnosed as a PA while 17 were "recurrent" tumours. Of the 181 GBs originally included, the 21 "recurrent" tumours were excluded. Thus, the final results presented in **Paper III** are based on 221 patients (160 GB, 41 AA and 20 AII).

To summarize, the total number of astrocytic glioma patients included in **Paper I-III** was 246 (172 GB, 54 AA and 20 AII). **Paper I** included 130 GB, **Paper II** 37 AA. Compared to these patient series, additional cases, including AII patients, were included for **Paper III**. Meanwhile were 12 GB and 13 AA patients studied in **Paper I** and **Paper II** excluded in **Paper III**, mainly because that study only focused on "primary", "non-recurrent" patients.

All tumours were extensively sampled (up to 15 blocks). Each tumour was analyzed comparing tumour DNA with the individual patient's white blood cell DNA. Tumour DNA was isolated form tumour pieces with high tumour cell count, generally at least 80% tumour cells as judged histologically. Each piece of tissue used for genetic analysis was histologically characterized to ensure that tumour tissue was being studied. This was confirmed by microsatellite analysis of the tumour DNA, which showed genetic abnormalities in all cases (*e.g.* loss of heterozygousity, LOH, at a number of sites) (Ichimura et al., 1998; Miyakawa et al., 2000).

The clinical data was in general collected from the individual patients' medical records from the different hospitals where they were treated and/or followed-up. Clinical data included age and sex of the patient, tumour localization, duration and type of symptoms before diagnosis, date of operation(s), radiotherapy and chemotherapy given and date of death (which was obtained from the SCR). In general the operations were gross total, no cases with only biopsy were included. The minimum follow-up time of GB patients alive at end of follow-up was 6.7 years, of AA patients 10.3 years and of AII patients 8.9 years. We also intended to collect the following clinical data: previous medical history, family history and performance status of the patients at the time of diagnosis and residual tumour volume after surgery. These factors had not been routinely documented and could therefore not be included in the retrospective approach applied.

Definition of "primary", "secondary" and "recurrent" tumours

In **Paper I-III**, and in this thesis, the terms "primary", "secondary" and "recurrent" are used. As mentioned, GBs are often divided into "primary" or "secondary", referring to whether a patient when diagnosed with a GB had a history of a prior histological diagnosis of an astrocytic tumour of lower grade (="secondary" GB) or not (="primary" GB). **Paper I** included 122 "primary" and 8 "secondary" GBs. In **Paper II** a similar definition of "primary" and "secondary" to that for GB in **Paper I** was used for AA. Of the 37 AA patients included 25 (68%) were "primary" AA. Six patients (16%) were "secondary" AA, *i.e.* they had a prior histological diagnosis of an AII. Six (16%) patients previously operated for an AA were also included and these were referred to as "recurrent" AA. **Paper III** included only "primary" AII, AA and GB.

Genetic analysis (Paper I-III)

By comparing tumour cell DNA with the individuals' white blood cell DNA, deletions, mutations, rearrangements and amplifications of genes were identified. In **Paper I** and **II** the tumour suppressor genes (TSGs) *CDKN2A, CDKN2B, RB1, TP53*, $p14^{ARF}$ and *PTEN* were first analysed for allele copy number by either Southern blot analysis, microsatellite analysis or multiplex Polymerase Chain Reaction (PCR). The latter method was necessary for *PTEN* analysis due to pseudogenes. The allele copy number analysis was followed by mutation screening using single-strand conformational polymorphism (SSCP) or denaturing gradient gel electrophoresis (DGGE) and sequencing to identify the somatic mutations in the retained TSG copies (Ichimura et al., 2000; Ichimura et al., 1996;

Schmidt et al., 1999). This allowed an assessment of the status of both alleles of each tumour suppressor gene. In addition the presence or absence of amplification of the proto-oncogenes *CDK4*, *MDM2* and *EGFR* was determined using microsatellite analysis and Southern blotting with densitometry (Ichimura et al., 1994; Liu et al., 2000; Reifenberger et al., 1993).

To characterize *EGFR* in **Paper III**, some additional methods were used. To determine the *EGFR* copy number was, in addition to Southern blotting, real time quantitative PCR performed on tumours where there was limited DNA. To differentiate the wild-type transcript from the EGFRvIII transcript we performed discriminative RT-PCR, and Ribonuclease Protection Assay was used in selected cases (particularly in those where there was evidence of an EGFRvIII transcript in the absence of amplification).

Definitions of gene status as "normal" or "abnormal"

To perform a statistical analysis correlating gene status with patient age and survival all genetic data in **Paper I-III** were categorized as "normal" or "abnormal" (or "missing"):

In **Paper I and II** the tumour suppressor genes *CDKN2A, CDKN2B, RB1, p14^{ARF}* and *PTEN* were considered "abnormal" when there was no wild-type allele present, either due to homozygous deletion or deletion of one allele and mutation of the other. Hemizygous deletion and retention of one wild-type allele was categorized as "normal", based on the generally unknown impact of a hemizygous deletion on the gene product levels. *TP53* was considered "abnormal" whenever a somatic mutation was identified in one or more alleles. As the protein functions as a tetramer a single mutated monomer included in such a tetramer may disrupt tetramer function (Levine, 1997; Willis et al., 2004). Hemizygous deletion of *TP53* without detectable mutation of the retained allele was categorized as "normal" as in the case of the other TSGs.

For the oncogenes*EGFR, CDK4* and *MDM2*, gene amplification was categorized as "abnormal". In **Paper I and II** the border ≥5 copies/genome in average for amplification was chosen to exclude the risk of falsely assessing rapidly proliferating or tetraploid tumours as having amplification (due to many cells in late S phase or beginning of M phase with 4 copies of each autosome). In **Paper III**, *EGFR* was considered amplified and categorized as "abnormal" if ≥7 wild-type copies/genome, also considering the fact that trisomy of chromosome 7 is common in astrocytic glioma. However, in the majority of tumours with gene amplification, the copy number exceeded these borders widely. In the case of *EGFR* an average of >100 copies per genome was not unusual. Regarding expression of the EGFRvIII transcript was presence classified as "abnormal", absence of EGFRvIII as "normal" in **Paper III**.

When categorizing the pathways, if at least one of the genes coding for a protein involved in the p53 pathway (*p14^{ARF}, MDM2* and *TP53*) or the Rb1 pathway (*CDKN2A*, *CDKN2B*, *RB1*, and *CDK4*) was "abnormal" the pathway was categorized as "abnormal". Only when all genes studied in a pathway were categorized as "normal" was the pathway categorized as "normal".

