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Receptor tyrosine kinase gene copy numbers and protein expression in astrocytic brain tumors

-With special reference to *KIT, PDGFRA, VEGFR2* and *EGFR*

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To my family,
Olavi, "Aarni" and Ilkka

CONTENTS

RECEPTOR TYROSINE KINASE GENE COPY NUMBERS AND PROTEIN EXPRESSION IN ASTROCYTIC BRAIN TUMORS WITH SPECIAL REFERENCE TO *KIT, PDGFRA, VEGFR2* AND *EGFR* **1**

ABBREVIATIONS **6**

LIST OF ORIGINAL PUBLICATIONS **8**

ABSTRACT **9**

INTRODUCTION **11**

REVIEW OF THE LITERATURE **13**

1. RECEPTOR TYROSINE KINASES 13
1.1 RTK signalling pathways *14*
1.2 Epidermal Growth Factor Receptor–family (RTK Subclass I) *15*
1.3 Platelet Derived Growth Factor Receptor Alpha-family (RTK subclass III) *16*
1.3.1 Stem Cell Growth Factor Receptor (KIT) 17
1.3.2 Platelet Derived Growth Factor Alpha (PDGFRA) 19
1.4 The Vascular Endothelial Growth Factor Receptor-family (RTK subclass V) *20*
2. CELLS AND TUMORS OF THE CENTRAL NERVOUS SYSTEM 21
2.1 Astrocytic tumors *23*
2.1.1 Molecular pathogenesis of glioblastomas and grade 2 and 3 astrocytomas 24
2.1.1.1 Isocitrate dehydrogenase-1 mutations 25
2.1.1.2 Intratumoral heterogeneity of EGFR amplification in glioblastomas 26
2.2 Paediatric CNS tumors *26*
2.2.1 Pilocytic astrocytoma 27
2.2.1.1 Genetic aberrations in pilocytic astrocytoma 27
2.2.2. Ependymoma 28
2.2.3 Medulloblastoma 29
3. GENE AMPLIFICATIONS IN CANCER 29
3.1 Gene amplifications at the locus 4q12 ..... *31*
4. ANGIOGENESIS IN GLIOMAS 31
5. RTK ACTIVATION AND TARGETED THERAPIES 33

AIMS OF THE STUDY **35**

MATERIALS AND METHODS **35**

1. PATIENTS AND TUMOUR SAMPLES 35
2. TISSUE MICROARRAY (III) 36
3. POLYMERASE CHAIN REACTION (I-III) 36
4. DENATURING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (I-III) 37
5. DNA SEQUENCING (I-III) 38
6. FLUORESCENCE *IN SITU* HYBRIDIZATION (I-III) 38
7. CHROMOGENIC *IN SITU* HYBRIDIZATION (III-V) 39
8. IMMUNOHISTOCHEMICAL STAINING (II-V) 39
9. LOCKED NUCLEIC ACID MRNA *IN SITU* HYBRIDIZATION FOR KIT (IV) 41
10. STATISTICAL ANALYSES (I-V) 41
11. ETHICAL ASPECTS (I-V) 41

RESULTS **42**

1. *KIT*, *PDGFRA*, *VEGFR2* AND *EGFR* AMPLIFICATIONS IN PRIMARY AND SECONDARY GLIOBLASTOMAS (II) 42
2. *KIT*, *PDGFRA*, *VEGFR2* AND *EGFR* AMPLIFICATIONS IN DIFFUSE GRADE 2 AND 3 GLIOMAS AND THEIR RECURRENCES (III) 42
3. COPY NUMBER ANALYSIS OF *KIT* IN PAEDIATRIC BRAIN TUMORS (IV) 43
4. HETEROGENEITY OF *EGFR* AND *KIT* AMPLIFICATIONS IN GLIOMAS AND GLIOBLASTOMAS (V). 43
5. *KIT*, *PDGFRA* AND *EGFR* MUTATIONS IN PRIMARY AND SECONDARY GLIOBLASTOMAS (I, II) 44
6. *TP53* MUTATIONS IN DIFFUSE GLIOMAS AND THEIR RECURRENCES (III) 44
7. PROTEIN EXPRESSION IN PRIMARY AND SECONDARY GLIOBLASTOMAS (II) 44
8. EXPRESSIONS OF KIT, PDGFRA, VEGFR2 AND EGFR PROTEIN IN DIFFUSE GRADE 2 OR 3 GLIOMAS AND THEIR RECURRENCES (III) 45
9. NESTIN AND PROMININ-1 EXPRESSION IN DIFFUSE GRADE 2 OR 3 GLIOMAS AND THEIR RECURRENCES (III) . 45
10. KIT, PHOSPHORYLATED KIT, SCF, VEGFR2 AND HIF-1-ALPHA EXPRESSION IN ENDOTHELIAL CELLS OF PAEDIATRIC BRAIN TUMORS, AND IN NON-MALIGNANT FETAL AND POSTNATAL TISSUES (IV) 46

11. HETEROGENEITY OF EGFR EGFRvIII, KIT AND PHOSPHO-KIT PROTEIN EXPRESSION IN GLIOMAS AND GLIOBLASTOMAS (V)..... 46
DISCUSSION..... 47
CONCLUSIONS..... 51
ACKNOWLEDGEMENTS..... 53
REFERENCES..... 54
ORIGINAL PUBLICATIONS..... 68

ABBREVIATIONS

µM	micromolar
AKT	v-akt murine thymoma viral oncogene homolog
ANG (-1 and -2)	angiopoietin (-1 and -2)
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
BAD	BCL2-antagonist of cell death
BBB	blood brain barrier
BCR-ABL	breakpoint cluster region for Abelson murine leukemia viral (v-abl) oncogene homolog 1
BFB	breakage-fusion-bridge
BRAF	Serine/threonine-protein kinase B-Raf
CaCl2	calcium chloride
CD113	prominin 1
CD34	cluster of differentiation 34
CDKN	cyclin-dependent kinase inhibitor
CEP4	centromere-specific probe for chromosome 4
CEP7	centromere-specific probe for chromosome 7
CGH	comparative genomic hybridization
CISH	chromogenic *in situ* hybridization
CML	chronic myeloid leukemia
C-MYC/ N-MYC	myc proto-oncogene / neuroblastoma MYC oncogene
CNS	central nervous system
CSF-1	colony stimulating factor-1
CSF1R/ fms	colony stimulating factor-1 receptor
DAB	3,3'-diaminobenzidine tetrahydrochloride
DCC	deleted in colorectal carcinoma
DHPLC	denaturizing high performance liquid chromatography
Dll4	Delta-like protein 4
Dmin	double minute
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphate
EDTA	ethylene diamine tetra acetic acid
EGF	epidermal growth factor
EGFR/ HER1/ErbB-1	epidermal growth factor receptor
EGFRvIII	truncated variant of epidermal growth factor receptor
ErbB2/HER2/Neu	human epidermal growth factor receptor 2
ERK	extracellular signal regulated kinase
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiosyanate
FLT3	fms-related tyrosine kinase 3
GIST	gastrointestinal stromal tumour
HIF	hypoxia inducible factor
HRP	horseradish peroxidase
HSR	homogeneously staining region
ICC	interstitial cells of Cajal
IHC	immunohistochemistry
JAK	janus kinase
JNK	c-Jun N-terminal kinase
KI67	proliferation-related Ki-67 antigen
KIT (SCFR)	stem cell growth factor receptor (KIT)
LOH	loss of heterozygosity
MAPK	mitogen-activated protein kinase
MDM2	murine double minute-2 p53 binding protein homolog
MEK	MAP kinase kinase
$MgCl_2$	magnesium chloride

mTOR	mammalian target of rapamycin
NAM	National Authority for Medicolegal Affairs
NSCLC	non-small cell lung cancer
PBS	phosphate buffered citrate
PCR	polymerase chain reaction
PDGF (A, B and C)	platelet derived growth factor (A, B and C)
PDGFR (A and B)	platelet derived growth factor receptor (A and B)
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PLCγ	phospholipase C-gamma
PlGF	placental growth factor
PTEN	phosphatase and tensin homolog
RAC	ras-related C3 botulinum toxin substrate-1
RAF	raf proto-oncogene serine/threonine protein kinase
RAS	mammalian ras gene family
RTK	receptor tyrosine kinase
SCF	stem cell factor
SMO	Smoothened, G protein-coupled receptor
SRC	sarcoma family of protein kinases
SSC	standard saline citrate
STAT	signal transducer and activator of transcription
TIE2	tyrosine kinase with immunoglobulin-like and EGF-like domains-1 (TEK)
TMA	tissue microarray
VEGF (A, C, D and E)	vascular endothelial growth factor (A, C, D and E)
VEGFR (1, 2 and 3)	vascular endothelial growth factor receptor (1, 2 and 3)
WHO	World Health Organization
WNT	wingless and int-signalling pathway
$\chi 2$	chi square

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications that are referred to in the text by their Roman numerals (I to IV):

(I) Sihto H, **Puputti M**, Pulli L, Tynninen O, Koskinen W, Aaltonen LM, Tanner M, Bohling T, Visakorpi T, Butzow R, Knuuttila A, Nupponen NN, Joensuu H. Epidermal growth factor receptor domain II, IV, and kinase domain mutations in human solid tumors (2005). J Mol Med 83: 976-983.

(II) Joensuu H, **Puputti M**, Sihto H, Tynninen O, Nupponen NN. Amplification of genes encoding *KIT*, *PDGFR*alpha and *VEGFR2* receptor tyrosine kinases is frequent in glioblastoma multiforme (2005). J Pathol 207:224-231.

(III) **Puputti M**, Tynninen O, Sihto H, Blom T, Mäenpää H, Isola J, Paetau A, Joensuu H, Nupponen NN. Amplification of *KIT*, *PDGFRA*, *VEGFR2*, and *EGFR* in gliomas (2006). Mol Cancer Res 4:927-934.

(IV) **Puputti M**, Tynninen O; Pernilä P; Salmi; Jalkanen S, Paetau A; Sihto H, Joensuu H. Expression of KIT receptor tyrosine kinase in endothelial cells of juvenile brain tumors (2010). Brain Pathol 20(4):763-70.

(V) **Puputti M**, Tynninen O, Paetau A, Joensuu H. Heterogeneity of EGFR and KIT Amplification and Their Protein Product Expression in Gliomas (2011). Manuscript submitted for publication.

ABSTRACT

The annual incidence rate of cancers of the central nervous system (CNS) is approximately 100 per million in the Northern European countries. CNS tumors are the most common solid tumors in the childhood. More than one half of malignant gliomas are eventually fatal. Gliomas, tumors of the glial cell origin, are the most common adult brain tumors. Gliomas are often divided into four malignancy grades, of which the grade four gliomas, glioblastomas, are the most aggressive ones. Glioblastomas are further divided into two categories, the primary and the secondary glioblastomas. Primary glioblastomas arise *de novo* without a pre-existing lesion, whereas secondary glioblastomas arise from a pre-existing lower grade astrocytic tumour. At present, there is no curative treatment available for glioblastoma.

Gene amplification, a gene copy number increase at a specific region of a chromosomal locus, is one of the molecular mechanisms associated with tumour progression. In general, gene amplifications are more common in advanced, widely metastatic cancers as compared to local ones. Receptor tyrosine kinase gene aberrations, such as amplifications of *EGFR* and *PDGFRA*, are also implicated in the genesis of gliomas, as are mutations of some genes.

The receptor tyrosine kinases KIT, PDGFRs and VEGFRs are currently important clinical targets for tyrosine kinase inhibitors. In the present study we investigated expression and amplification of *KIT*, *PDGFRA*, *VEGFR2* and *EGFR* in glioblastomas and in lower grade gliomas, and analyzed the hot spot mutation sites of *KIT, PDGFRA* and *EGFR* genes for presence of mutations in glioblastoma. Furthermore, we evaluated expression of KIT, SCF and VEGFR2 in paediatric brain tumors and in tumour endothelial cells, and studied the intratumoral heterogeneity of *EGFR* and *KIT* amplifications in primary glioblastomas and astrocytomas.

Mutations turned out to be infrequent in these genes suggesting that neither primary nor secondary glioblastomas are usually driven by *KIT* or *PDGFRA* mutations, or by *EGFR* kinase domain mutations. No mutations were detected with denaturing high-performance liquid chromatography (DHLPC) in *KIT* exons 9, 11, 13 or 17, *PDGFRA* exons 12 or 18, or *EGFR* kinase domain exons 18, 19 or 21 in a series of 57 glioblastomas. Similarly, no mutations were found in exons coding for the extracellular domain II *EGFR*, and only one mutation was found in an exon coding EGFR domain IV among 57 glioblastomas (I).

Unlike gene mutations, amplifications of *KIT, VEGFR2, PDGFRA* and *EGFR* turned out to be frequent in glioblastoma. *KIT* was amplified in 47% and *VEGFR2* in 39% out of the 43 primary glioblastomas investigated, and *PDGFRA* in 29% (II). As many as 35 (81%) out of the 43 glioblastomas had either *KIT* or *EGFR* amplification. Presence of *KIT*, *PDGFRA* and *VEGFR2* amplifications were strongly associated ($p < 0.0001$ for each pair wise comparison) suggesting co-

amplification, whereas no significant associations were found between amplifications of these genes and *EGFR* amplification. All four secondary glioblastomas investigated had *KIT* amplification, whereas none harboured *EGFR* amplification (II).

We investigated presence of gene amplifications also in other types of gliomas, namely in astrocytomas (n = 55), anaplastic astrocytomas (n = 39), oligodendrogliomas (n = 37) and oligoastrocytomas (n = 27) either in tumour samples collected at the time of the diagnosis or in samples collected at the time of tumour recurrence (III). In tumour tissue samples collected at the time of the diagnosis *KIT* and *PDGFRA* amplifications turned out to be more frequent in anaplastic astrocytomas than in astrocytomas, oligodendrogliomas and oligoastrocytomas [28% versus 5% ($p = 0.012$) and 33% versus 2% ($p = 0.0008$), respectively]. *VEGFR2* amplifications occurred in 6% to 17% of the gliomas sampled at the time of the diagnosis, and *EGFR* amplifications in 0% to 12%. Amplified *KIT* was more frequently present in recurrent gliomas than in newly diagnosed gliomas (19 out 71 vs. 8 out of 81, respectively; $p = 0.0066$). *KIT* amplification was associated with KIT protein expression and with presence of *PDGFRA* and *EGFR* amplifications both at the time of the first diagnosis of glioma and at the time of tumour recurrence, and with *VEGFR2* amplification at the time of tumour recurrence. Eighty-three samples taken at the first diagnosis of glioma and 74 samples collected at the time of tumour recurrence were available for assessment of coamplification of *KIT*, *PDGFRA* and *VEGFR2*. Amplification of at least two of the three genes was present more often in recurrent gliomas (ten out of 74, 14%) as compared to gliomas studied at the time of the diagnosis (three of 83, 4%; $p = 0.025$). The amplicon contained amplified *KIT* in all 13 cases (III).

None of the 9 paediatric pilocytic astrocytomas studied with *in situ* hybridization harboured amplification of *KIT* (IV). In contrast, KIT expression was common in tumour endothelial cells in pilocytic astrocytomas, and endothelial cell KIT was frequently activated (phosphorylated). Tumour endothelial cell KIT expression was associated with a young age at the time of the diagnosis. Ependymomas also frequently expressed KIT in endothelial cells, and its expression tended to be associated with a young age at the time of the diagnosis.