The genetic data for **Paper I** was complete for 7 of the studied genes, but for 4 GB *PTEN* data was missing and 3 of these also had missing *EGFR* data (due to insufficient amounts of tumour DNA). The genetic data for **Paper II** was complete. For **Paper III** the *EGFR* amplification data was complete but in 4 GB EGFRvIII could not be assessed (due to lack of tumour RNA). Whenever data, either clinical or genetic, was missing the case was coded as having "missing data" (and not as "normal").

Clinical follow-up (Paper I-III)

The starting point for patient survival was generally the date of the first operation. Exceptions in **Paper I** were the "secondary" GBs included and in **Paper II** the 6 "secondary" and 6 "recurrent" patients included. The starting point for patient follow-up for these particular cases was instead the date of the operation from which the tumour tissue used in the genetic analysis was collected, as we did not know the genetic status of the tumour removed at earlier operations. However, this factor

was taken into account using stratification (**Paper I**) or in multivariate analysis (**Paper II**). To further consider the potential bias of including the "recurrent" cases in **Paper II**, was a separate survival analysis performed whenever a statistically significant association with survival had been found. In this additional analysis the survival of the 6 patients was calculated from the date of the first AA operation (to confirm significant associations found, as if the 6 "recurrent" cases were known to have had the same genetic profile at the first operation). In **Paper III** were, as mentioned, only "primary" AII, AA and GB included, and thus survival was reckoned from the date of the first operation.

Statistical analysis (Paper I-III)

To study the potential influence of single clinical or genetic factors on patient survival in **Paper I-III** we used Wilcoxon-Gehan statistic, even when comparing different age groups (Gehan, 1965). Wilcoxon-Gehan statistic is one of many methods used in univariate survival analysis and is comparable to the log rank statistic. These methods are used to compare the survival curves for two or more groups of patients, taking censored observations into account. When analysing age considered as a continuous variable Cox' regression analysis was used. When applicable, Cox' regression was also used as a multivariate method with relevant factors put into the model. In **Paper I and III** Cox' regression analysis was used when adjusting for age, considered as a continuous variable (thus as a "bivariate" rather than as a "multivariate" analysis), and in **Paper II** when adjusting for age (as a continuous variable) plus whether the tumour studied was "primary" or not (thus, as a binary variable). In **Paper I** Cox' regression multivariate analysis was also used to test the main finding (the association between *PTEN* and Rb1 pathway abnormalities and survival) compared with all other factors that appeared as significant predictors of survival in univariate analyses in the study: age, radiotherapy given or not and right- or left-sided tumour location. The main finding was also tested in defined sub-groups using Wilcoxon-Gehan statistic.

Age was included in all multivariate models due to its well-recognized significance as a strong, negative prognostic factor. This has been clearly shown for GB in our material and in numerous other studies (Burger & Green, 1987; Curran et al., 1993; Ohgaki et al., 2004; Rasheed et al., 2002; Salminen et al., 1996; Shinojima et al., 2003; Smith et al., 2001). With the comparably small numbers of AAs in **Paper II** the impact of age could not be established. In **Paper II** age was anyway included in the multivariate analysis because of the extensive literature on age and survival even for AA patients (Perry et al., 1999; Rasheed et al., 2002; Smith et al., 2001; Waha et al., 1996). Comparing age distribution of patients with or without single gene abnormalities was done using the student's t-test (**Paper I**) or ANOVA test (**Paper III)**, which is basically the same methodology when comparing only 2 groups. The, by the time, latest version of the Statistical Package for the Social Sciences (SPSS) for Windows was used for all statistical analyses (release 11.0.5 or 12.0.1).

In studies like these, there are always potential problems regarding "multiple comparisons". Here we have many variables, both clinical and genetic. The collection of detailed information about the individual patients allowed a comparison with other cohorts to establish how the representative our material was, as well as to be able to adjust for potential confounding factors. Analyzing as many as 9 genes underlines the "explorative" dimension of the studies since there was a lack of known genetic prognostic markers. However, the genes used for correlation analysis were not randomly chosen, but chosen for their high mutation frequency in these tumours and because they are generally considered as biologically highly significant. The alternative would be to decide to analyse fewer genes, but then the question arises: which genes to choose? Entering an era of micro-array analysis generating data on thousands of genes in the hope of identifying significant patterns of prognostic importance and indicators of good treatment response the problems with statistical analyses and their complexity is increasing exponentially. In that context the number of comparisons made by us is limited and possible to control. The results indicated by our studies must, however, be interpreted cautiously, taking into account that one or more of the significant findings may have been generated by random. It is however not possible to say which, if any.

Statistical analysis (Paper IV)

Compared to **Paper I-III**, the methodology was entirely different in **Paper IV**. We conducted a cohort study using the Swedish Cancer Registry (SCR). From the SCR we could identify all individuals that had been operated for a parathyroid adenoma (PA) from 1958 to January 31st, 1999. After the exclusion of duplicates, cases diagnosed at autopsy, patients that could not be followed-up and all subjects who had died before the 1st of January 1970 (as different CNS tumours could not be identified by underlying histopathological code in the SCR until 1970) there remained 12,468 subjects. Using the national registration number we identified 121 individuals who also had a diagnosis of a CNS tumour in the SCR. Of these, 70 had been diagnosed with the parathyroid adenoma prior to the CNS tumour and risk ratios could thus be estimated comparing the findings with the expected incidence rate based on CNS tumour rates in the Swedish population during the same period. In order to confirm the reported diagnosis, we retrieved the underlying reports of the CNS tumours sent to the Registry and updated the tumour nomenclature in line with the most recent WHO classification (Kleihues & Cavanee, 2000). All but 2 reports were possible to retrieve.

The Swedish Cancer Registry (SCR) was started in 1958. All newly diagnosed malignant tumours, all tumours of the endocrine system and all tumours of the CNS have to be reported to the SCR by both the diagnosing physician and the pathologist. Every Swedish resident has an individual, unique, national registration number, which ensures accurate registration. The personal identity number was introduced in 1947. It is used to index almost all registers in Sweden, including the SCR and the Register of total population (from which emigrants can be identified).

We estimated the risk of persons diagnosed with PA to develop a primary CNS tumour in comparison to the normal population. Person-time at risk was calculated for each member of the cohort from the date of diagnosis of parathyroid adenoma until diagnosis of the CNS tumour or death, emigration, or end of follow-up, whichever occurred first. The expected number of CNS tumours in the cohort was calculated by multiplying the five-year age interval and calendar-year-specific cancer incidence rates of the general Swedish population between 1958 and 1999 by person-years accumulated in each interval of the exposed population. The standardized incidence ratio (SIR) was calculated as the rate of observed CNS tumours to that expected and 95% confidence intervals (CI) around the SIR was calculated, assuming that the observed number of cancers followed a Poisson distribution (Breslow & Day, 1987). Analyses stratifying by gender and age of the subjects, calendar period and birth cohort, were also performed.

To overcome the potential influence of intensified diagnostic activities of persons with a newly diagnosed disease, so called “surveillance bias”, a separate analysis was performed excluding cases that were diagnosed with a CNS tumour within one year after the parathyroid adenoma.

RESULTS AND DISCUSSION

Background to Paper I

GB is the commonest and most malignant and therapy-resistant form of primary tumour of the CNS and occurs in all age groups, but the incidence peaks in late middle age. Median survival is less than 1 year with current standard surgical and irradiation therapy (Barker et al., 1996b; Curran et al., 1993; Ohgaki et al., 2004). The most important prognostic determinants are age, which is negatively correlated with survival (Burger & Green, 1987; Ohgaki et al., 2004; Salminen et al., 1996), followed by performance status (Curran et al., 1993; Salminen et al., 1996), extensive surgery and high-dose post-operative radiation (Bleehen & Stenning, 1991; Walker et al., 1979), which are all positively associated with survival. GB is also the CNS tumour type best characterized on a molecular genetic basis and many gene abnormalities have been reported (Kitange et al., 2003; Louis, 1997; Rao & James, 2004). Detailed correlation studies of the status of up to 6 genes and patient outcome have been reported (James et al., 1999; Ohgaki et al., 2004; Smith et al., 2001; Zhou et al., 1999). A study of paediatric astrocytomas showed an association between loss of wild-type PTEN and poor outcome, but no separate analysis of GB was reported (Raffel et al., 1999). Unfortunately there have been seemingly few clinically relevant and indisputable findings coming out of the large amount of genetic data produced so far and no clear associations between histological or molecular genetic markers and GB patient outcome. This contrasts to advances in identifying prognostic and/or treatment predictive indices in other types of malignancies, including oligodendrocytic tumours (Bauman et al., 2000; Cairncross et al., 1998).

Paper I

In an attempt to establish whether knowledge of the presence or absence of abnormalities of the genes most frequently found aberrant in GB could be clinically useful, we compared results from the detailed analysis of 9 genes with patient survival in 129 histologically well-defined GBs, both "primary" and "secondary". The genes studied were 3 genes coding for proteins in the p53 pathway (*i.e. TP53, p14^{ARF}*and *MDM2*), 4 in the Rb1 pathway (*i.e. CDKN2A, CDKN2B, RB1* and *CDK4*) as well as *PTEN* and *EGFR*. This was the largest study to-date regarding number of patients and number of genes analyzed in detail.

In order to statistically analyze the complex data, with many clinical and genetic factors to consider, in a way we believed was clinically and tumour biologically relevant, we followed some general strategies:

1. We focused exclusively on GB as defined by the latest edition of the WHO classification of tumours of the nervous system (Kleihues & Cavanee, 2000).
2. We wanted the data to be as complete as possible, both regarding the clinical and the genetic data. This helped us in evaluating the representativity of the patients included and of the tumour material. It also helped us to identify potential confounding factors.
3. Being aware of the potential drawbacks of multiple statistical analyses and having such a complex data set, we wanted to avoid doing every possible comparison between genetic data and survival. For the genetic data we therefore set up clear definitions for "normal" and "abnormal".
4. To handle the potential bias related to the strongest prognostic factor, age, we performed a bivariate analysis for each genetic factor adjusting for the age factor.
5. We also choose to stratify for the second strongest factor known, post-operative radiotherapy. This was done in order to explore whether the main findings in the study (the association between Rb1 pathway abnormality + loss of wild-type *PTEN* and short survival) was restricted to irradiated or non-irradiated patients, *i.e.* if this combination could be more of relevance for predicting treatment outcome rather than over-all survival.

6. Additionally, we performed a multivariate analysis of the 5 factors with the strongest associations with survival found forced into the model, in order to test how much each factor contributed to the survival differences.

Before starting the correlation analysis, exploring potential associations between gene defects and survival, we determined a strict definition of which groups to compare, i.e. what should be considered "normal" and "abnormal", both regarding pathways and single genes (see also Materials and Methods section). Based on the generally accepted hypothesis of TSGs, that for a tumour cell to significantly loose the tumour suppression function of a TSG, both wild-type copies to have to be lost, we decided to classify only bi-allelically affected cases as "abnormal". Due to the function of p53 protein as a tetramer (discussed under "p53 pathway" in Introduction), an exception was made regarding the TP53 gene. Thus, even mono-allelic mutations, where a mutated protein could be produced and where there was also a wild-type allele, were in some statistical analysis classified as "abnormal". Oncogenes were considered amplified, and coded as "abnormal", only when there was an average of >5 copies/genome present, excluding the possibility of a large amount of tetraploid cells (in G2 or early M phase of the cell cycle) contributing to false positive results.

These strict definitions provided us with dichotomous genetic variables and, most often, a large enough number of patients in each group for analysis. This diminished the otherwise overwhelming problems of "mass-significance". The drawback was of course that we potentially missed some relevant associations.

Adjusted for age, no single gene was significantly associated with survival, although loss of both copies of wild-type *PTEN* showed borderline significance ($p<0.078$) with median survival for the *PTEN* "normal" group of 289 days compared to 198 days for the *PTEN* "abnormal" group. Considering the number of previous inconclusive reports on single genes and survival in GB was significantly associated with survival we also considered it relevant to explore the possibility of an association between combinations of genetic abnormalities and survival. At first we combined data on the genes involved in the same cellular pathways p53 and Rb1 pathways in concordance with the general knowledge of the biological significance of disruption of these pathways in cancer cells and the fact that in the majority of tumours with Rb1 or p53 pathway classified as "abnormal" only one gene defect per pathway was found. Aberrations of the individual pathways were in univariate analysis found to clearly associate with patient outcome, but in multivariate analysis only the Rb1 pathway abnormalities were significantly associated with survival.