Finally, we investigated heterogeneity of *KIT* and *EGFR* amplification and their protein products in gliomas by studying several tissue blocks from each tumour using FISH and immunohistochemistry, respectively (V). *EGFR* amplification was found in ten (67%) out of the 15 glioblastomas studied when CISH analysis was carried out from only one tissue block, and in 11 (73%) cases when all available tissue blocks were analyzed. This finding suggests that *EGFR* amplification is not markedly heterogeneous in glioblastoma. In contrast, *KIT* was amplified in six (40%) out of the 15 index glioblastoma tissue blocks, but in 10 (67%) glioblastomas when all tissue blocks were analyzed. EGFR, EGFRvIII, KIT and phospho-KIT expression was heterogeneous in immunohistochemical staining of the tumors, and occurred at least in one part of tumour in 93%, 33%, 93% and 100% of glioblastomas, respectively. None of the seven astrocytomas contained amplified *EGFR* and only one

harboured amplified *KIT* even when all blocks were examined. These findings suggest that that glioblastomas show marked heterogeneity in *KIT* amplifications and KIT and phospho-KIT expression, and that heterogeneity is less for *EGFR* amplifications and for EGFR and EGFRvIII expression. The proportion of glioblastomas with *EGFR* or *KIT* amplification may thus be larger than reported in the literature when tumour heterogeneity is accounted for.

INTRODUCTION

Tumors of the CNS range from benign neoplasms, such as meningioma, to almost invariably lethal cancer, glioblastoma multiforme. Approximately 900 CNS neoplasms are diagnosed in Finland annually, and the annual incidence rate of CNS tumors has slowly increased (http://www.cancerregistry.fi/). In the Nordic countries, the annual incidence rate of gliomas is approximately 10 cases per 100,000 in the age-group of over 40 years (Deltour *et al*. 2009). Malignant gliomas account for approximately 70% of new cases of malignant primary brain tumors (Wen and Kesari 2008), and the CNS tumors are the most common solid tumors diagnosed in children.

The CNS consists mainly of neurons and glial cells. Neurons conduct electrical signals within the neural system, and glial cells, such as astrocytes, provide nutrition, insulation and structural support to neurons and their axons to facilitate signal conduction (DeAngelis 2001, Kleihues *et al.* 2002). Gliomas are often classified into four malignancy grades depending on histological findings that include cell mitotic activity, presence of tumour necrosis accompanied by areas of palisading cells, cell atypia and endothelial proliferation. High mitotic activity, presence of tumour necrosis, marked cell atypia and angiogenesis are characteristic features of the most malignant (grade IV) glioma, glioblastoma, whereas pilocytic astrocytomas (grade I), astrocytomas (grade II) and anaplastic astrocytomas (grade III) do not have all of these features. Most glioblastomas are considered to arise *de novo* without presence of a pre-existing lesion (primary glioblastomas), but they can arise also from pre-existing lower grade astrocytoma (secondary glioblastomas).

The median age of patients at the time of the diagnosis glioblastoma is approximately 60 years, and 45 years at the time when secondary glioblastoma is diagnosed (Fisher *et al*. 2007; Wen and Kesari 2008). Patients diagnosed with glioblastoma have a median survival time barely exceeding one year following the diagnosis (Kleihues *et al.* 2002, Ohgaki and Kleihues 2005a), and at present no curative treatment is available. In contrast, pilocytic astrocytomas that occur predominantly in children are tumors of low malignancy potential can usually be removed completely by surgery and are associated with approximately 95% 5-year survival (Fisher et al. 2008).

The factors that cause genesis of gliomas are incompletely understood. The only established risk factor is ionizing radiation (Fisher *et al*. 2007). Suggested risk factors for glioma include exposure to

electromagnetic fields, use of cellular telephones, some food ingredients, immunological factors, viral infections and occupational risk factors, but none is currently supported by firm evidence. A small proportion (approximately 5%) of patients diagnosed with malignant glioma has a family history of gliomas, but most of the glioma patients have no known genetic cause. Gliomas may rarely be associated with a genetic syndrome, such as neurofibromatosis type 1 or type 2, Turcot's syndrome (intestinal polyposis and a brain tumour) or the Li–Fraumeni syndrome (a germ-line *TP53* mutation) (Farrell *et al*. 2007).

Cancers often harbour many genetic aberrations that eventually lead to tumour invasiveness, unlimited growth and metastasis (Hanahan and Weinberg 2000). In gliomas, several genetic and epigenetic errors leading to tumour development have been characterized. The involved molecular networks include receptor tyrosine kinase (RTK) signalling through the Ras–MAPK and PI3K–AKT–mTOR pathways, and Wnt and sonic hedgehog (SHH) signalling, along with the cell cycle-regulating RB and p53 pathways (reviewed in Huse and Holand, 2010). Recently, mutations in *isocitrate dehydrogenase I* (*IDH1*) were found to occur in the majority of grade II and grade III gliomas and secondary glioblastomas (Dang *et al*. 2009, Smeitink 2010).

Gene amplifications are frequent in cancer. Amplifications and activating mutations of the genes encoding RTKs are of particular interest, since these can now be selectively targeted by monoclonal antibodies and small molecule tyrosine kinase inhibitors. Activated RTKs pass cell survival promoting signals to the nucleus, and they may direct cells to invasion and metastasis. Primary glioblastomas often harbour amplification of the gene encoding for the epidermal growth factor receptor (*EGFR;* Albertson 2006, Collins 2004), but amplification of this gene likely presents only one of the many genetic aberrations involved in glioma progression (Behin *et al*. 2003, Collins 2004, Huse and Holand, 2010).

The aim of the present study was to investigate human gliomas, glioblastomas in particular, for genetic aberrations in *KIT* (encodes the receptor of the stem cell factor, SCF), the gene encoding the platelet-derived growth factor receptor-alpha protein (*PDGFRA*), *VEGFR2* (encodes vascular endothelial growth factor receptor-2) and *EGFR*. A common nominator of these four genes is that the proteins encoded by them are now important therapeutic targets for small molecule tyrosine kinase inhibitors in the treatment of several types of human cancer.

REVIEW OF THE LITERATURE

1. Receptor tyrosine kinases

Receptor tyrosine kinases (RTKs) are a class of proteins that are important in the development, maintenance and function of the normal tissues, but they may also play a key role in the genesis of some cancers. RTKs are transmembrane growth factor receptors that mediate signals from the cell environment into the cell. They regulate fundamental cellular processes, such as the cell cycle, migration, metabolism, survival, proliferation and differentiation. During evolution, the first RTK likely arose from a fusion of an epidermal growth factor (EGF)–like domain and a cytoplasmic tyrosine kinase, which took place before appearance of animals (King and Carroll 2001, Robinson *et al.* 2000a). RTKs share two major functional domains: an extracellular ligand-binding domain and an intracellular domain that has tyrosine kinase function (Grassot *et al*. 2006).

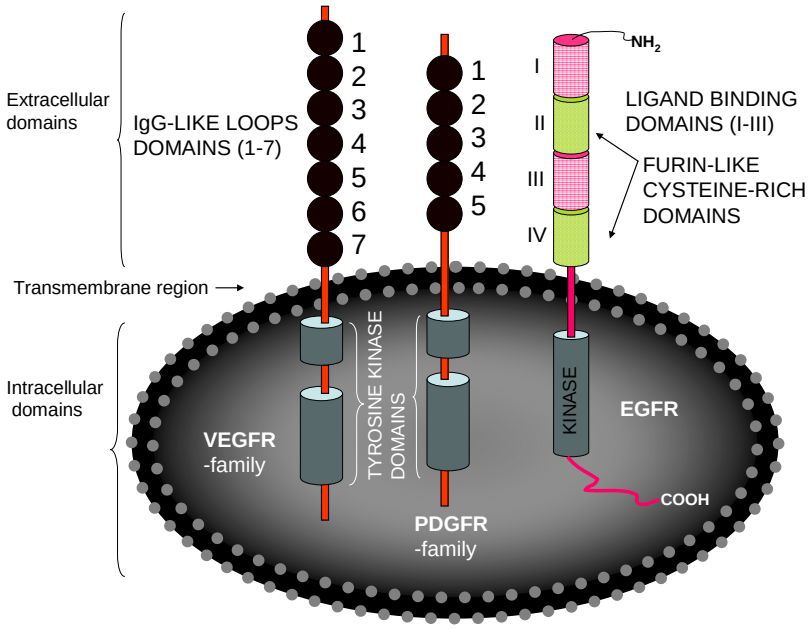


Figure 1. A schematic illustration of receptor tyrosine kinases of the VEGFR, PDGFR and EGFR-families. These families share a common overall structure consisting of an extracellular domain, a transmembrane region and an intracellular domain, but the ligand-binding extracellular domain, in particular, shows structural differences.

RTKs are activated (phosphorylated) following binding of a specific soluble ligand. Phosphorylation of tyrosine residues increases kinase enzymatic activity and activates downstream signalling pathways (Grassot *et al.* 2006, Hubbard and Till 2000, Kostich *et al.* 2002, Robinson *et al.* 2000). Since RTKs are involved in the regulation of the cell, an imbalance in the RTK signalling homeostasis may give rise to neoplastic transformation, and dysregulation of RTK signalling occurs in various types of human cancer. Several molecular mechanisms may influence this homeostasis.

These mechanisms include an increased dose of RTK-specific ligands; an increased number of RTKs on the cell membrane; gene amplification, mutation or aberrant regulation of the gene that encodes the RTK; abnormal post-translational regulation of messenger-RNAs transcribed to an RTK; defective down regulation of RTKs; and abnormal interaction between RTKs and the proteins that bind to them including proteinases that cleave of the extracellular portion of the RTK; and intracellular phosphatase activity.

1.1 RTK signalling pathways

Intracellular cell signalling is mediated by various proteins, micro-RNA and short interfering RNA (siRNA) that interact with each other and the DNA within a cell (Feero WG *et al.* 2010). RTK signal is conducted downstream via a cascade of proteins, and eventually the signal is delivered into the nucleus where it induces transcription of the target genes. Ligand binding to the cognate RTK causes receptor dimerization, autophosphorylation of the adjacent intracellular kinase domains, and subsequently activation of one or more signalling pathways, such as the RAS-RAF-MEK-ERK, PI3K-AKT, PLCγ-PKC, SRC/JNK or JAK/STAT pathways (Blume-Jensen *et al.* 1998, Jiang & Liu 2008, Reber *et al.* 2006, Roberts & Der 2007, Roskoski 2005a, Wong *et al.* 2007).

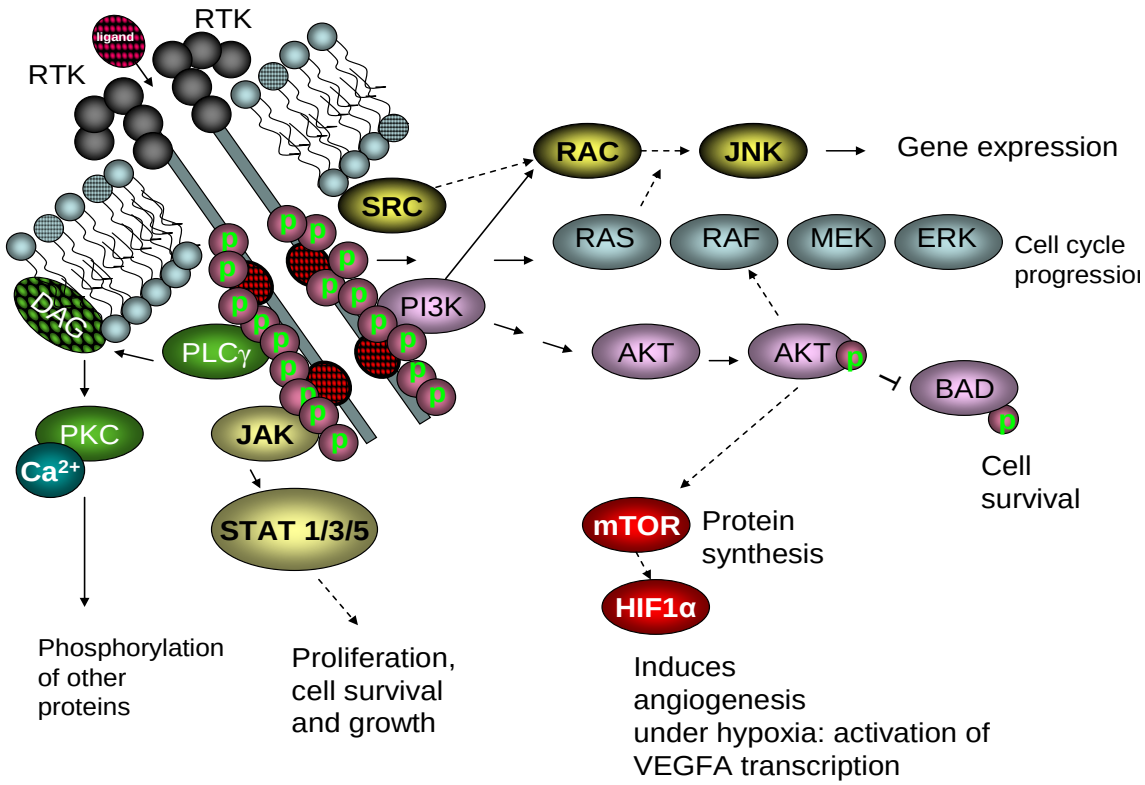


Figure 2. Schematic presentation of basic signalling pathways downstream of an RTK. Upon ligand binding and dimerization of the RTK, PI3K/AKT, RAS/RAF/ERK, SRC/JNK, JAK/STAT and/or PLCγ/PKC signalling pathways are activated resulting eventually in alteration of gene expression and cell function.

1.2 Epidermal Growth Factor Receptor–family (RTK Subclass I)

The epidermal growth factor receptor (EGFR, also known as ErbB-1 or HER-1) is the cell-surface receptor for the members of the EGF-family of extracellular protein ligands. The gene encoding this protein is located at the chromosomal locus 7p12. EGFR is a member of the ErbB family of receptors (www.genome.ad.jp) (Grassot *et al.* 2006). It resides on the plasma membrane and is activated following binding of one of its 11 known ligands, which can be classified into three groups (a) ligands that specifically bind to EGFR (EGF, transforming growth factor-a, amphiregulin and epigen); (b) those that bind to EGFR and ERBB4 (betacellulin, heparin-binding EGF and epiregulin); and (c) neuregulin (NRG) that binds to ERBB3 and ERBB4. NRG1 and NRG2 bind to both ERBB3 and ERBB4, whereas NRG3 and NRG4 only to ERBB4 (Mitsudomi and Yatabe 2010). In the absence of a bound ligand EGFR is a monomer, but ligand binding leads to formation of a receptor homodimer. EGFR may dimerize with another member of the ErbB receptor family, such as ErbB2/HER2/neu, to create a heterodimer. Ligand-induced EGFR dimerization leads to autophosphorylation of several tyrosine residues in the cytoplasm domain of each receptor monomer. The best characterized downstream signalling pathways include the RAS-RAF-MEK-ERK, PI3K-AKT and PLCγ-PKC pathways, which upon activation stimulate cell proliferation, motility and survival (Boonstra *et al.* 1995, Citri & Yarden 2006, Jiang & Liu. 2008, Roberts & Der 2007, Wong *et al.* 2007, Zandi *et al.* 2007).