We then carried out an analysis based on the Rb1 and p53 pathways results also adding the factors *PTEN* and *EGFR*, yielding 6 possible "pairs". We wished to examine whether combinations of aberrant cellular pathways could have additive or even synergistic effects. If so, we would expect a trend in the survival figures from the "normal"/"normal" group in one end of the spectrum and the "abnormal"/"abnormal" group in the other end, with the "normal"/"abnormal" groups having intermediate survival figures. In univariate analysis, the *PTEN* and Rb1 pathway "pair" showed median survival figures best fitting with such an additional effect. We found that abnormalities in any of the four genes coding for components of the Rb1 pathway were associated with shorter survival ($p=0.002$) and in combination with loss of wild-type *PTEN* the association was even stronger ($p<0.001$), the median survival being 166 days compared with the group of GB patients that did not have these abnormalities (median survival 437 days). Adjusted for age, the survival difference was also statistically significant using Cox' regression analysis ($p=0.012$).

This finding was also tested in the treatment sub-groups, and the tendencies were the same in all cases. One sub-group analysis was, based on the extensive literature suggesting different mutation patterns in "primary" and "secondary" GB, excluding the "secondary" GBs. The tendencies were the same regardless of how the material was stratified (Table 4). When all factors that showed a significant association with survival in univariate analysis was forced into a multivariate model,

disruption of the Rb1 pathway, but not loss of wild-type *PTEN*, showed independent impact on survival (Table 5).

One could speculate in why this combination so strongly associates with short survival, indicative of an extra aggressive tumour phenotype. A dysfunctional Rb1 pathway would lead to inappropriate progression from G1 into S phase of the cell cycle. Combining that high-proliferative feature with lost inhibition of the PI3K–Akt pathway secondary to loss of PTEN, would promote the cells to survive, over-coming pro-apoptotic signals. In addition, PTEN can affect the stability and transcriptional activities of the p53 protein directly, both by a phosphatase-dependent mechanism and by PTEN forming complexes with p53 in the absence of MDM2 (Freeman et al., 2003). However, it is notable that *PTEN* and *TP53* mutations do not tend to be mutually exclusive in these tumours. In our study 27 (22%) of the GBs had both *TP53 PTEN* mutations, in concordance with previous GB studies (James et al., 1999; Kato et al., 2000).

While the analysis of gene abnormalities and their potential influence on patient outcome was the primary purpose of the study, we did make some other observations:

• We found that right-sided tumours were associated with shorter survival. This was surprising and we have found no major support for this in the literature. Interestingly, the opposite, borderline significant, association was found among the 37 AAs studied for **Paper II**. There are few studies on the potential association between survival and the location of the tumour. Two of them show that frontal tumours are associated with a prolonged survival (Jeremic et al., 1994; Simpson et al., 1993), which was not confirmed in our series (Table 1).

• In this set of GBs we had few "secondary" GBs (n=8) as compared to "primary" GBs (n=122). "Primary" GBs are in the most recent WHO classification described to typically have occurred after a period of symptoms of <3 months (Kleihues & Cavanee, 2000). We found that the median time of symptoms before diagnosis was 2 months, but >1/3 of our cases (44/122) had a clinical history of 3 months or more.

• Regarding the distribution of mutations in our small series of "secondary" GBs we observed a frequency of *MDM2* amplification and loss of wild-type *CDKN2A* that was similar to the "primary" GBs, which did not agree with previous reports (Biernat et al., 1997a; Biernat et al., 1997b; Nakamura et al., 2001).

Background to Paper II

AA is, second to GB, the most common and most malignant type of adult CNS tumours. AA patients have a median survival of about 3 years, though with a wide range (Herfarth et al., 2001; Lin et al., 2003). As for GB, young age is generally accepted as a positive prognostic factor for AA (Perry et al., 1999; Rasheed et al., 2002; Smith et al., 2001; Waha et al., 1996). Several genes have been documented as being aberrant in astrocytic tumours and these abnormalities are generally believed to accumulate in a particular order in the different malignancy grades (Kitange et al., 2003; Louis, 1997). The gene defects observed in AA are largely the same as in GB, but in general they occur at lower frequencies. One exception is *TP53* mutations, which is the most commonly aberrant gene in AA – approximately 50% of these tumours have no wild-type *TP53* and a further 10-15% have one mutated and one wild-type allele (Ichimura et al., 2000; Kato et al., 2000; Rasheed et al., 1994). Another exception is loss of both wild-type copies of *RB1*, which has not been reported in AA (Ichimura et al., 1996; Ueki et al., 1996).

In general AA is, compared to GB, less well characterized with regard to mutation rates. There are also often difficulties with comparisons between recent and older series of AA, since the WHO criteria have changed over the years (Kleihues, 1993; Kleihues & Cavanee, 2000; Zülch, 1979). In the 1993 version the presence of necrosis and microvascular proliferation automatically put a tumour into the GB category, stating about AA: "Vascular proliferation and necrosis are absent." Thus, there has been a shift in the diagnostic criteria so that a fraction of tumours that previously might have been diagnosed as an AA, despite having small areas of / limited necrosis and vascular proliferation after 1993 would have to be classified as a GB.

Attempts to correlate the genetic findings in AA with patient outcome have to-date not provided any convincing correlations with survival (Galanis et al., 1998; James et al., 1999; Olson et al., 1998; Rasheed et al., 2002). Most studies have suffered from including more than one tumour type and/or malignancy grade in their analyses and used various definitions of anomalies of tumour suppressor genes and of amplification of oncogenes. One study of alterations of the *EGFR*, *PTEN* and *TP53* genes in 63 AA (including tumours only biopsied) have shown a negative association between loss of a functional *PTEN* and survival and a positive association between *TP53* mutations and survival (Smith et al., 2001).

Paper II

The survival data on 37 AA was correlated with the results of a detailed analysis of the 9 genes involved in astrocytic tumour development (*i.e.* the same genes as were studied in Paper I). Of the 37 AA included 25 were from the patients' first operation and 12 were not. These 12 patients had either been operated for an AA (6 patients) before or had tumours that had progressed from an AII (6 patients). For the statistical analysis we again followed some general strategies. As in **Paper I** we wanted the data to be as complete as possible both regarding clinical and genetic data, using the same definitions of "normal" and "abnormal" genes and including the strongest prognostic factor known, age, in the multivariate analysis of each genetic factor. Two aspects of the study, however, differed from Paper I:

1. We focused exclusively on AA as defined by (Kleihues & Cavanee, 2000).
2. We included the 6"recurrent" and the 6"progressed" cases in this study so as not loose "power". To handle the potential bias related to this inclusion we adjusted for this (together with age) in the multivariate analysis of each genetic factor. We choose this strategy instead of stratification, since we then would have had to small groups to compare.

The material was found to be representative regarding age distribution and the location of the tumours, while we had a comparatively high number of females in the series (Kleihues & Cavanee, 2000). A linear relationship between age and survival was not demonstrated but when the patients were divided into three groups the subjects in the oldest group had the shortest and the youngest

age group the longest survival. This was only borderline significant but in line with other reports (Herfarth et al., 2001; Lin et al., 2003; Perry et al., 1999). The survival range (from a few months to >13 years) widely overlapped that usually observed for both AII and GB (Herfarth et al., 2001). Minimum follow-up was 10.3 years, which is necessary in studies where survival can be long.

The main finding was that any abnormality in the four genes (*CDKN2A*, *CDKN2B*, *RB1*, *CDK4*) coding for components of the Rb1 pathway were associated with shorter survival (median survival 1.4 compared to 5.8 years) (p=0.009). This finding was consistent in multivariate analysis, adjusting for age and whether the tumour was "primary" or "recurrent"/"progressed" (p=0.013). While only 2/25 "primary" AA with the Rb1 pathway classified as "abnormal", Rb1 pathway was found "abnormal" in 3/6 "recurrent" and 3/6 "progressed" cases. This "imbalance" in the material is difficult to explain. It could maybe be a consequence of the "recurrent"/"progressed" being biologically different, having developed genetic defects in a different order than the "primary" cases or just be a chance finding. However, it is also a fact that 5 of the 6 "recurrent"/"progressed" cases with the Rb1 pathway "abnormal" had received radiotherapy after a previous operation, which might have affected the clonal evolution within these tumours.

Despite the strong correlations between gene defects and survival observed, the genetic data presented here do not seem to be predictive regarding which tumour will progress to a GB which is an expected scenario for a substantial part of successively treated AA patients. Case AA69, with one mutated *TP53* allele as the only aberration in the 9 genes studied, progressed to GB after 1.8 years, while the "recurrent" case AA51, that had multiple genetic abnormalities (loss of both wild-type copies of *CDKN2A*, *CDKN2B*, $p14^{ARF}$, *TP53* and hemizygous deletion of *PTEN*), had at a third operation 2.8 years later still not progressed to GB.

Comparing the results with those for GB presented in **Paper I** it is important to note that no AA had the genetic profile that characterized the large GB sub-group (38%) with the worst prognosis, *i.e.* disruption of the Rb1 pathway and loss of wild-type *PTEN*. Regarding the study of AA by Smith, *et al*, (Smith et al., 2001), their finding of a positive association between *TP53* mutation and survival and an opposite association between *PTEN* mutation and survival was not confirmed by our results. There are some obstacles for direct comparisons to our data, which may explain this. Smith, *et al*, included a large number of AA diagnosed with biopsy only (48%), making a reliable histological classification more difficult. The seemingly high frequency of point mutations of *PTEN* (18%) could be explained by some of the included tumours actually being GBs not adequately sampled. In other AA series *PTEN* mutations are found from 0 to 7.4% (Kato et al., 2000; Raffel et al., 1999; Rasheed et al., 2002; Smith et al., 2001)except for one other study, which also included biopsy cases, where 20% of the studied AAs carried *PTEN* mutations (Rasheed et al., 1997). In **Paper I** we found loss of both wild-type copies of *PTEN* in 48% of the GBs.

In fact, the only case (AA29) with loss of both wild-type copies of *PTEN* in our series had a point mutation of the stop codon on the retained allele, resulting in the addition of 8 amino acids to the C-terminal. The consequence of this *PTEN* mutation is unclear as all other coding regions were wild-type. Further characterization of the *PTEN* gene, including epigenetic and expression aspects, would be of interest since 22 of our 37 AA (59%) had hemizygous deletions of *PTEN* (but 21/22 had retention of the other allele as shown in Table 2), of which we do not know the biological significance. Interestingly these cases showed a median survival of 2.0 years, as compared to 7.9 years of the cases with 2 retained wild-type copies.

Background to Paper III

The wild-type *EGFR* gene is expressed in many types of cells, both normal and malignant (Moscatello et al., 1995). In malignant cells is *EGFR* amplification is often accompanied by gene rearrangements, of which the most common results in an aberrant transcript coding for a truncated receptor known as EGFRvIII (or ΔEGFR). The product of this transcript variant (with an in-frame deletion of 801 bases) is constitutively active (Humphrey et al., 1990; Sugawa et al., 1990; Wong et al., 1992). In GB over-expression is found in up to 1/2 of the cases (Ekstrand et al., 1991; Libermann et al., 1985; Liu et al., 1998) and *EGFR* amplification in about 1/3, of which approximately 1/2 have rearrangements (Ekstrand et al., 1992; Frederick et al., 2000). Although expressed to some extent, *EGFR* is rarely found amplified in AII and only in 5-18% of AA (Olson, 1998; Smith, 2001). Numerous studies of *EGFR* gene amplification in GB have been published and some studies have focused on its potential impact on response to radiotherapy or patient survival. The reported results on the prognostic impact have so far been contradictory. Furthermore, there is only one large study that considers both *EGFR* amplification and expression of EGFRvIII and survival (Shinojima et al., 2003).

Paper III

Based on the previous lack of comprehensive studies on *EGFR* amplification, rearrangements and survival, the primary purposes of this study were to characterize grade II-IV astrocytic tumours regarding combined *EGFR* gene abnormalities and subsequently examine their potential influence on survival. We focused on the 2 most common types of EGFR aberrations; gene amplification and expression of the EGFRvIII transcript. Before starting to statistically analyze the data, we agreed on some general strategies:

1. To simplify the survival analysis we decided to only include “primary”, non-recurrent cases (160 GB, 41 AA and 20 AII)
2. We wished to explore the potential impact of *EGFR* amplification on patient survival and the potential impact of expression of the EGFRvIII transcript, since it codes for a constitutively active receptor variant.
3. We also wanted to explore whether a combination of gene amplification and presence of EGFRvIII could contribute to a particularly aggressive tumour variant. If so, we would expect a trend in the survival figures from the “normal”/”normal” group in one end and the “abnormal”/”abnormal” group in the other, with the “normal”/“abnormal” group showing intermediate survival. To test this hypothesis, we set up the cases in 3 groups:

- Neither *EGFR* amplification nor expression of the EGFRvIII transcript
- Either *EGFR* amplification or expression of the EGFRvIII transcript
- Both *EGFR* amplification and expression of the EGFRvIII transcript.