Elevated tumour EGFR content is associated with unfavourable survival in several human types of human cancer, such as carcinomas of the head and neck, bladder, ovary, uterine cervix and oesophagus (Nicholson *et al.* 2001). Amplification of *EGFR*, often resulting in increased tumour EGFR levels, has been detected in several types of human cancer, such as breast carcinoma, non-small-cell lung cancer (NSCLC), oesophageal squamous cell carcinoma and in primary glioblastoma (Bhargava *et al.* 2005, Hanawa *et al.* 2006, Suzuki *et al.* 2005, Wong *et al.* 1992). In primary glioblastoma, *EGFR* amplification is present in approximately 30 to 50 percent of the tumors (Ekstrand *et al.* 1991, Frederick *et al.* 2000, Hui *et al.* 2001). In the absence of gene amplification EGFR overexpression may result from a variety of molecular mechanisms that include increased activity of the *EGFR* promoter or dysregulation at the translational or the post-translational level.

Mutations of *EGFR* are frequent in human cancers. These can be divided into three main groups based on location of the mutation: mutations in the part of the gene that encodes for the RTK extracellular domain, those found in the intracellular tyrosine kinase domain, and mutations found in the gene sequences that encode the rest of the protein (Zandi *et al.* 2007). *EGFR* kinase domain mutations are relatively rare; small deletions and point mutations have been detected in NSCLC (Huang *et al.* 2004, Mitsudomi *et al.* 2005, Paez *et al.* 2004). In contrast, deletions in the extracellular domain involving exons 2 to 7 of *EGFR* are frequent in glioblastoma, but can be found also in other types of cancer, such as prostate cancer, breast cancer, NSCLC, ovarian cancer, and head and neck cancer (Fenstermaker & Ciesielski 2000, Frederick *et al.* 2000, Moscatello *et al.* 1995, Olapade-

Olaopa *et al.* 2000, Okamoto *et al.* 2003, Sok *et al.* 2006, Wong *et al.* 1992). Such a partially deleted gene gives rise to truncated, constitutively activated receptors that do not bind ligands and are thus not under normal regulation (Zandi *et al.* 2007).

In glioblastoma, expression of the truncated form of EGFR (EGFRvIII) occurs in approximately 20% to 30% of the cases (Gan *et al.* 2009). EGFRvIII activates not only the same downstream effectors pathways as EGFR, but also the c-Jun N-terminal kinase (JNK) via PI3K (Antonyak *et al.* 1998, Chu *et al.* 1997, Pedersen *et al.* 2005).

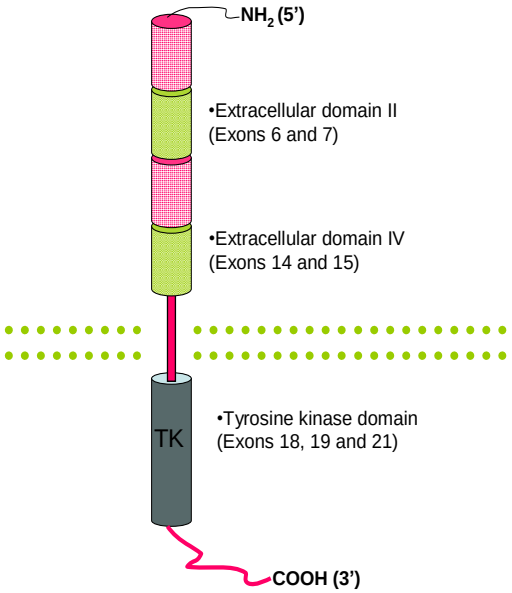


Figure 3. EGFR: A schematic presentation indicating the exons that code for the extracellular domains II and IV, and the tyrosine kinase domain.

1.3 Platelet Derived Growth Factor Receptor Alpha-family (RTK subclass III)

Type III receptor protein-tyrosine kinases include the stem cell factor (SCF) receptor (SCFR/KIT), the platelet-derived growth factor (PDGF) receptor (alpha and beta), the macrophage colony-stimulating-factor receptor-1 (CSF1R) and the fms-related tyrosine kinase-3 (FLT3). These receptor protein tyrosine kinases all share similar features: an extracellular immunoglobulin-like ligand-binding domain, a single transmembrane segment and a spilt cytoplasmic kinase domain. The class III receptors are characterized by presence of five immunoglobulin-like domains in their extracellular portion. *KIT* and *PDGFRA* genes are located adjacent at the chromosomal locus 4q12 next to the vascular endothelial growth factor receptor-2 (*VEGFR2*) gene, which belongs to the RTK subclass V (Grassot *et al.* 2006).

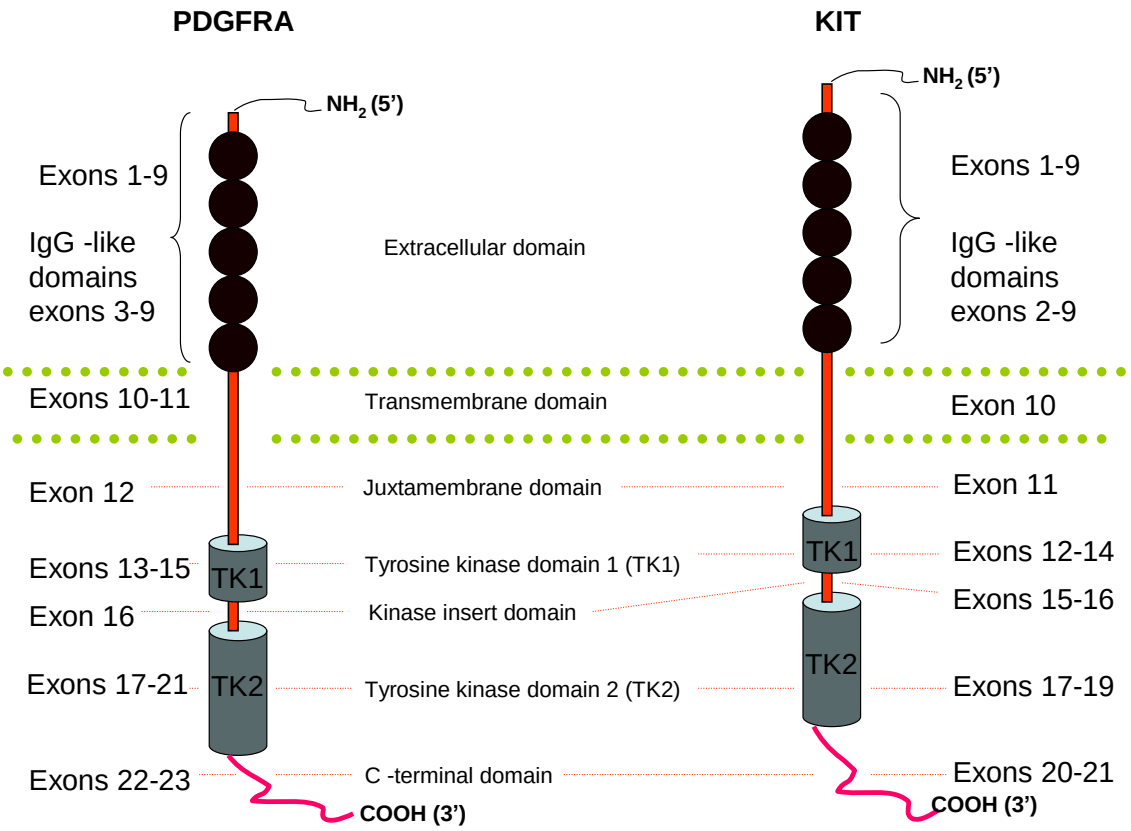


Figure 4. PDGFRA and KIT RTKs: Exons coding extracellular, transmembrane and intracellular tyrosine kinase domains in PDGFRA and KIT receptors.

1.3.1 Stem Cell Growth Factor Receptor (KIT)

SCF is the ligand of the KIT receptor tyrosine kinase. KIT binds the SCF with the first three of the five immunoglobulin-like loops in the extracellular domain of the receptor. Physiologically SFC may exist as a membrane-bound form or as a soluble form. Interaction of the SCF with KIT rapidly induces KIT dimerization and increases its autophosphorylation activity. SCF and KIT are critical for survival and development of stem cells involved in haematopoiesis, skin pigmentation and reproduction, as well as in mast cell development. KIT is also expressed in epithelial cells of the breast and in basal cells of the skin. SCF and KIT play a role in the development of the interstitial cells of Cajal (ICC) in the intestine, and in the learning functions in the hippocampus region of the brain.

Upon KIT dimerization, downstream of KIT multiple signal transduction components are activated. These components include PI3K, the SRC family members, the JAK/STAT pathway and the RAS-RAF-MAP kinase cascade (Grassot *et al.* 2006, Linnekin. 1999, Reber *et al.* 2006, Roskoski. 2005b,

Tsuura *et al.* 1994). In addition, the KIT receptor promotes cell survival via activation of PI3K/AKT-mediated phosphorylation of BAD that regulates cell apoptosis (Blume-Jensen *et al.* 1998).

Deficiency in the KIT function impairs erythropoiesis, pigmentation of the skin, fertility and gastrointestinal peristalsis. KIT may be activated through a gain-of function mutation that may result in a malignant phenotype in cells dependent on KIT function. Abundant KIT expression has been reported to occur in some sarcomas (notably in GIST), small cell carcinoma of the lung, adenoid cystic carcinoma of the head and neck, renal chromophobe carcinoma, as well as in some thymic, ovarian and breast carcinomas and in glioblastoma (Miettinen &Lasota 2005, Sihto H *et al.* 2007).

KIT is often mutated and constitutively activated in gastrointestinal stromal tumors (GISTs), systemic mastocytosis and mast cell leukemia. *KIT* amplifications are rare in GISTs, but high levels of KIT protein are detected in most (95%) of these tumors. Approximately 80% of GISTs have activating *KIT* mutations that are most commonly located in exons 11 and 9 of the gene. Rarely, *KIT* exon 13 and 17 mutations are found in GISTs that have not been exposed to imatinib or other tyrosine kinase inhibitors (Hornick & Fletcher 2002, Joensuu *et al.* 2002, Miettinen & Lasota 2005, Sihto *et al.* 2005, Tabone *et al.* 2005). Most GISTs are probably either KIT or PDGFRA driven tumors. They probably originate from a precursor cell of the ICC based on their phenotypic resemblance with this cell phenotype (Corless *et al.* 2004, Hirota *et al.* 1998, Kindblom *et al.* 1998, Sarlomo-Rikala *et al.* 1998). The ICCs are located in the gastrointestinal tract, where they regulate peristalsis and mediate autonomic nervous system functions (Hirota *et al.* 1998, Huizinga *et al.* 1995, Kindblom *et al.* 1998, Maeda *et al.* 1992, Miettinen & Lasota 2005, Sarlomo-Rikala *et al.* 1998, Torihashi *et al.* 1999).

GIST may rarely arise also in children and young adults. In paediatric GISTs *KIT* and *PDGFRA* mutations are far less common than in adult tumors, and imatinib is generally less effective than in the treatment of adult GISTs. Despite lack of *KIT* mutations, these tumors have often activated KIT, but their molecular pathogenesis is likely heterogeneous (Janeway *et al.* 2007).

Epidermal melanocytes and mast cells express KIT constitutionally, and KIT is likely important in their development and function. Primary malignant melanomas and their metastasis show variable KIT expression. Imatinib has recently been found to be effective in a proportion of patients who have either mucosal melanoma, acral melanoma or melanoma arising in sun exposed skin. In one study, *KIT* mutations were found in 38% of mucosal and in 6% of acral melanomas, and their presence may likely be used as a guide for treatment selection (Handolias *et al*. 2010). *KIT* activating mutations have been detected in human mast cell neoplasms, most of them in exon 17. Of germ cell tumors KIT may be expressed in seminomas, and a majority of classic testicular seminomas express KIT. One third of seminomas and their extra testicular analogs harbour *KIT* mutations. The reported point mutations are located in exon 17. In addition, a few point mutations and deletions of exon 11 have been reported in testicular tumors (Hawkins E *et al.* 1997, Kemmer K *et al.* 2004, Looijenga LH *et al.*

2003, Przygodzki *et al.* 2002, Rapley *et al.* 2004, Sakuma *et al.* 2003, Sakuma *et al.* 2004, Tian *et al.* 1999). A gain in the copy number of *KIT* and KIT overexpression may be associated with progression of carcinoma *in situ* to an invasive testicular germ cell tumour (McIntyre *et al.* 2005).

KIT is expressed in a subset of hematopoietic stem cells, and it likely has an important role in erythropoiesis. KIT-deficient mice develop hypoplastic bone marrow and macrocytic anaemia (Waskow *et al.* 2004). Approximately 70% of acute myeloid leukemias (AMLs) express KIT (Bene *et al.* 1998, Escribano *et al.* 1998, Kanakura *et al.* 1993, Schwartz *et al.* 1999, Valverde *et al.* 1996). Some adult sarcomas, including angiosarcomas, are also occasionally KIT-positive in immunostaining, although mutations in *KIT* exons 11 or 17 are lacking (Miettinen et al. 2000; Hornick & Fletcher 2002). Hemangioendotheliomas and hemangiomas are usually KIT-negative, but KIT may be expressed in juvenile hemangiomas of the immature phenotype (Miettinen *et al.* 2000, Miettinen & Lasota 2005).

In the normal kidney, KIT is expressed in distal nephrons, and it is expressed in normal fetal and adult renal tubules (Kato *et al*. 2005, Miliaras *et al.* 2004). KIT is expressed in some malignant renal tissues, such as renal chromophobe cell carcinomas and very frequently in oncocytomas, but mutations of *KIT* or *PDGFRA* have not been found (Kato *et al.* 2005, Miliaras *et al.* 2004). KIT may be expressed in paediatric renal tumors, but *KIT* mutations or amplifications are not present. KIT expression may be associated with unfavourable outcome in a subset of Wilm's tumors and clear cell sarcomas of the kidney, and it is expressed in a proportion of paediatric synovial sarcomas, osteosarcomas and Ewing sarcomas (Jones *et al.* 2007, Smithey *et al.* 2002).

1.3.2 Platelet Derived Growth Factor Alpha (PDGFRA)

PDGFRs and their ligands, platelet derived growth factors (PDGFs), are important in mesenchymal cell migration and proliferation. During embryogenesis the PDGF/PDGFR system is essential e.g. in the development of the cardiovascular system, the brain and the lungs. In adults, PDGF/PDGFR signalling is involved in angiogenesis, wound healing and inflammation.

The PDGF family consists of PDGF-A, -B, -C and -D ligands. The PDGFs bind to the protein tyrosine kinase receptors PDGFR-alpha and -beta (PDGFRA and PDGFRB, respectively). These two receptors dimerize upon binding of the PDGF dimer, leading to three possible receptor combinations (AA, BB or AB) (Heidaran *et al.* 1990, Matsui *et al.* 1989).

Abnormalities in PDGF/PDGFR signalling contribute to several human diseases and malignancies (Jones & Cross 2004). Constitutive activation of PDGFRA is present in some myeloid malignancies as a consequence of formation of a fusion gene. Activating mutations of *PDGFRA* are present in a subset of GISTs.

The structure of PDGFRA is very similar to KIT: five immunoglobulin–like extracellular domains, a transmembrane domain, an intracellular juxtamembrane domain, a split tyrosine kinase domain with a kinase insert and a C-terminal tail. Activating mutations of *PDGFRA* occur most commonly in the activation loop (exon 18) and in the juxtamembrane region coding exon 12 (Blume-Jensen & Hunter 2001, Heinrich *et al.* 2003, Jones & Cross 2004, Kawagishi *et al.* 1995, Sihto *et al.* 2005).

In low grade astrocytomas, one of the earliest molecular alterations includes overexpression of PDGFA/PDGFRA, which creates an autocrine growth-factor stimulation loop. Amplification of *PDGFRA* is a common molecular genetic aberration associated with diffusely infiltrating astrocytic gliomas and secondary glioblastomas (Behin *et al.* 2003, Reifenberger & Collins. 2004).

1.4 The Vascular Endothelial Growth Factor Receptor-family (RTK subclass V)

Vascular endothelial growth factors (VEGFs) mediate angiogenic signals to the vascular endothelium. They are classified as class V receptor tyrosine kinases, and show structural similarities to the subclass III RTKs except that they have seven immunoglobulin-like loops in the extracellular domain (Figure 1). Three receptors have been identified that specifically bind VEGF. These receptors have been named VEGFR1, VEGFR2 and VEGFR3. In the adult, VEGFR1 and VEGFR2 are expressed mainly in the blood vascular endothelium, whereas expression of VEGFR3 is restricted largely to the lymphatic endothelial cells (Grassot *et al.* 2006, Veikkola *et al.* 2000). VEGFR2 is a high-affinity receptor for the potent angiogenic ligands VEGF-A and VEGF-B, and the processed forms of VEGF-C and VEGF-D.

VEGFs and their endothelial receptors are key regulators of angiogenesis, vasculogenesis and lymphangiogenesis. VEGFR-1 may act as a stimulator of pathological angiogenesis when activated by its ligands VEGF-B and PlGF (placental growth factor). PlGF recruits angiogenic macrophages to tumors. VEGF-C and VEGF-D induce lymphangiogenesis via VEGFR-3, but VEGFR-3 is important also for angiogenesis acting together with VEGF/VEGFR-2 and Dll4 (Delta-like protein 4)/Notch signalling to control angiogenic sprouting (Lohela *et al*. 2009). Angiopoietin-2 and the Tie2-receptor (tyrosine kinase with immunoglobulin-like and EGF-like domains-1) are also involved in tumour angiogenesis, where Tie2 utilizes a unique signalling mechanism at the endothelial cell-cell junctions (Saharinen *et al*. 2010).

2. Cells and tumors of the central nervous system

Tumors of the nervous system account for less than 2% of all human malignancies. Yet, approximately 175,000 new cases are diagnosed worldwide annually, probably without a large variance in the incidence between geographic regions or populations. Gliomas are the most common brain tumors, with an annual incidence of 5 to 10 cases per 100,000 persons. Gliomas are believed to arise from the glial cells of the CNS that provide nutrition, insulation and structural support for neurons and neuronal axons, and thus facilitate signal conduction (DeAngelis. 2001, Kleihues *et al.* 2002).

Glial cells or neuroglia consists of ependymal cells, oligodendrocytes and astrocytes; the latter are also known collectively as astroglia. Radial glial cells, Schwann cells and satellite cells have various functions ranging from insulation and to microenvironmental control within the brain. Microglia are glial cells that act in active immune defence of the brain. Ependymal cells form the lining of the ventricular system, and ependymal cells are believed to contribute to the cerebrospinal fluid flow and homeostasis (Meiniel 2007). Oligodendrocytes are myelin-forming glial cells. The neurons are dependent on the myelin sheath provided by the oligodendroglial cells, since myelin provides insulation and allows fast neurotransmission. The Schwann cells are responsible for the myelin production in the peripheral nervous system.

The most abundant type of glial cells are astrocytes, star-shaped cells that give support to the endothelial cells at the blood-brain-barrier (BBB), provide nutrition to the nervous tissue and participate in repair of the brain tissue. Astrocytoma is a tumour likely derived from these cells, and the tumour cells in astrocytoma resemble morphologically normal astrocytes. Similarly, oligodendrogliomas are defined by the presence a major oligodendrocyte population in the tumour. Mixed forms of these tumour types exist, and the most malignant glial tumors rarely consist of one homogeneous cell population. Based on histological classification, astrocytomas, oligodendrogliomas and ependymomas belong to tumors of neuroepithelial origin (Bene *et al.* 1998, Cavaliere *et al.* 2005, Collins. 2004, DeAngelis 2001, Kleihues *et al.* 2002, Louis *et al.* 2007).

Tumour stem cells have been implicated in formation of glial tumors. Oligodendrocytes and astrocytes may have a common progenitor cell in the subventricular zone (Carmen *et al*. 2007), and neuroglial precursors are thought to emigrate from this spatially restricted germinal zone to colonize the hemispheres (Levison *et al.* 1993). In case of ependymal tumors, the radial glial cells are thought to be candidate stem cells that give rise to this tumour type (Taylor et al. 2005, Poppleton & Gilbertson 2007, Vescovi *et al.* 2006). Neural stem cells have been identified in the mature CNS, and new neurons and glial cells are produced throughout life from these neural stem cell niches (Galli *et al.* 2004, Galli *et al.* 2008).

Glial tumors are often composed of heterogeneous cell populations containing also small subpopulations of highly proliferative immature precursor cells that may or may not be neural CD133-positive stem cells, which may contribute to tumour initiation (Chen *et al*. 2010). The etiology and causes of gliomas are still unclear. Variable environmental factors have been suggested as risk factors for glioma, but the data are mostly contradictory or inconclusive. Few studies suggest an increase in the incidence of malignant gliomas over time, but these findings may be confounded by prolonged life expectancy and increased use of brain imaging. High-dose ionizing radiation, some genetic syndromes (such as neurofibromatosis, tuberous sclerosis and Li-Fraumeni syndrome) and old age are the only generally accepted risk factor for glial neoplasms, and some hereditary syndromes for menigeomas (Bondy *et al*. 2008). Gliomas hardly ever spread outside of the CNS or give rise to metastases in other internal organs. However, gliomas often have a diffuse growth pattern within the tissue, and, hence, their surgical removal usually remains partial (Cavaliere *et al.* 2007, DeAngelis 2001)

Table 1A. Histological classification of selected CNS tumors: location, age at clinical manifestation and 5-year survival.*

Tumors of neuroepithelial tissue	**Typical location**	Age at manifestation (% of cases)			5-year survival (%)
		0-20 years	20-45 years	>45 years	
Astrocytic tumors					
Pilocytic astrocytoma (grade 1)	Cerebellum, optic nerve	74	20	6	>85
Astrocytoma (grade 2)	Cerebral hemispheres	10	61	29	>50
Anaplastic astrocytoma (grade 3)	Cerebral hemispheres	11	34	55	22
Glioblastoma multiforme (grade 4)	Cerebral hemispheres	3	25	72	<3
Oligodendroglial tumors					
Oligodendroglioma/ Anaplastic oligodendroglioma	Cerebral hemispheres	8	46	46	>50
Ependymal tumors					
Ependymoma (grade 2)	Ventricles, spinal cord	37	38	25	<30
Medulloblastoma (grade 4)	Cerebellum	74	23	3	>50

Table 1B. Other histological types of CNS tumors.*

Astrocytic tumors	***Pineal parenchymal tumors***
	Pineocytoma
Pleomorphic xanthoastrocytoma	Pineoblastoma
Subependymal giant-cell astrocytoma	***Embryonal tumors***
Oligodendroglial tumors	Primitive neuroectodermal tumour
Mixed gliomas	***Meningeal tumors***
Oligoastrocytoma	Meningioma
Anaplastic oligoastrocytoma	Hemangiopericytoma
Ependymal tumors	Melanocytic tumour
Anaplastic ependymoma	Hemangioblastoma
Myxopapillary ependymoma	***Primary central nervous system lymphomas***
Subependymoma	***Germ-cell tumors***
Choroid-plexus tumors	Germinoma
Choroid-plexus papilloma	Embryonal carcinoma
Choroid-plexus carcinoma	Yolk-sac tumour (endodermal-sinus tumour)
Neuronal and mixed neuronal–glial tumors	Choriocarcinoma
Gangliocytoma	Teratoma
Dysembryoplastic neuroepithelial tumour	Mixed-germ-cell tumors
Ganglioglioma	***Tumors of the sellar region***
Anaplastic ganglioglioma	Pituitary adenoma
Central neurocytoma	Pituitary carcinoma
Metastatic tumors	Craniopharyngioma

*Modified from (DeAngelis. 2001, Kleihues *et al.* 2002 Ohgaki & Kleihues 2005a and b, Surawicz *et al.* 1998).

2.1 Astrocytic tumors

Astrocytomas are usually found in the cerebral hemispheres. Adult gliomas may show histological variations that may make their classification demanding. The WHO classification grading is based on the number of mitoses, presence of nuclear atypia, microvascular proliferation and tumour necrosis (DeAngelis 2001, Kleihues *et al.* 2002). Diffuse low grade astrocytomas (WHO grade 2) are well differentiated tumors with a low density of tumour cells that infiltrate diffusely into the brain parenchyma. The mitotic activity is low, and necrosis and microvascular proliferation are absent. Diffuse low-grade astrocytomas tend to progress into anaplastic astrocytomas (WHO grade 3) and eventually to glioblastoma (WHO grade 4) (Behin *et al.* 2003). The grade 4 gliomas are also called "glioblastoma multiforme" reflecting the heterogeneity of these tumors. Glioblastomas may present with varying cell morphology within the tumour, where high grade malignant areas with atypical tumour cells may be adjacent to morphologically less anaplastic tumour cells or endothelial proliferations.

Primary glioblastomas are usually diagnosed among older patients, whereas the lower grade gliomas tend to affect younger adults, although the age distribution at presentation is wide. The mean age of adult glioma patients is 40 to 45 years at the time of the diagnosis and that of glioblastoma patients 55 to 60 years (DeAngelis 2001, Kleihues *et al.* 2000, Ohgaki & Kleihues 2005a). Patients diagnosed with anaplastic astrocytoma have a median survival time of approximately 3 years after the diagnosis, but the median survival time is only approximately 1 year following the diagnosis of glioblastoma (DeAngelis 2001).

The current treatment of malignant gliomas is surgical resection, radiation therapy and in selected cases chemotherapy, notably temozolomide in glioblastoma. Unfortunately, none of these treatments is curative. Small tyrosine kinase inhibitors such as imatinib, geftinib and erlotinib have limited activity in the treatment of gliomas (Behin *et al.* 2003, DeAngelis. 2001, Dresemann. 2005, Reardon *et al.* 2005, Rich *et al.* 2004).

2.1.1 Molecular pathogenesis of glioblastomas and grade 2 and 3 astrocytomas

EGFR amplifications, mutations and re-arrangements are frequently present in glioblastoma. Amplification of *EGFR* and subsequent over-expression of the EGFR protein are probably the most common genetic alterations in primary glioblastoma, and occur at a frequency of about 40% (Gan HK *et al*. 2009). Approximately 70% of the glioblastomas that over-express EGFR have re-arrangement of *EGFR* (Ekstrand *et al*. 1991). The most common of *EGFR* mutations is the deletion variant EGFRvIII, where 267 amino acid residues have been deleted from the extracellular domain of EGFR as a result from deletion of exons 2 to 7 from the gene. This results in inability of the truncated protein to bind any known growth factor, but the truncated receptor remains constitutively active and signals for a prolonged time as a result from impaired protein endocytosis and degradation due to deficient ubiquitination (Huang *et al*. 1997). Most glioblastomas (50% to 60%) that show amplification of *EGFR* express EGFRvIII (20% to 30% of all unselected glioblastomas). EGFRvIII can signal through homodimers or through heterodimers with e.g. EGFR or HER2. Glioma cells have been found also to secrete microvesicles expressing EGFRvIII that are released into cellular surroundings and the blood of tumour-bearing mice, and can merge with the plasma membranes of cancer cells lacking EGFRvIII. These events lead to the transfer of oncogenic activity via the microvesicles (Al-Nedawi *et al*. 2008).

EGFR is located on chromosome 7. Yet, the most frequent genetic alteration in primary glioblastomas (occurs up to 80% of cases) is LOH on chromosome 10 (Ohgaki & Kleihues 2009), which finding underlines presence of many genetic rearrangements in glioblastoma. Several genetic alterations may occur when diffuse astrocytoma is transformed to anaplastic astrocytoma, and eventually to secondary glioblastoma. EGFRvIII is less prevalent in secondary glioblastomas compared to primary glioblastomas (Gan *et al*. 2009). At a population level, one of the early genetic rearrangements in

secondary glioblastoma includes mutation of *TP53*, which is found in over 60% of the tumors. *TP53* mutations exist in primary glioblastomas, but not as frequently as in secondary glioblastomas (28% vs. 65%) (Ohgaki & Kleihues 2009). Amplification *MDM2* is not frequent in glioblastoma (<15%), and occurs exclusively in primary glioblastomas that lack *TP53* mutation (Ohgaki & Kleihues 2009, Rao *et al*. 2010). In secondary glioblastomas *DCC* (Deleted in Colorectal Carcinoma) tumour suppressor gene expression is frequently lost, and tumors often have amplified *PDGFR* (Reifenberger & Collins 2004).

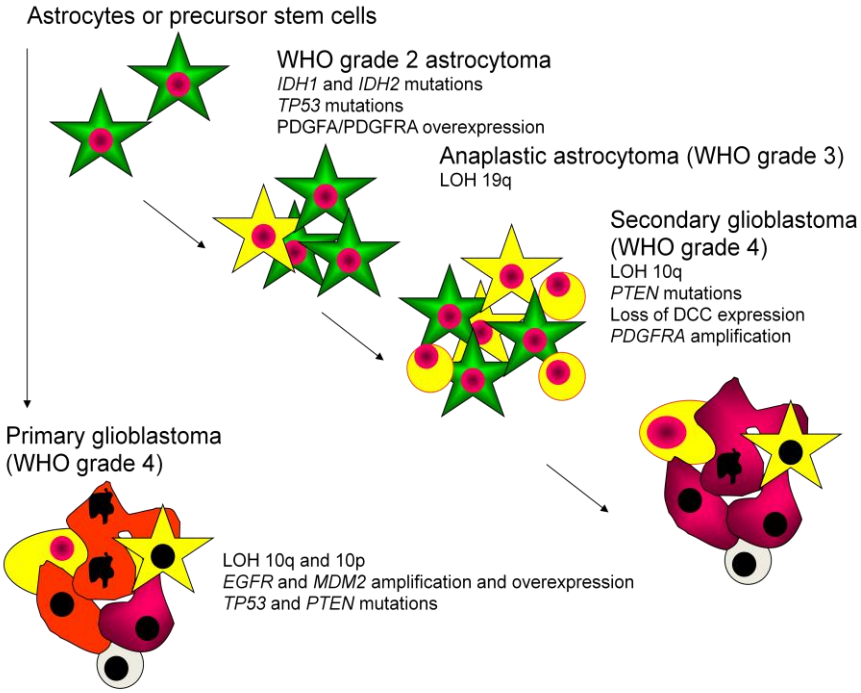


Figure 5. Genetic pathways to secondary and primary glioblastoma. Glioblastoma may develop *de novo* without a pre-existing lower grade tumour. These tumors are called primary glioblastomas, and they frequently contain *ERGR* amplification and express EGFR. The alternative pathway entails gradual glioma development from a pre-existing lower grade lesion. Such glioblastomas are called secondary, and are characterized by presence of the PDGFA growth factor and its receptor (PDGFRA), and IDH1 and IDH2 mutations. These two pathways are distinct but not mutually exclusive (Behin *et al*. 2003, Ohgaki & Kleihues 2007, Reifenberger & Collins 2004).