Except for 4 GB cases with missing EGFRvIII data, the gene and transcript data was complete in the 221 cases. Amplification was found in 65 of 160 GB (41%), 4 of 41 AA (9.8%) and none of the 20 AII. The mean copy number in the amplified GB was 44 copies (ranging from an average of 8 to 151 copies per genome). Of the amplified cases 46% of the GBs and 25% of the AAs (1 of 4) with *EGFR* amplification showed no evidence of the EGFRvIII transcript. In total there were 42 GBs, 5 AAs and no AIIs that expressed EGFRvIII. Of these, 34 GBs (81%) and 3 AAs (60%) also fulfilled the criteria for *EGFR* amplification.

In the 160 GB no significant associations between the *EGFR* abnormalities, or combination of abnormalities, and survival was found, or between *EGFR* abnormalities and patient age. To explore if any associations between the *EGFR* data and survival could be restricted to a particular group of patients sub-group analysis was performed stratifying for radiotherapy. No associations with EGFR status and survival in the patient group not treated with post-operative radiotherapy, nor any associations predictive of the outcome in the post-operatively irradiated group, were found.

Among the 41 Anaplastic Astrocytoma (AA) cases we found a tendency towards worse survival in cases with tumours with *EGFR* amplification in comparison to those without and in AAs with presence of EGFRvIII transcript compared to those not expressing it. Presence of the EGFRvIII transcript was detected in 5 of 41 AAs (12.2%) and 3 of these also had evidence of amplification. All 5 patients with EGFRvIII died before end of follow-up (compared to 25 of the 36 AA with no EGFRvIII) with a median survival of 1.3 years (compared to 5.6 years for the 37 cases with no EGFR abnormalities). The survival differences were borderline significant in univariate analysis (p=0.061), but non-significant in multivariate analysis adjusting for age. The patients with tumours with presence of EGFRvIII were significantly older than those without (p=0.023).

We conclude from these data that *EGFR,* as a single factor is an insufficient predictor of patient outcome in GB (or in AII). In AA, while uncommon, *EGFR* aberrations tend to be associated with shorter survival. The comparably small number of AAs, and the lack of strong and independent associations with survival, demands a cautious interpretation of the findings but they could reflect that there is a fraction of AA patients, although having a tumour with a clear AA histology and without the histological characteristics of a GB, showing a higher level of malignancy that could be identified by EGFR analysis.

Attempts to correlate *EGFR* analysis with outcome in GB patients treated with conventional therapy, surgery followed by radiotherapy, have to-date provided inconclusive or contradictory results, see **Paper I** and (Huncharek & Kupelnick, 2000; Ohgaki et al., 2004). In one recent publication on 87 GB patients, association between *EGFR* amplification and shorter median survival (1.2 compared to 1.7 years) was reported. Although the survival difference was small it was statistically significant in univariate analysis and multivariate analysis adjusting for age. Further dividing the amplified group revealed an association between EGFRvIII expression and shorter median survival (Shinojima et al., 2003). The study also included patients not operated, only biopsied, which is a potential bias when it comes to comparing diagnoses and survival figures. Shinojima, et al, also found *EGFR* amplification to be associated with high patient age, a finding with borderline statistical significance. As mentioned, in our series of GB patients we could not see any such associations, but within the AA series patients with presence of EGFRvIII were older than those without (p=0.023).

During the last 2 decades, knowing that there are high frequencies of genetic alterations and/or over-expression of EGFR in GB and other tumours combined with the lack of success with many conventional therapies, molecules targeting EGFR, particularly monoclonal antibodies and tyrosine kinase inhibitors, have been tried. There are monoclonal antibodies developed both against EGFR and EGFRvIII, or both (Jungbluth et al., 2003; Masui et al., 1984; Sampson et al., 2000). Monoclonal antibodies can also be used in radio-immunotherapy, *i.e.* with a radioactive substance attached (Quang & Brady, 2004). Also EGFR-binding ligands, either radiolabeled or toxin-conjugated, have been tested (Brady et al., 1992; Phillips et al., 1994). In addition, a number of small-molecule tyrosine kinase inhibitors targeting the EGFR, inhibiting its tyrosine kinase activity by competing with ATP for specific sites, have been developed (Han et al., 1996; Han et al., 1997). In a study by JG Paez, *et al*, a specific point mutation in the region coding for the tyrosine kinase domain of the EGFR was found associated with a favourable treatment response to one of these tyrosine kinase inhibitors, gefinitib (Paez et al., 2004).

Thus, EGFR analyses may become essential for individualized therapy, rather than used as a general prognostic factor. It is of major importance to further elucidate the complexity of combinations of amplification, rearrangements and point mutations of the *EGFR* gene, in order understand how this affects the response to the developed EGFR-targeting drugs as well as to guide the ongoing therapeutic developments.

Background to Paper IV

The vast majority of primary tumours of the CNS are believed to be sporadic and, with the exception of therapeutic radiation, no strong risk factors have been identified despite that a wide range of potential factors have been evaluated (Gurney & Kadan-Lottick, 2001). The general lack of knowledge on the aetiology of CNS tumours strongly motivates further investigations, both regarding environmental and hereditary factors.

In order to learn more we wanted to explore if there was an association between parathyroid adenoma (PA) and CNS tumours. This was performed as one part of a larger study on the relationship between PAs and other tumour types, including breast cancer (Michels et al., 2004), since PA aetiology is also unclear. The identification of associated tumour types can generate hypothesis for either shared environmental factors, providing suggestions for preventive measures, or genetic factors, which can eventually help to locate previously undetected cancer genes.

Paper IV

To investigate if there was an association between PA and CNS tumours we used Swedish registry data. In the Swedish Cancer Registry (SCR) all individuals operated for PA between 1958 and 1999 were identified (n=12,468) and through linkage to the reported CNS tumours in the SCR we could do follow-up of the PA patients for the subsequent development of a primary CNS tumour. We found 70 such cases. To confirm the type of the CNS tumours diagnosis reported to the Registry we retrieved the underlying reports and updated the tumour nomenclature in line with the most recent WHO classification (Kleihues & Cavanee, 2000). As compared to the 35 expected, the standard incidence ratio (SIR) was 2.0 (95% confidence interval (CI) 1.5-2.5). This increased risk was independent of duration of follow-up and was confined to meningiomas (SIR=2.4, 95%CI 1.7-3.4) and neurinomas (SIR=3.4, 95%CI 1.5-6.8). For astrocytic tumours, after excluding the first year, the observed number was close to the expected (SIR=0.8, 95% CI 0.4-1.6).