2.1.1.1 Isocitrate dehydrogenase-1 mutations

Isocitrate dehydrogenase-1 (*IDH1*) mutations were recently discovered as a frequent and early alteration in human brain tumors (Dang *et al*. 2009). Interestingly, these data suggest for the first time that the molecular pathway to low grade astrocytomas, oligodendroglial tumors and secondary glioblastomas may have a common origin. Primary glioblastomas rarely harbour *IDH1* mutations, suggesting that primary and secondary glioblastomas originate from a different cell. The histological criteria for the differential diagnosis between low-grade astrocytomas, oligodendrogliomas and oligoastrocytomas are subjective, and since most of these low-grade gliomas are can now be genetically characterized either by the presence of *IDH1* mutation and *TP53* mutation (secondary glioblastomas, low grade astrocytomas) or an *IDH1* mutation and 1p/19q loss (oligodendrogliomas

and anaplastic oligodendrogliomas).The molecular classification may augment or even eventually replace the histological classification of diffuse gliomas (Ohgaki & Kleihues 2009).

Isocitrate dehydrogenase-1 (encoded by *IDH1* at 2q33) is normally in the cytosol and peroxisomes, and catalyzes oxidative carboxylation of isocitrate to α-ketoglutarate, generating NADPH from $NADP^+$ (Smeitink 2010). Forced expression of mutant IDH1 reduces formation of α-ketoglutarate in cultured cells and increases HIF1α levels. This may facilitate tumour growth (Zhao *et al*. 2009).

IDH1 mutations were first identified by Parsons *et al.* in glioblastoma multiforme (Parsons *et al*. 2008), and they were subsequently found in over 80% of anaplastic astrocytomas, low-grade astrocytomas, anaplastic oligodendrogliomas, oligodendrogliomas, oligoastrocytomas, anaplastic oligoastrocytomas and secondary glioblastomas (Yan *et al*. 2008, Blass *et al*. 2009, Watanabe *et al*. 2009). Of note, *IDH1* mutations are infrequent (<5%) or absent in primary glioblastomas, pilocytic astrocytomas, ependymomas and other CNS tumors. The *IDH1* mutation frequency mapping thus suggests that low grade astrocytomas, secondary glioblastomas, oligogendrogliomas and oligoastrocytomas may share the same cell of origin. Since *IDH1* mutations occur predominantly in younger patients and are associated with a better prognosis than is the case with tumors that do not carry the *IDH1* mutation, D-2-hydroxyglutarate may become a biomarker serving diagnostic, prognostic and therapeutic purposes (Smeitink 2010).

2.1.1.2 Intratumoral heterogeneity of EGFR amplification in glioblastomas

Glioblastomas frequently display morphological, cytological and genetic heterogeneity (Furnari *et al*. 2007, Jung *et al*. 1999, Kleihues *et al*. 2002, Maher *et al*. 2001), and genetic intratumoral heterogeneity has long been known to occur in glioblastomas (Harada *et al.* 1998, Ren *et al.* 2007). *EGFR* amplification has been found in approximately 40% of glioblastomas when studied usually by *in situ* hybridization techniques and from one tissue block, but the observed frequency varies substantially in single studies ranging from 24% to 73% Fischer *et al*. 2008, Guillaudeau *et al*. 2009, Kramar *et al.* 2007, Layfield *et al*. 2006, Lopez-Gines *et al*. 2005, Miyanaga *et al*. 2008, Necesalová *et al*. 2007). A Systematic study on intratumoral heterogeneity of *EGFR, PDGFRA* and *MET* amplification reveal that in gliobalstomas, subpopulations with different RTK amplifications exist but cells with different amplifications are actually subclones with common CDKN2A homozygous deletions and TP53 mutations, and thus cells with different RTK amplifications do not originate independently but are derived from the same precursor (Snuderl *et al.* 2011).

2.2 Paediatric CNS tumors

Tumors of the brain or the spinal cord are the most common solid tumors of the childhood. The most common histological tumour type is astrocytoma that accounts for approximately one third of all paediatric brain tumors (Larouche *et al.* 2007, Rickert *et al*. 1997). Most of the astrocytic tumors are low grade (Rickert *et al*. 1997). Medulloblastomas and ependymomas each comprise approximately

10% of the paediatric brain tumors. The most important prognostic factors are tumour histology (whether classified malignant or not) and completeness of resection (Larouche *et al.* 2007).

2.2.1 Pilocytic astrocytoma

Pilocytic astrocytomas (WHO grade 1) account for more than 20% of all CNS tumors in children aged less than 15, and they mainly occur in children and in young adults (Jones *et al.* 2006). Pilocytic astrocytomas are highly angiogenic showing endothelial-cell proliferation and haemorrhages, but still usually slow-growing, and are considered benign tumors (Gesundheit *et al.* 2003, Reifenberger & Collins. 2004). These tumors are typically well-circumscribed and occur in the cerebellum or at the optic tract (Reifenberger & Collins. 2004). Histologically, they exhibit a biphasic pattern with bipolar, highly fibrillated astrocytes accompanied by Rosenthal fibers adjacent to microcystic regions. The astrocytes frequently show granular bodies. Cell atypia, mitoses and necrotic areas that are characteristic to high grade gliomas are lacking, reflecting their benign nature (Reifenberger & Collins. 2004).

Pilocytic astrocytomas usually do not contain the molecular genetic alterations involved in the progression of diffuse astrocytomas, such as *TP53* mutations (Haapasalo *et al.* 1999, James & Collins 1992, Ohgaki *et al.* 1993 Ransom *et al.* 1992 von Deimling *et al.* 1994). In classic pilocytic astrocytomas wild type TP53 most likely drives apoptosis, which is compatible with their generally indolent clinical course. Patients diagnosed with pilocytic astrocytoma with classic features have usually better survival than patients whose pilocytic astrocytoma has mixed tumour components (Haapasalo *et al.* 1999). Pilocytic astrocytoma can be cured by resection, but they recur if surgical extirpation remains partial (Haapasalo *et al.* 1999).

2.2.1.1 Genetic aberrations in pilocytic astrocytoma

Pilocytic astrocytomas have a generally favourable outcome, and the genetic rearrangements that promote aggressive tumour growth and behaviour are thus expected to be relatively scarce. There is some evidence suggesting that adult and juvenile pilocytic astrocytomas differ genetically. Gains of chromosomes 5 and 7, and gain of 9q have been detected more frequently in pilocytic astrocytomas of patients older than 15 at the time of the diagnosis (Jones *et al.* 2006, Sanoudou *et al.* 2000). Intratumoral analysis of pilocytic astrocytomas has revealed chromosomal heterogeneity associated with variations in histological morphology, e.g. gains of chromosomal material in 4q and 6q in tumour regions consisting of the classic biphasic pattern (Wemmert *et al.* 2006). Overall, chromosomal polyploidy of pilocytic astrocytomas is rare, and usually detected in those tumors that have undergone transformation into a more malignant direction (Mathew *et al.* 1996, Tomlinson *et al.* 1994b).

However, pilocytic astrocytomas have some distinctive genetic features compared to diffusely growing astrocytic tumors. These features include relatively rare RTK overexpression on tumour cells and down regulation of *EGFR*. Gene expression profiles of pilocytic astrocytomas have revealed few

genes that are distinct from higher grade gliomas and also from the normal brain tissue. When pilocytic astrocytomas were compared to the normal brain tissue and grade 2 astrocytomas, a strong overexpression of *TIMP4* (tissue inhibitor of metalloproteinases 4) gene was detected. TIMP4 is able to inhibit directly a group of matrix metalloproteinases that act as proteolytic enzymes. Similar findings were done with *IGFBp2* (insulin-like growth factor binding protein 2) gene and with some genes involved in the maintenance of the extracellular environment and lipid metabolism. IGFBp2 is produced in various regions in the CNS and is thought to inhibit IGF (insuline-like growth factor)-stimulated events such as mitogenesis and cell migration. Comparison of gene expression between glioblastomas and pilocytic astrocytomas indicated upregulation of a subset of genes involved in invasion and angiogenesis in glioblastomas, whereas pilocytic astrocytomas were characterized by the expression of genes involved in metabolism, proteolysis, signal transduction and cell adhesion (Colin *et al.* 2006, Rorive *et al.* 2006).

2.2.2. Ependymoma

Ependymomas are tumors of the neuroectodermal origin, and are typically located in the spinal cord or adjacent to the ventricles. In addition, ependymomas can sometimes be found in the brain parenchyma (Centeno *et al.* 1986, Oppenheim *et al.* 1994). Although ependymomas occur in all age groups (Reni *et al*. 2007), the median age at presentation is approximately 4 years. Ependymomas are somewhat more common in males than females (McGuire *et al*. 2009). Surgery is the standard treatment for ependymoma. Postoperative radiotherapy is indicated in the treatment of high-grade ependymoma and in case where surgical resection is incomplete (Reni *et al.* 2007).

Prognosis of spinal ependymomas is more favourable than that of infratentorial or supratentorial ependymomas. 5-year survival of paediatric ependymomas generally improves with increasing age. In the U.S. Surveillance Epidemiology End Results (SEER) database, 45.5% of the patients diagnosed with ependymoma survived for 5 years after the diagnosis in the age group of 0-4, whereas the survival rate improved up to 77.0% when ependymoma was diagnosed at the age of 16 to 18 (McGuire *et al*. 2009) and the oldest age-group (over 45) and infants had the least favourable survival rates (Reni *et al*. 2007).

Ependymomal tumors are classified by the WHO into grades 1 to 3. The WHO grade 1 and 2 ependymomas have a low mitotic activity and regular nuclear morphology, whereas anaplastic ependymomas (the WHO grade 3) are highly cellular, show cell and nuclear pleomorphism and endothelial cell proliferation, have frequent mitoses and contain tumour necroses. Perivascular rosettes are a histological hallmark of ependymoma (Reni *et al*. 2007). Anaplastic ependymomas exhibit more rapid growth than grade 1 or 2 ependymomas (Kleihues *et al.* 2002, Schiffer *et al.* 1991).

Little is known about the risk factors or about the genetic basis of ependymomas. A possible relationship between polyomavirus SV40 infection and ependymoma has been suggested, but has not been confirmed (Bergsagel *et al.* 1992, Lednicky *et al.* 1995). Data about the cytogenetic features of ependymoma are also scarce. Frequent mutations or amplifications of receptor tyrosine kinases have not been described. *TP53* has probably only a minor role in tumour progression (Bijlsma *et al.* 1995, Metzger *et al.* 1991, Sato *et al.* 1996). Amplification of *MDM2* was detected in 35% of ependymomas, and MDM2 overexpression was present in as many as up to 96% of the tumors analyzed (Suzuki & Iwaki 2000).

2.2.3 Medulloblastoma

Medulloblastomas originate from embryonal or fetal precursor cells, and are typically diagnosed at the age of 3 to 6. These tumors are highly malignant, but they respond to radio- and chemotherapy (Kleihues *et al.* 2002). Long-term survival can be achieved in approximately 85% of the standard risk patients and 70% of the high risk patients with a combination of chemotherapy and irradiation (Dhall 2009). The late adverse effects of multimodality treatment (particularly those of radiation therapy) may be substantial, and, therefore, novel approaches such as proton therapy are under evaluation with promising early results (Yock et al. 2010).

The wingless (WNT) pathway coordinates several developmental processes, and mutations in proteins of the WNT pathway occur in approximately 15% of sporadic medulloblastomas (Gilbertson 2004, Wechsler-Reya & Scott 2001). Aberrant signalling of the sonic hedgehog pathway is implicated in causing medulloblastoma in approximately 20% of children with this disease, and orally active inhibitors that bind to the SMO receptor are currently being tested in the treatment of medulloblastoma (Gajjar et al. 2010). Approximately 30% of childhood medulloblastomas contain amplified *N-MYC* (Lengauer C *et al.* 1998).

3. Gene amplifications in cancer

Gene amplification is defined as a greater than the normal number of gene copy numbers at a specific chromosomal locus. In cancer, presence of gene amplification is one molecular mechanism that may enhance tumour growth and progression. The amplicon usually contains at least one gene that is strongly expressed abetting amplifications also in adjacent genes that may be irrelevant to tumour progression and that may not be expressed (Knuutila 2004).

Recent studies where the entire tumour genome was sequenced typically reveal presence of numerous genetic aberrations in a single tumour including gene amplifications and gene deletions (Stephens *et al*. 2009). Amplifications of growth promoting genes are frequent in late stage cancers. Some tumour types have characteristic gene amplifications that are associated with overexpression of the gene product. For instance, approximately 15 to 20% of breast cancers harbour amplification of *HER2* oncogene, overexpress the protein and are associated with generally unfavourable outcome unless treated with anti-HER2 targeted therapy (Ross & Fletcher 1998, Slamon & Press 2009). Another

example is amplification of *N-MYC* (V-myc myelocytomatosis viral oncogene homolog) in neuroblastoma. This gene belongs to the MYC family of transcription factors and was one of the first tumour amplifications studied in a detail. Amplification of *N-MYC* has been reported to occur in approximately 20% of neuroblastomas, where it is associated with aggressive clinical behaviour of the disease (Savelyeva & Schwab 2001).

The size of the amplified genetic material may range from only one gene or few genes to whole chromosomal arms, or may span even an entire chromosome. Gene amplification can present as a homogeneously staining region (HSR) that consists of DNA sequence repeats within a chromosome, or the amplified DNA can exist as extrachromosomal bodies called double minutes (Dmins). The double minutes are readily detectable by molecular biological methods (Albertson 2006).

Some chromosomal regions may be prone to undergo amplification. Genomic sequences that have received much attention are the chromosomal fragile sites, which are chromosomal regions that replicate late during the S-phase and are prone to breakage under conditions of replication stress. Gene amplifications are abundant in tumour cells with inactive *TP53*. Factors such as radiation, hypoxia and chemotherapy agents may also induce chromosomal breakage. There is a heterogeneous group of approximately 100 fragile sites in the human genome, identified by treating cells with agents that interfere with DNA replication (Glover *et al.* 2005, Wahl *et al.* 1984). Fragile sites can be divided into common and rare sites based on their prevalence in a population. Common fragile sites are present in all individuals, and they coincidence with the amplification boundaries found in cell lines. The amplification hot spots occur at the same loci as many of the known amplification-activated oncogenes (Myllykangas & Knuutila 2006)

Amplification may be initiated by a DNA double strand break that is present at a frailty check point of the cell cycle. A double-strand DNA break in a replicating cell can be generated by collapse of the replication fork, which is unable to progress as a result of a rearrangement in the DNA. In addition to specific breakage-promoting sequences at the fragile sites, errors in DNA replication and telomerase dysfunction may also be involved in the genesis of DNA amplifications (Albertson. 2006, Chernova *et al.* 1998, Coquelle *et al.* 1997, Paulson *et al.* 1998, Pipiras *et al.* 1998). Amplification resulting from telomere dysfunction is thought to occur through breakage–fusion–bridge (BFB) events as originally proposed based on observations made in studies on maize (McClintock 1942, Murnane & Sabatier 2004).

Dmins are thought loop out from the chromosomes following breakage of replication bubbles. Dmins containing tandem repeats may also be formed in HSR breakdown, where DNA fragments are clipped out and fused into circular elements. Dmins may be integrated back into chromosomes and constitute a HSR. The HSR gene amplifications are not necessarily restricted to these chromosomal loci of amplified genes, but they can be present also elsewhere in the genome as distributed insertions

(Albertson 2006). According to the BFB model suggested by Barbara McClintock (1942), the initiating event in formation of a HSR is uncapping of a DNA sequence by a double strand break or telomere erosion, and post-replication fusion of the two sister chromosomes probably by DNA repair proteins. The resulting dicentric fused chromosome forms an anaphase bridge when it is drawn by the two centromeres of the fusion chromosome to the opposite poles of the mitotic spindle. This stress can cause a secondary breakage of the chromatin and uneven segregation of the DNA material into the daughter cells, and a repeat of the BFB in every cell division eventually leads to gene amplification (Myllykangas & Knuutila 2006).

3.1 Gene amplifications at the locus 4q12

PDGFRA, KIT and *VEGFR2* genes are located at the chromosome locus 4q12. Amplification of *PDGFRA* has long been known to be present in some diffuse gliomas, but recently an adjacent gene, *KIT*, was found to be amplified in some glioblastomas and paediatric osteosarcomas, although no activating mutations of *KIT* were reported (Entz-Werle *et al.* 2007, Sihto *et al.* 2005). *PDGFRA* is thought to be amplified as Dmins (Muleris *et al.* 1994), and recent findings suggest that in glioblastomas the amplified region may include a number of adjacent genes at the 4q12 locus (Holtkamp *et al.* 2007). In adult and adolescent seminomas *KIT* amplification has been accompanied with mutations in *KIT* exon 17 (McIntyre *et al.* 2005), and *PDGFRA* and *VEGFR2* were also occasionally co-amplified. Based on genome-wide mapping of genetic alternations in human gliomas, the glioma phenotype may in part reflect its gene amplification and expression profiles (Bredel *et al.* 2005).

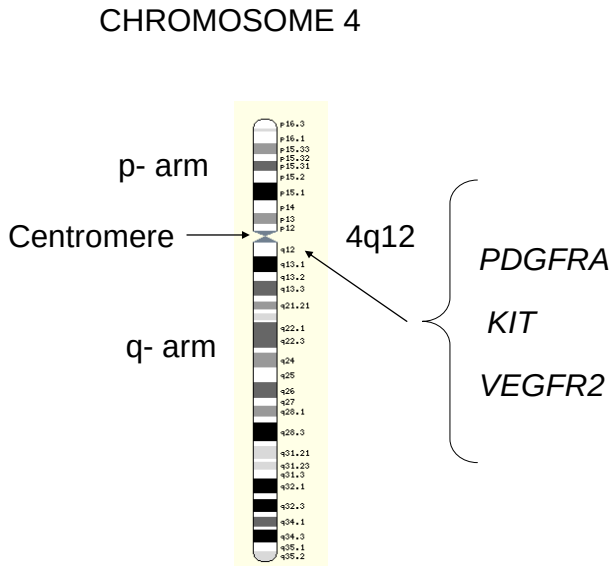


Figure 6. Chromosome 4 showing the location of *PDGFRA*, *KIT* and *VEGFR2*.

4. Angiogenesis in gliomas

Tumors may produce pro-angiogenic proteins to overcome hypoxia, which results in activation of the transcription factor HIF-1-α (hypoxia inducible transcription factor-1-alpha). This induces expression

of VEGF and results in activation of genes that are involved in angiogenesis induction (Carmeliet *et al.* 1998, Kerbel & Folkman 2002, Lohela *et al.* 2009, Semenza 2003, Shweiki *et al.* 1992). VEGF and other proangiogenic factors are required for blood vessel and tumour growth, but tumour angiogenic activity may not always reflect its biological aggressiveness (Bergers *et al.* 2003).

ANG-1 (angiopoietin-1) is a ligand of the TIE-2 RTK that is expressed on endothelial cells. It interacts with other angiogenic molecules to stabilize the vascular structures by promoting survival of differentiated endothelial cells (Audero *et al.* 2001, Hanahan 1997, Oliner *et al.* 2004, Papapetropoulos *et al.* 1999, Yancopoulos *et al.* 2000, Saharinen *et al*. 2010). ANG-2 is an antagonist ligand for TIE-2 in endothelial cells, and like VEGF-A, it is produced under hypoxic conditions trough induction by HIFα (Maisonpierre et al 1997, Pouyssergur *et al*. 2006). Initiation of vascular sprouting requires destabilization of capillaries, an action mediated by ANG-2 and ANG-1/TIE-2 signalling, which allows VEGF-A-induced cell migration and division (Pouyssegur *et al.* 2006). Hypoxic stress also leads to attenuation of protein synthesis by regulating the mTOR and other pathways (Pouyssegur *et al.* 2006).

Some diffuse astrocytic tumors, especially primary glioblastomas, may grow fast, which may lead to tissue hypoxia and tumour necrosis. VEGF is an important angiogenic factor also in gliomas. Glioblastomas are typically highly vascular, and VEGF-A, VEGFR-1 and VEGFR-2 are upregulated and phosphorylated in the tumour vasculature (Carroll *et al.* 1999). VEFG is expressed in the palisading tumour cells around necrotic areas and around tumour vessels that express VEGFR-1 and VEGFR-2 (Berkman *et al.* 1993, Hatva *et al.* 1995, Plate *et al.* 1994a, Plate *et al.* 1994b, Plate & Mennel 1995, Plate & Risau 1995, Samoto *et al.* 1995, Shweiki *et al.* 1992, Takano *et al.* 1996). VEGFR-2 is expressed not only in the vascular endothelia of glioblastoma, but sometimes on the glioblastoma cells as well (Steiner *et al.* 2004). Astrocytomas first depend on the existing normal brain blood vessels, but with further tumour growth hypoxia induce angiogenesis via HIFs, VEGFs, VEGFR-2 and ANG-2 (Bergers & Benjamin 2003).

Although pilocytic astrocytomas differ greatly from their malignant counterparts, they also have a high blood vessel density. Pilocytic astrocytomas show features of more mature, stable, thick and multi-layered vessels compared to anaplastic astrocytomas. In pilocytic astrocytomas VEGFR-2 and VEGFR-1 are expressed in the tumour vasculature in coiled young proliferating vessels together with VEGF. This suggests presence of a paracrine loop. Unlike diffuse gliomas, progressing pilocytic astrocytomas do not develop the characteristic necroses with palisading tumour cells (Gesundheit *et al.* 2003, Haapasalo *et al.* 1999, Leung *et al.* 1997, Takeuchi *et al.* 2004).

PDGF and its receptors are also important for angiogenesis. PDGFRs are expressed in several cell types, and PDGFRA, for example, in the vascular smooth muscle cells and megakaryocytes. PDGF has some angiogenic effect, and its role in wound healing is evident (Heldin & Westermark 1999).

Angiogenesis may be assisted by homing of bone marrow-derived cells on the developing vessels. Cells of hematopoietic origin have been reported to navigate they way to the sites where new blood vessels are formed and contribute to neovascularization, and express endothelial cell markers such as E-cadherin, VEGFR-1, VEGFR-2 and TIE-2 (Bertolini *et al.* 2006, Conejo-Garcia *et al.* 2005, De Palma *et al.* 2005, Grunewald *et al.* 2006, Okazaki *et al.* 2006, Venneri *et al.* 2007). The putative cancer stem cells of brain tumors might localize close to an adjacent blood vessel (Asahara *et al.* 1997, Calabrese *et al.* 2007). However, the mechanisms by which bone marrow-derived stem cells might contribute to angiogenesis are incompletely understood, the origin of neovascular endothelial cells are controversial and some recent results suggest that bone marrow-derived circulating endothelial precursors do not contribute to vascular endothelium and are not needed for tumour growth (Purhonen *et al*. 2008).

Besides the VEGF/ VEGFR pathway, hypoxic conditions may promote expression of the genes involved in oxygen supply and tissue maintenance. Secretion of the SCF and signalling via the KIT-AKT pathway has been implied under hypoxia (Berdel *et al.* 1992, Cockman *et al.* 2000, Erlandsson *et al.* 2004, Jin *et al.* 2002, Jogi *et al.* 2002, Litz & Krystal 2006, Ohh *et al.* 2000, Sihto *et al.* 2007).
KIT may have a role in angiogenesis as well. SCF-KIT signalling enhances tube formation of developing capillary vessels (Matsui *et al.* 2004). KIT is expressed during ontogenesis in the alveolar capillaries and in capillary endothelia of the placenta (Miettinen *et al.* 2000). KIT may be expressed in tumour endothelial cells, and endothelial cells of primary glioblastomas were recently found to express KIT frequently (Sihto *et al*. 2007). The functional role of endothelial cell KIT remains speculative, but in pathological angiogenesis activated KIT may recruit endothelial progenitors to the inflamed endothelium (Dentelli *et al.* 2007). Little is known about endothelial cell KIT expression in lower grade gliomas and other CNS tumors.

5. RTK activation and targeted therapies

RTKs are activated upon ligand binding and formation of homo- or heterodimers, and consecutive trans-autophosphorylation (Blume-Jensen & Hunter 2001). Certain tyrosine amino acid residues in the RTK kinase domain serve as phosphoacceptors activating the receptor by a conformational change. Autophosphorylation of one or two homologous juxtamembrane tyrosine residues is required for full kinase activation in RTKs, and a mutation of a critical codon encoding a tyrosine residue to, for example, a phenylalanine encoding residue may significantly reduce the kinase activation (Blume-Jensen & Hunter 2001, Hashimoto *et al.* 2003, Pawson & Scott. 2005, Ueda *et al.* 2002).

Several ATP-mimetic drugs are currently being used in cancer therapy, and many more are being developed. Imatinib mesylate was the first small molecule RTK inhibitor that was approved for cancer treatment, first for CML and later for GIST. Imatinib is a selective inhibitor of KIT, PDGFRA,

PDGFRB, BCR-ABL, ABL, ARG and c-fms (Capdeville *et al.* 2002, Desjardins *et al.* 2007, Druker *et al.* 2006).

KIT is activated notably in GISTs due to mutations as discussed above (1.3.1). Most GISTs are highly responsive to imatinib, in particular those that harbour mutation in *KIT* exon 11 (Heinrich *et al*. 2003). Imatinib is now the standard treatment of advanced GIST (Joensuu 2006), and it is now approved also for adjuvant treatment of high-to-moderate risk GIST patients (DeMatteo et al. 2009). *KIT* mutations (D816V) are found also in mastocytosis, some cases of acute myeloid leukemia (Fritsche-Polanz *et al.* 2010) and seminomas (McIntyre *et al.* 2005). Recently, *KIT* mutations and amplifications were found also in 10 to 40% of mucosal and acral melanomas, and in some melanomas located in sun-exposed sites. Interestingly, many of these melanomas respond to imatinib and other RTK inhibitors (Handolias *et al.* 2010). However, many human tumour types that express KIT in immunohistochemistry do not contain activated or mutated KIT, and KIT immunohistochemistry is inadequate in selection of patients for targeted therapy (Sihto *et al.* 2005).

Small molecule oral tyrosine kinase inhibitors have recently been studied in the treatment of glioblastoma, but the success has remained limited. The agents tested include inhibitors of the EGFR, such as gefitinib (Franceschi *et al*. 2007, Uhm *et al*. 2010), and inhibitors of the PDGFRs and KIT, such as imatinib (Dresemann et al. 2010). Factors that limit efficacy of tyrosine kinase inhibitors may include pharmacological factors, such as poor drug penetration into the CNS, and early development of drug resistance (Mellinghoff et al. 2005), but tumour heterogeneity might also have a role.

AIMS OF THE STUDY

To study *EGFR* mutations and their association with *EGFR* amplification in various histological types of human cancer, including brain tumors (I).

To investigate amplifications of *KIT*, *PDGFRA*, *VEGFR2* and *EGFR* genes and their mutations in glioblastoma (II).

To investigate amplifications of *KIT*, *PDGFRA*, *VEGFR2* and *EGFR* genes in low grade glioma, anaplastic astrocytoma and their recurrences (III).

To evaluate KIT and VEGFR2 expression in endothelial and tumour cells in paediatric brain tumors, and to investigate *KIT* gene copy numbers in these tumors (IV).

To study the heterogeneity of *EGFR* and *KIT* gene amplification and their protein products in gliomas and glioblastoma (V).

MATERIALS AND METHODS

1. Patients and tumour samples

The tissue diagnoses in the current studies were made by a professional pathologist, who also selected the tumour samples and ascertained their representativeness from hematoxylin and eosin stained slides. The following series were studied:

(I) Tumour tissues from a series of 560 neoplasms consisting of several histological types of tumors were selected at random from the archives of the Departments of Pathology, Helsinki University Central Hospital, Tampere University Central Hospital, and the Institute of Medical Technology, University of Tampere. In addition, tumour samples from six NSCLC patients treated with gefitinib were analyzed.

(II) Formalin-fixed paraffin-embedded tumour tissue from 48 patients who underwent craniotomy for a brain tumour and who were subsequently diagnosed with glioblastoma multiforme at the Department of Pathology, Helsinki University Central Hospital, between October 2000 and December 2003.

(III) Formalin-fixed paraffin-embedded tumour tissue from 119 patients who had craniotomy for a primary glioma in 1979 to 2000, and who had brain tumour tissue available for analysis both from the primary tumour and its recurrence, were retrieved from the

archives of the Department of Pathology, Helsinki University Central Hospital. The tumors were originally diagnosed as diffuse astrocytoma (grade 2, 3 or 4), oligodendroglioma (grade 2 or 3) or oligoastrocytoma (grade 2 or 3).

(IV) Formalin-fixed, paraffin-embedded tissue was retrieved from 35 patients with juvenile pilocytic astrocytoma diagnosed at the age of 18 or younger, and from 49 paediatric brain tumors consisting of various histological types diagnosed at the Helsinki University Central Hospital, Helsinki, Finland, in 1985 to 2007. Samples containing histologically normal tissue of several organs (n=16) were collected from aborted fetuses, from autopsies and from surgical operations. Fetal samples from fetuses younger than 20 weeks were obtained from spontaneous or induced abortions performed by the treating obstetricians according to the Finnish law at the Turku University Central Hospital. Other fetal samples were from stillbirths. The non-neoplastic and dysplastic cortical and subcortical tissues from brain resection for epileptogenic lesions were retrieved at random from the archives of the Department of Pathology, Helsinki University Central Hospital, Finland.