Our main finding was this significant association between a history of PA and a meningioma or neurinoma among subjects operated on after the age of 40, independent of calendar year, duration and sex. This was a novel finding pointing towards the existence of a shared aetiology of PA and meningioma/neurinoma, which could be genetic or environmental or a combination of both. If there exists a causal connection one cannot say, but only speculate on some putative factors:

Radiation/X-Ray

There is compelling evidence that therapeutic radiation to the head/neck region increases the risk to develop a CNS tumour. Particularly radiation given in childhood seems to elevate the risks, as has been seen in an Israeli cohort treated for tinea capitis with as low doses as 1-2 Gy, later developing meningiomas, neurinomas and astrocytic tumours more than expected (Ron et al., 1988) and a Swedish cohort treated for skin haemangioma (Karlsson et al., 1998). Accordingly there are reports of CNS tumours developed after higher doses, especially after irradiation of ALL (Salvati et al., 1996; Salvati et al., 1990) and in atomic bomb survivors (Preston et al., 2002).

As radiation has also been identified as a risk factor for the development of PA (Holmberg et al., 2002), one could speculate whether chest X-rays for children and adults in the Swedish tuberculosis screening programs during the 1930'ies up to the 1960'ies may have affected the incidence of PAs. The steady increase in incidence of hyperparathyroidism during the last 40 years, a pattern similar to that seen for meningiomas and neurinomas, may at least partially be explained by this exposure (Lonn et al., 2004b). It is therefore of interest that our study did not show any excess risk for astrocytic tumours after a PA operation, since ionizing radiation has been implicated as a risk factor also for these tumours. The absence of such an association could either be due to the fact that ionizing radiation is not as strongly associated with astrocytomas as for meningiomas and

neurinomas, which would be supported by the study by E Ron, *et al* (Ron et al., 1988), or that other aetiological factors are shared between the outcomes.

We found no evidence from what was reported to the SCR that the subjects in our study had any history of any paediatric malignancy that would have been treated with radiotherapy. On the other hand, we cannot from any of the registries used exclude that radiation had been used for treating some benign condition or that they had been extensively X-rayed.

Parathyroid hormone (PTH)/calcium

PAs most often overproduce parathyroid hormone (PTH), which is the most common cause of primary hyperparathyroidism and subsequent hypercalcemia. PAs are usually single and benign and often asymptomatic. If left untreated they may lead to increasing serum calcium levels, osteoporosis and kidney stones but the majority stay asymptomatic (Silverberg et al., 1999). A potential risk factor explaining why CNS tumours are overrepresented among PA patients would be if elevated of the PTH and/or calcium levels prior to the operation could be carcinogenic or have promoted the growth of a development CNS tumour. There are some evidence that the parathyroid hormone-related protein (PTH-rp), produced by some cancer cell types, can increase the malignant potential and metastatic capacities, apart from causing hypercalcemia (Dougherty et al., 1999; Manenti et al., 2000; Massfelder et al., 2004; Nishihara et al., 1999). However, dietary intake of another calcium-modulating hormone, vitamin D, is found associated with protection against the development of prostate cancer (Zhao & Feldman, 2001) and both vitamin D and calcium itself are considered protective against colon cancer (Lamprecht & Lipkin, 2003). This is of course very speculative, but could perhaps these findings be interconnected through a PTH-depletion induced by a high intake of vitamin D?

HRT

Another potential etiologic factor is the increased use of oestrogen as hormone replacement therapy (HRT) in postmenopausal women and/or as treatment against osteoporosis. Hormone replacement therapy or other hormonal involvement has been implicated as a risk factor for meningiomas since females are overrepresented and steroid hormone receptors are expressed in meningiomas (Hsu et al., 1997). If women diagnosed with hyperparathyroidism are found to be osteoporotic they might undergo oestrogen treatment to a higher extent than the normal population, which then would contribute to an elevated risk for hormone-related tumours. Our observation that there is an elevated risk for meningiomas but not for astrocytic gliomas after a PA diagnosis could be explained by the fact that progesterone and oestrogen receptors are expressed to a lesser extent in astrocytic tumours compared to meningiomas (Carroll et al., 1993; Longstreth et al., 1993; Paoletti et al., 1990). However, there was a remarkable similarity in the risk estimates among males and females indicating that this can at least not be the only explanation.

Hereditary factors

If the identified association between PA and meningioma (and neurinoma) in this study is not to be explained by the above, or any other undiscovered, environmental risk factor, a shared genetic background could be suggested. What is known until now, hereditary factors explain only a few percent of the incidence of PA (al Zahrani & Levine, 1997; Marsh & Zori, 2002) as well as of primary CNS tumours. Of the many well-characterized cancer syndromes some include PA, while other syndromes include CNS tumours. There are few indications in the literature of any cancer syndrome explaining the association we describe.

Multiple Endocrine Neoplasia (MEN) is generally divided into 4 types, MEN type 1 and type 2A, B and C of which the MEN 1 and 2A include parathyroid disorders. Another syndrome associated with parathyroid disorders, the hyperparathyroidism–jaw tumour (HPT-JT) syndrome, is characterized by an aggressive type of PA or carcinoma in conjunction with fibro-osseous tumours, but not with any

CNS tumours (Carling & Udelsman, 2003). MEN type 1 is characterized by hyperparathyroidism due to parathyroid hyperplasia, occurring in young adults, and several other tumour types involving the endocrine system (Zarnegar et al., 2002). There are also case reports of MEN1-patients who have developed an ependymoma (Giraud et al., 1997; Kato et al., 1996) and meningiomas (Banik et al., 1984; Grinblat et al., 1976). In one prospective study, meningiomas were found in 8% of MEN type 1 patients (Asgharian et al., 2004). MEN type 2A, B and C are all characterized by medullary thyroid carcinoma, but no associations with CNS tumours are reported.

As has been introduced earlier in this thesis, there are several familial cancer syndromes that include high frequencies of primary CNS tumours. For the von Hippel–Lindau disease (VHL), characterized by CNS haemangioblastomas and renal cell carcinomas, there are anecdotal reports of primary hyperparathyroidism, while other well-described cancer syndromes associated with CNS tumours, such as Neurofibromatosis type 1 (NF1) and 2 (NF2), Cowden's disease, Turcot's syndrome and LiFraumeni syndrome not have been found associated with any parathyroid disorders (Marsh & Zori, 2002).