(V) Brain tumour tissue from 22 patients who underwent craniotomy and extensive resection at the Helsinki University Central Hospital (HUCH), Helsinki, Finland, in 1998 to 2006 was used as the starting material in the study. Since we investigated tumour heterogeneity, we required that five or more tumour tissue blocks were available for analysis at a computerized search of the files of the Department of Pathology, HUCH. Otherwise, the tumors were selected at random for the study.

2. Tissue microarray (III)

Routinely fixed paraffin-embedded tumour samples of representative tumour regions were used for construction of tumour tissue microarray (TMA) blocks, each containing approximately 100 tumour samples. Five µm sections were cut from the TMAs and processed for immunohistochemistry.

3. Polymerase chain reaction (I-III)

Fifty nanograms of the genomic DNA was amplified in a polymerase chain reaction (PCR) reaction containing 0.6 x Platinium PCR Buffer (Invitrogen), 1.4 to 2.4 µM of $MgCl_2$, 160 µM of dNTPs (Clontech), 0.3 µM of forward and reverse primers, and DNA polymerases AmpliTaq Gold (Applied Biosystems), and Platinium Taq (Invitrogen) in a volume of 50µL. The PCR cycling conditions consisted of an initial denaturation step at 94°C for 14 minutes, after 35 cycles at 94°C for 30 seconds, annealing (annealing temperatures in Table 2) for 45 seconds and 2 minutes at 72°C, and final extension for 10 minutes at 72°C. Heteroduplex formation was created by denaturing the PCR products for 5 minutes at 95°C and then allowing the samples to reanneal by decreasing the temperature 1°C per minute from 95°C to 40°C.

4. Denaturing high-performance liquid chromatography (I-III)

Denaturing high-performance liquid chromatography (DHPLC) is a high-throughput mutation-screening method (Xiao & Oefner 2001). In this technique, a PCR amplicon from the target gene is denatured in order to separate the existing homoduplexes and then allowed slowly to cool (reanneal). This results in formation of both homo- and heteroduplexes. The DHPLC running temperature is carefully selected so that partial denaturation begins to occur in the area around the polymorphic mismatch. Heteroduplexes elute before the homoduplexes in a specifically designed dsDNA HPLC column. DHPLC has the sensitivity for detecting genetic changes in heterogeneous cell populations, e.g. mutant cells within their normal cell background/contamination. Five to 10 µL of the PCR product was injected on the HPLC Column and eluted at a flow rate of 0.45 mL/min within a linear acetonitrile gradient consisting a mixture of buffer A (100mmol/L triethyl ammonium acetate and 0.1 mmol/L EDTA; Varian), and buffer B (100 mmol/L triethyl ammonium acetate, 0.1 mmol/L EDTA and 25% acetonitrile; Varian). PCR products from normal DNA were used as controls. The elution temperatures for each amplicon were obtained from the DHPLC Melt Program (http://insertion.stanford.edu/melt.html) and then optimized by studying alterations in the elution profiles of the samples within a temperature range of 2°C under and above the suggested melting temperature. The temperature that best separated homoduplexes was used for DHPLC analysis.

Table 2. The used primer sequences and the annealing temperatures for different genes and their exons, and the elution temperatures used in DHPLC analyses.

GENE		Primer sequences (5'->3')		Temperature (°C)	
Study (I)	Exons	Forward	Reverse	Annealing	Elution
EGFR	4	GCTTAATTGCGGGACTCTTGT	TAGAGCTGTCCCCCATAGGA	57	
EGFR	4.2	AAAGAGTGCTCACCGCAGTT	CATAGGAGCTGGAGGCAGAG	57	62
EGFR	5	AGCCAGCCAAACAATCAGAG	CCACCCTGGATTAACTGCAT	57	
EGFR	5.2	GAAAGGGCGTCATCAGTTTC	TTTGGAGTCTTCAGAGGGAAAA	57	58
EGFR	6	CCCTGGGAAATGATCCTACC	AAGTCTTCTGTCCTGGTGTGG	56	
EGFR	6.2	CAGGGAACCTTTGCTCTTTTT	CAGCCTGCTCCAGGGAGG	59	63
EGFR	7	GCTGAGTGTACTTACCTCAC	GGACAAGGATGCCTGACCAG	61	
EGFR	7.2	CCAGCGTGTCCTCTCTCCT	CAGTTAGAGGGCCCACAGAG	59	62
EGFR	8	CTTTCCATCACCCCTCAAGA	CTCAGCAGCCGAGAACAAG	59	
EGFR	8.2	CACCGTCATCACCTTCCTTT	CCGAGAACAAGCCTCGTC	60	62
EGFR	12	TCAATCAAAGGTGGTCTGGA	GGACCCATTAGAACCAACTCC	57	
EGFR	12.2	TCTGGAGAAACAAAGTTTTCAGG	AAACAGAAGCGGTGACTTACT	61	57
EGFR	13	CATGTCTGTGTCACCCAAGG	CAGAAAACCCAAAACCTCCA	61	57
EGFR	14	GGTGATTTGTGTTCCTGCAA	TCATCACTGTTCGGCTTCTG	57	
EGFR	14.2	CTGACGGGTTTCCTCTTCCT	CCCTTCCCATGAAATGGAC	57	61
EGFR	15	ATCATTTGGCTTTCCCCACT	ATTTGTTGCCGGAAAACTTG	59	
EGFR	15.2	GCATGAACATTTTTCTCCACCT	TGAATTTGCAAGAGAGGAAATG	59	60
EGFR	17	CATGGAATCTGTCAGCAACC	ACATACACAACTGCTAATGG	56	58
EGFR	18	CAAATGAGCTGGCAAGTGCCGT	GAGTTTCCCAAACACTCAGTGA	61	
EGFR	18.2	CAAGTGCCGTGTCCTGGCACCCAAGC	CCAAACACTCAGTGAAACAAAG	52	58
EGFR	19	GGCACCATCTCACAATTGCC	CCCCACACAGCAAAGCAGAA	57	
EGFR	19.2	TGCCAGTTAACGTCTTCCTTC	CCACACAGCAAAGCAGAAAC	60	59

EGFR	21	CACTAACGTTCGCCAGCCAT	CTCCCCTGCATGTGTTAAAC	61	
EGFR	21.2	CACTAACGTTCGCCAGCCAT	CCTGGTGTCAGGAAAATGCT	60	63
EGFR	23	CATTCATGATCCCACTGCCT	CAAGGCTCAGCTAGGCAGT	52	60
Study (II)					
EGFR	18	CAAATGAGCTGGCAAGTGCCGTGT	GAGTTTCCCAAACACTCAGTGAAA	56	
EGFR	18.2	CAAGTGCCGTGTCCTGGCACCCAAGC	CCAAACACTCAGTGAAACAAAGA	56	56
EGFR	19	GGCACCATCTCACAATTGCC	CCCCACACAGCAAAGCAGAA,	56	
EGFR	19.2	TGCCAGTTAACGTCTTCCTTCTC	CCACACAGCAAAGCAGAAACTC	56	58
EGFR	21	CACTAACGTTCGCCAGCCATA	TGTTTAACACATGCAGGGGAG,	56	
EGFR	21.2	CCTCACAGCAGGGTCTTCTCT	CAGCATTTTCCTGACACCAGG	56	60
Study (II)					
KIT	9	GTATGCCACATCCCAAGTGT	CATGACTGATATGGTAGACA	57	57
KIT	11	CCAGAGTGCTCTAATGACTG	GGAAGCCACTGGAGTTCCTT	57	57
KIT	13	GACATCAGTTTGCCAGTTGT	TGTTTTGATAACCTGACAGAC	57	58
KIT	17	GCAACACTATAGTATTAAAAAG	CCTTTGCAGGACTGTCAAGCA	57	57
Study (II)					
PDGFRA	11	ATGTGGAGTGAACGTTGTTGG	CTAGTTCTTACTAAGCACAAGC	57	59
PDGFRA	17	CAGGGGTGATGCTATTCAGC	TTAAAGTGAAGGAGGATGAGCC	60	59
Study (III)					
TP53	6	AGGGTCCCCAGGCCTCTGAT	CCACTGACAACCACCCTTAA	58	62
TP53	7	TGCTTGCCACAGGTCTCC	TGTGCAGGGTGGCAAGTGGC	64	62
TP53	8-9	AGTAGATGGAGCCTGGTTTTT	RAGAAAACGGCATTTTGAGTG	63	63

5. DNA sequencing (I-III)

Samples with an abnormal elution profile (eluation peak shape or shift in eluation time deviating from normal control sample) in DHPLC compared with a control consisting of lymphocyte DNA were subjected to automated sequencing located at Biomedicum Helsinki. The PCR products were first purified, using a QiAquick PCR purification kit (Qiagen). Direct sequencing of the PCR products was performed using BigDye3 termination chemistry (Applied Biosystems) and an ABI 3100 Genetic Analyser (Applied Biosystems).

6. Fluorescence *in situ* hybridization (I-III)

Centromere-specific probes for chromosomes 4 and 7 (CEP4 and CEP7, Spectrum Green, Vysis) were purchased, and the gene copy numbers were determined using BAC (bacterial artificial clones) probes for *KIT* (clone RP11-586A2), *PDGFRA* (clone RP11-231C18), *VEGFR2* (clone RP11-662M13) and *EGFR* (clone RP11-815K24) (Invitrogen). The correct probe identity was confirmed using specific PCR-primers for each gene. The BAC DNA was isolated using standard techniques, and labeled with the digoxigen-nick and biotin-nick translation mix (Roche). Interphase nuclei were prepared using standard techniques and viewed under a fluorescence microscopy equipped with an ISIS digital image analysis system (MetaSystems). Dual-color hybridizations were performed. After hybridization the probes were detected immunochemically with avidin-fluorescein isothiocyanate and anti-digoxigenin rhodamine, and the slides were counterstained with 0.1 µmol/L 4,6-diaminido-2-

phehylindole in an antifade solution. Signal copy numbers were counted approximately from 50 randomly chosen non-overlapping nuclei.

7. Chromogenic *in situ* hybridization (III-V)

Whole tissue sections of 3µm mounted on glass slides were deparaffinized and incubated in 0.05M Tris-HCl/ 0.001M EDTA solution (pH 9) in a temperature controlled microwave oven (at +92°C for 10 minutes), followed by cooling down for 20 minutes at room temperature. After wash with H_2O, enzymatic digestion was carried out by applying 100µL of 10µg/µL proteinase K (Roche) in 2mM $CaCl_2$/ 20mM TRIS solution on to tissue sections for 10 to 20 minutes at room temperature or +37°C following with wash with H_2O and dehydration with an ascending gradient of ethanol. Digoxigen-labelled BAC probes were prepared as described above and applied onto slides. Sections were denatured in the presence of BAC probes on a heat plate (+75°C to +85°C). Hybridization was expected to occur during over night incubation at +37°C. After incubation the slides were washed with 0.5xSSC for 5 minutes at +75°C followed by PBS wash. The probes were detected by FITC-sheep anti-digoxigen, HRP-goat anti-FITC and DAB chromogen according to the manufacturer's protocol (Spot-Light CISH detection kit, Zymed). Alternatively, the enzymatic digestion was carried out by applying 100 µL of digestion enzyme onto the slides for 10 to 15 min at room temperature (Digest-All III solution, Zymed), and the probes were detected with a mouse anti-digoxigenin antibody (diluted 1:300; Roche Biochemicals, Mannheim, Germany), an antimouse-peroxidase polymer (Powervision+, ImmunoVision Technologies), and diaminobenzidine chromogen according to the manufacturer's protocol. Tissue sections were counterstained with hematoxylin. Gene amplification was considered to be present when six or more signals or clusters of signals in one nucleus were detected in more than 10% of the counted nuclei. Cases with three to five signals per nucleus were considered as aneuploid and those with 2 signals as diploid (normal).

8. Immunohistochemical staining (II-V)

Five µm tissue sections were cut and mounted on glass slides. Tissue sections were deparaffinized and examined for KIT (CD117), phosphorylated KIT, SCF, PDGFRA, VEGFR2, HIF-1-alpha, EGFR, EGFRvII, phosphorylated EGFR, TP53, Ki67, CD34, nestin and prominin 1 (CD133). The primary antibodies were diluted in PowerVision pre-antibody blocking solution and incubated overnight at 4 ◦C. Binding of the primary antibody was detected with a Powervision+ Poly-HRP histostaining kit (Immunovision Technologies), using an Envision Detection KIT (Dako) or with the Ultravision Immunohistochemical Detection System (Lab Vision). The antibodies used, their dilution concentrations, pre-treatment buffers and the heat pre-treatments given are listed in Table 3. Tissue samples with known expression of the studied protein were used as positive controls. The immunostainings were graded as negative (-), faintly positive (+), moderately positive (++), or markedly positive (+++) when viewed under a consultation microscope (Nikon Eclipse E600).

Table 3. A list of antibodies and their dilution concentrations, pre-treatment buffers and heat pre-treatments used.