The study by Ashgarian, *et al*, of the occurrence of meningioma in MEN type 1 patients is interesting but could not in a simple way be compared to our data. They prospectively followed 74 MEN type 1 patients for the occurrence of meningioma with serial brain imaging. They detected meningiomas, all asymptomatic, in 6 of 74 patients (8%), which was significantly more than in the control group (Asgharian et al., 2004). Their results indicate that meningiomas are over-represented in MEN type 1 patients and this could to some extent explain our finding. However, from the Cancer Registry data available we, as shown in Table 3, identified comparably few patients with multiple tumours. This decreases the likeliness of an underlying cancer syndrome. If MEN type 1 patients were overrepresented among the identified cases in our study we would have expected to have found also other endocrine tumours to have been reported to the SCR, for some cases in our cohort. As shown in Table 3 of **Paper IV** the single patient that after the PA operation was diagnosed with an additional endocrine tumour (pancreatic) did not have a meningioma, but a "low-grade astrocytoma". Some epidemiological reports though indicate that as yet unidentified hereditary components may be important (Grossman et al., 1999; Malmer et al., 2003).

Surveillance bias is always a concern in registry-based studies of this kind. The very strong association between parathyroid adenomas and all CNS tumours during the first year after operation is probably due to such bias combined with confounding by indication, *i.e.* the symptoms from an undiagnosed CNS tumour will lead to the diagnosis of an PA. However, consistent elevated risk still present after 15 years or more strongly argues against surveillance bias explaining our findings. There would appear to be an underlying biological explanation that should be the subject for future aetiological studies. Our findings also have clinical implications. Clinicians with a patient with a newly diagnosed meningioma should be aware of the possibility of an underlying hyperparathyroidism and likewise be aware of the association when patients with a history of hyperparathyroidism present themselves with symptoms from the CNS.

Finding out what causes a disease and knowing its detailed biology is fundamental and permits the introduction of preventive measures where possible, as well as provides data for the design of innovative specific therapies.

CONCLUSIONS AND FUTURE PROSPECTS

We are only beginning to understand the complexity of the regulation of even the most fundamental activities of normal mammalian cells. While the study of cancer cells has led to great leaps forwards in our understanding of normal cellular functions we are probably still just "scratching the surface" of tumour biology. An individual tumour cell is a small part of a complex interactive environment in an unfortunate host. The aim of the studies forming the basis for this thesis, was to contribute to our understanding of the aetiological factors and genetic changes associated with the development of CNS tumours and whether these changes can be used as indicators of therapy response and prognosis.

The main finding of **Paper I** was that abnormalities in any of the four genes (*CDKN2A*, *CDKN2B*, *RB1*, *CDK4*), coding for components of the Rb1 pathway, in combination with loss of both wild-type copies of *PTEN* was associated with a particularly poor outcome in GB patients. The median post-operative survival in the 48 cases with this combination of genetic defects was 166 days as compared to the 11 cases without these abnormalities whose median survival was 437 days. Patients with either Rb1 pathway or *PTEN* abnormalities had a median survival in-between ($p<0.001$). The survival difference was also statistically significant in Cox' regression bivariate analysis adjusting for age ($p=0.012$), which is generally believed to be the strongest indicator of prognosis. In conclusion this finding indicates that knowledge of the genetic abnormalities in GB provides clinically useful prognostic information.

The main finding of **Paper II** was that abnormalities in any of the Rb1 pathway-related genes *CDKN2A*, *CDKN2B*, *RB1* or *CDK4*, were associated with shorter survival in AA patients ($p=0.009$). The median post-operative survival in the 8 cases with Rb1 pathway-related gene abnormalities was 1.4 years and in the 29 cases without such abnormalities 5.8 years. The survival difference was statistically significant also in Cox' regression multivariate analysis adjusting for age and whether cases were "primary" AA or not ($p=0.013$). To conclude, this finding indicates that analysis of genetic abnormalities in AA can help to identify a fraction of AA patients who have a survival similar to GB patients rather than to the typical AA patient.

Some further concluding remarks can be made regarding **Papers I-II**:

- Although the associations found have strong statistical significance and thus are potentially of clinical importance, the data should be interpreted with caution due to the multiple comparisons performed. Ideally, our findings should be tested in prospective studies, performed in large cohorts of uniformly treated patients.
- As new developments provide technologies that permit fast and high throughput molecular analysis, much more genetic and molecular data will become available in the near future. Translational research of this type will face an even greater hazard of "mass-significance" due to multiple comparisons. A parallel development of bioinformatics and statistical techniques will be necessary in order to analyze the potentially huge amounts of data available.
- As further advances in our understanding of the molecular genetics and cell biology of gliomas are made, in addition to prognostic information, the data may also provide targets for innovative therapy in the individual case.

The main finding of **Paper III** was that, while analysis of neither *EGFR* amplification status nor the presence or absence of the EGFRvIII transcript predicted patient outcome in GB and AII, amplification and expression of EGFRvIII was associated with shorter survival in AA patients. As there were relatively few AAs in the study (n=41), and only 6 cases with *EGFR* amplification and/or expression of the EGFRvIII transcript, the statistical significance of the survival differences was fairly low. However, the point estimates (median survival for EGFRvIII expressing cases was 1.3 years compared to 5,4 years in the EGFRvIII non-expressing cases) suggest that this could be of clinical significance. Confirmation in large studies is necessary.

While the findings indicate that a molecular analysis of *EGFR* is of limited prognostic value, except possibly in AA, it should be noted that the study was done on patients conventionally treated. The ongoing development of novel therapies targeting tyrosine kinase receptors such as the EGFR may make this type of analysis essential. Oncologists may in the near future have access to a wide range of EGFR-targeting molecules, each with different effect(s). Choice of the appropriate molecule for an individual patient would then require a detailed analysis of the EGFR and its aberrant variants in the tumour tissue.

The main finding in **Paper IV** was the increased risk of developing a primary CNS tumour after surgical removal of a parathyroid adenoma. This general over-risk was found to be restricted to meningiomas (SIR: 2.4) and neurinomas (SIR: 3.4). This novel finding, while provocative and hypothesis generating, cannot be easily explained. Further study of an un-related population is required to establish the link. In addition it would be interesting to screen for meningiomas in parathyroid adenoma patients and compare the prevalence with that of the normal population or investigate newly diagnosed meningioma patients with regard to serum calcium and parathyroid hormone levels. However, not only epidemiological approaches could be applied, but also biological experiments setting up models to test the potentially cancer promoting effects of *e.g.* calcium or parathyroid hormone.

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