Target protein (study)	Antibody used	Dilution	Pre-treatment buffer	Heat pre-treatment
EGFR (II, III, IV)	Mouse monoclonal NCL-EGFR (Novocastra)	1:150	(II) and (III) Reveal buffer (Biocare Medical); (IV) 10mmol/L Sodium-citrate buffer, pH 6	Autoclave at 120 ◦C, 2 min
EGFRvIII (II,IV)	Mouse monoclonal NCL-EGFRT (Novocastra)	1:200	Reveal buffer (Biocare Medical)	Autoclave at 120 ◦C, 2 min
phosphorylated EGFR (II)	Monclonal mouse anti-EGFR (My10; BD Biosciences)	1:100	TRIS-EDTA, pH 9	Microwave owen 7min and 3x5 min, 850W
KIT (CD117) (II-IV)	Polyclonal rabbit anti-CD117 (A 4502; DAKO)	1:300	(II) and (III) TRIS-EDTA, pH 9; (IV) 10mmol/L Sodium-citrate buffer, pH 6	(II) Microwave owen 7min and 3x5 min, 850W; (III) 98°C waterbath, 15 min
phosphorylated KIT (IV)	Phospho-c-KIT (Tyr719) (#3391, Cell Signaling)	1:35	10mmol/L Sodium-citrate buffer, pH 6	98°C waterbath, 15 min
SCF (IV)	Rabbit polyclonal SCF (#2273 Cell Signaling)	1:200	10mmol/L Sodium-citrate buffer, pH 6	Autoclave at 120 ◦C, 2 min
PDGFRA (II)	Polyclonal rabbit anti-PDGFRalpha Ab-1 (RB-1691; NeoMarker)	1:150	Citrate buffer, pH 6	Microwave owen 2x4min, 850W
VEGFR2 (III,IV)	An epitope specific rabbit anti-VEGFR2 (Flk-1 Ab-1, Neomarkers)	1:100	10mmol/L Sodium-citrate buffer, pH 6	Autoclave at 120 ◦C, 2 min
Nestin (III)	Mouse monoclonal anti-nestin antibody (clone 10C2, Chemicon International)	1:500	10mmol/L Sodium-citrate buffer, pH 6	Autoclave at 120 ◦C, 2 min
Prominin-1 (CD133)(III)	Mouse anti-human CD133 antibody (AC133 pure, Miltenyi Biotec)	1:10	10mmol/L Sodium-citrate buffer, pH 6	Autoclave at 120 ◦C, 2 min
CD34 (II)	Monclonal mouse anti-CD34 (BD Biosciences)	1:50	TRIS-EDTA, pH 9	Microwave owen 7min and 3x5 min, 850W
Ki67 (II)	Mouse monoclonal NCL-Ki67-MM-1 (Novocastra)	1:1000	Reveal buffer (Biocare Medical, Walnut Creek, CA)	Autoclave at 120 ◦C, 2 min
HIF-1-alpha (IV)	Monoclonal mouse anti-HIF-1α antibody (Neomarkers)	1:100	10 mmol/L Sodium-citrate buffer, pH 6	98°C waterbath, 20 min
TP53 (II,III)	Mouse monoclonal NCL-P53-DO7 (Novocastra)	1:500	Reveal buffer (Biocare Medical, Walnut Creek, CA)	Autoclave at 120 ◦C, 2 min

9. Locked nucleic acid mRNA *in situ* hybridization for KIT (IV)

Digoxigenin-labeled locked nucleic acid probes (LNATM mRNA, Exiqon, Inc., Woburn, MA. USA) were designed against the KIT extracellular domain coding sequence 5'-gttgagaagagcctgtctggac-3'. Efficacy of hybridization was controlled with a polyT(25)Vn probe (LNATM, Exiqon) against a poly(A) tail. *In situ* hybridization was carried out as described earlier with minor modifications (Parikka *et al.* 2001). All reagents and instruments were treated either with diethyl pyrocarbonate (DEPC) or were RNAse-free. The sections were counterstained with 0.1% Nuclear Fast Red (Sigma-Aldrich Inc., Saints Louis, Missouri, USA). The hybridization results were graded as negative (-) or positive (+). Glioblastomas with known endothelial cell KIT expression in the tumour vasculature served as a positive control.

10. Statistical analyses (I-V)

Frequency tables were analyzed using the $\chi 2$ test or Fisher's exact test, and non-normal distributions between groups were compared using the Kruskal–Wallis or Mann-Whitney tests. Life-tables were computed using the Kaplan–Meier method. The log-rank test was used to compare time-to-event distributions. Overall survival was computed from the date of the diagnosis to death, and the patients alive were censored on the last date of follow-up. All p-values are two-sided. Statistical analyses were computed with either a Statview or an SPSS computer program.

11. Ethical aspects (I-V)

The tissue samples analyzed were originally collected for diagnostic or therapeutic purposes, and left-over tissue was used for the current studies after obtaining permission from the National Authority for Medicolegal Affairs (NAM) and an Institutional Ethics Committee. All clinical data were processed anonymously using a unique study identity code number for each sample and/or patient.

RESULTS

The main findings of the thesis are summarized below (a more detailed description of the results are available in the attached Studies I to V).

1. *KIT, PDGFRA, VEGFR2* and *EGFR* amplifications in primary and secondary glioblastomas (II)

Forty-three primary glioblastomas were analyzed for *KIT*, *VEGFR2*, *PDGFRA* and *EGFR* amplification using fluorescence *in situ* hybridization. *KIT* was amplified in 47% and *VEGFR2* in 39% of the glioblastomas, respectively, and *PDGFRA* in 29%. Thirty-five (81%) of the tumors had either *KIT* or *EGFR* amplification. *KIT*, *PDGFRA* and *VEGFR2* amplifications were strongly associated ($p < 0.0001$ for each pair wise comparison) suggesting co-amplification, whereas no significant association was found with presence of *EGFR* amplification. Glioblastomas with *KIT*, *PDGFR* or *VEGFR2* amplification were associated with similar outcome as other glioblastomas.

The four secondary glioblastomas arising from pre-existing lower grade astrocytic tumors investigated had each *KIT* amplification. None of the four analyzed secondary glioblastomas had amplified *EGFR* as compared with 30 (70%) out of the 43 primary glioblastomas ($p = 0.013$). Three out of the four secondary glioblastomas harboured *PDGFRA* amplification, and one had *VEGFR2* amplification.

2. *KIT, PDGFRA, VEGFR2* and *EGFR* amplifications in diffuse grade 2 and 3 gliomas and their recurrences (III)

KIT and *PDGFRA* amplifications were more frequent in anaplastic astrocytomas as compared to astrocytomas, oligodendrogliomas and oligoastrocytomas [28% versus 5% ($p = 0.012$) and 33% versus 2% ($p = 0.0008$), respectively]. *VEGFR2* amplifications occurred in 6% to 17% of the grade 2 or 3 gliomas at diagnosis, and *EGFR* amplification in 0% to 12%. Amplified *KIT* was more frequently present in recurrent gliomas than in newly diagnosed gliomas ($p = 0.0066$).

KIT amplification was associated with *KIT* protein expression and with presence of *PDGFRA* and *EGFR* amplifications both at the time of the first glioma diagnosis and at tumour recurrence, and with *VEGFR2* amplification at tumour recurrence. Three (4%) primary gliomas and 10 (14%) recurrent gliomas that were evaluable for co-amplification of *KIT*, *PDGFRA* and *VEGFR2* showed amplification of at least two of these genes; the amplicon contained amplified *KIT* in all 13 cases. In addition to astrocytomas, amplification of *KIT*, *PDGFRA* and *VEGFR2* was found frequently in recurrent oligoastrocytic tumors (in 23-33% of the cases).

The effect of gene amplifications on survival was investigated. Since gene amplifications tended to be associated with a low grade of glioma differentiation, *KIT*, *PDGFRA*, *VEGFR2* and *EGFR* amplifications were added as cofactors in a multivariate model that contained the histologic type of

glioma as another cofactor. Presence of *KIT* amplification tended to be associated with unfavourable survival based on this multivariate model that accounted for the histologic type of glioma (hazard ratio, 2.0; 95% confidence interval, 0.9-4.4; p = 0.075), whereas presence of *PDGFRA* and *VEGFR2* amplification was not associated with outcome in the model (p = 0.58 and p = 0.11, respectively).

3. Copy number analysis of *KIT* in paediatric brain tumors (IV)

KIT copy number was assessed using CISH in 9 pilocytic astrocytomas. None of the tumors had amplification of the gene. Two tumors showed *KIT* aneuploidy (three to five copies of *KIT* were present per one cell).

4. Heterogeneity of *EGFR* and *KIT* amplifications in gliomas and glioblastomas (V).

EGFR was amplified in 10 (67%) out of the 15 glioblastomas when only one (index) tissue block from each tumour was studied, whereas 11 (73%) glioblastomas were classified *EGFR*-amplified and the rest *EGFR* aneuploid when all blocks available were considered. *KIT* was amplified in six (40%) index glioblastoma tissue blocks, but in 10 (67%) when all blocks were analyzed. None of the seven astrocytomas contained amplified *EGFR* and only one harboured amplified *KIT* even when all blocks were examined.

Table 4. A summary of copy number analysis of astrocytic tumors (II, III, IV, V)

		Gene and the frequency of amplifications found (%)			
Tumour type	Studied samples (n)	*PDGFRA*	*KIT*	*VEGFR2*	*EGFR*
Astrocytoma grade II (III)	38	3	6	14	0
Astrocytoma grade II (V)	5	NA	0	NA	0
Anaplastic Astrocytoma (III)	18	33	28	17	12
Anaplastic Astrocytoma (V)	2	NA	1	NA	0
Secondary Gliobalstoma (III)	16	31	36	12	14
Primary Gliobalstoma (II)	43	29	47	39	70
Primary Gliobalstoma (V)	15	NA	67	NA	73
Pilocytic astrocytoma (IV)	9	NA	0	NA	NA

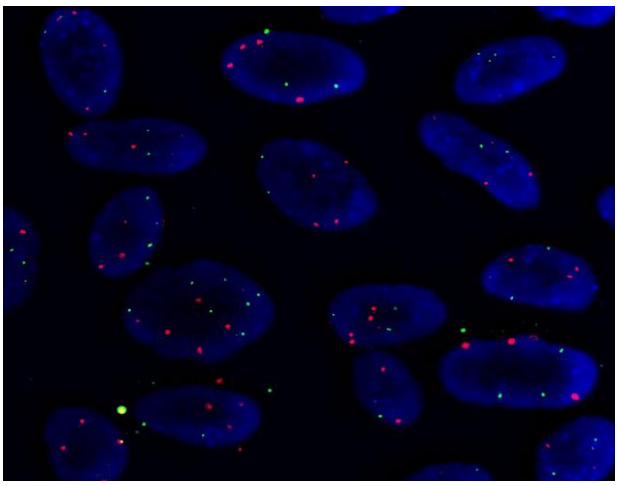


Figure 7. Glioma nuclei that present aneuploid copy numbers of the genes *KIT* and *PDGFR* detected by fluorescence *in situ* hybridization. Red signals are for *KIT* gene and green signals are for *PDGFRA* gene.

5. *KIT*, *PDGFRA* and *EGFR* mutations in primary and secondary glioblastomas (I, II)

Among the primary (n=43) and secondary (n=4) glioblastomas subjected to mutation screening, no mutations were detected with denaturing high-performance liquid chromatography in *KIT* exons 9, 11, 13 or 17, *PDGFRA* exons 12 or 18, or *EGFR* exons 18, 19 or 21 (II).

One mutation in an exon coding domain IV of *EGFR* was found in one out of the 57 glioblastomas in study I. This was a missense mutation C624F (substitution of cysteine by phenylalanine at the nucleotide position 1,871) in *EGFR* exon 15 (I).

Brain tumors are the topic of the present thesis, but we investigated in Study I also other tumors than brain tumors for presence of *EGFR* mutations. In brief, we found no *EGFR* kinase domain mutations in glioblastomas, but *EGFR* kinase domain mutations were found in lung adenocarcinomas and bronchioloalveolar carcinomas, of which 8 (11%) out of 73 tumors had a mutation. Six out of the eight mutations detected were in-frame deletions in exon 19, and two were leucine to arginine substitutions (L858R) at amino acid position 858 in exon 21. The deletions were clustered, and in each case, the amino acid residues LREA (leucine, arginine, glutamic acid and alanine) located at 747–750 were deleted. No *EGFR* kinase domain mutations occurred among the 493 tumors consisting of other histological cancer types than lung cancer, suggesting that *EGFR* kinase domain mutations are more common in lung adenocarcinomas and bronchioloalveolar carcinomas than in other types of human cancer.

6. *TP53* mutations in diffuse gliomas and their recurrences (III)

Mutation of *TP53* was present at the time of the diagnosis in 11 (29%) of the 38 astrocytomas, five (28%) out of the 18 anaplastic astrocytomas, four (20%) out of the 20 oligodendrogliomas and in three (27%) out of the 11 oligoastrocytomas studied. *TP53* mutations were not detected more frequently in recurrent tumors compared to newly detected tumors ($p = 0.25$); they were present in five (29%) out of the 17 recurrent astrocytomas, eight (38%) out of the 21 recurrent anaplastic astrocytomas, four (24%) out of the 17 recurrent oligodendrogliomas, seven (44%) out of the 16 recurrent oligoastrocytomas, and in six (38%) out of 16 secondary glioblastomas studied. We did not find any statistically significant associations between presence of *TP53* mutations and *KIT*, *PDGFRA*, *VEGFR2* or *EGFR* amplifications either in the subset of newly diagnosed gliomas or among recurrent tumors ($p > 0.10$ for all comparisons), although *TP53* mutations tended to be associated with *EGFR* amplifications in the subset of recurrent gliomas ($p = 0.075$).

7. Protein expression in primary and secondary glioblastomas (II)

EGFR protein was expressed more commonly in primary glioblastomas that harboured a high-level *EGFR* amplification (91%) than in glioblastomas that lacked such an amplification (91% vs. 60%,

respectively, p = 0.019). *EGFR* amplification was strongly associated with expression of phosphorylated EGFR. Fourteen (64%) of the 22 primary glioblastomas with high-level *EGFR* amplification expressed phosphorylated EGFR as compared with only one (5%) out of the 21 tumors that lacked high level amplification ($p < 0.0001$).

EGFRvIII expression was present in six (27%) out of the 22 primary glioblastomas with high-level *EGFR* amplification as compared to none of the rest of the tumors (n=21; p = 0.022). Marked KIT protein expression was rare, and occurred in only one out of the 43 primary and one out of 4 secondary glioblastomas, both of which had high-level *KIT* amplification. KIT protein expression tended to be more common in glioblastomas that had *KIT* amplification or aneuploidy than in tumors with a normal *KIT* copy number (p = 0.079). *KIT*, *PDGFRA* or *VEGFR2* amplifications were not associated with expression of EGFR, phosphorylated EGFR or truncated EGFR ($p > 0.10$ for all comparisons). Primary glioblastomas that had either *KIT*, *PDGFRA* or *VEGFR2* amplification tended to express Ki67 more often than tumors that lacked the gene amplification (p = 0.098, 0.060 and 0.097, respectively), whereas no such tendency was found for *EGFR* amplification (p = 0.49).

All 47 glioblastomas examined expressed the PDGFRA protein. TP53 expression was not associated with amplification of any of the four genes (*EGFR*, *KIT*, *PDGFRA* or *VEGFR2*) in primary glioblastomas ($p > 0.10$ for all comparisons). None of the four secondary glioblastomas expressed EGFR as compared to 33 (76%) out of the 43 primary glioblastomas (p = 0.0061), and none expressed phosphorylated EGFR or EGFRvIII. Three (75%) out of four secondary glioblastomas, but only four (9%) out of 43 primary glioblastomas, expressed TP53 (p = 0.0092).

8. Expressions of KIT, PDGFRA, VEGFR2 and EGFR protein in diffuse grade 2 or 3 gliomas and their recurrences (III)

KIT protein expression was rare in grade 2 or 3 gliomas. Moderate KIT expression was present in only one (1%) out of the 87 gliomas at the time of the diagnosis. This was a case of anaplastic astrocytoma that harboured *KIT* gene amplification. Strong KIT protein expression was detected in three (3%) recurrent gliomas (n=87); in two (13%) out of 16 secondary glioblastomas and in one (6%) out of 18 anaplastic astrocytomas. All recurrent gliomas with strong KIT expression had *KIT* gene amplification, and KIT protein expression was significantly associated with *KIT* amplification in the subset of recurred gliomas (p = 0.018). No associations were found between VEGFR2 protein expression and presence of *VEGFR2* amplification (p = 0.18), or with EGFR protein expression and *EGFR* amplification (p = 1.00).

9. Nestin and prominin-1 expression in diffuse grade 2 or 3 gliomas and their recurrences (III)

No association was found between *KIT* amplification, *TP53* mutation and prominin-1 expression (a suggested marker for brain stem cells) in the subset of newly diagnosed gliomas (n = 87), and, similarly, no association was present between prominin-1 expression and *PDGFRA*, *VEGFR2* or

EGFR gene amplifications ($p > 0.1$ for all comparisons). Nestin expression (another protein marker of neural stem cells) was associated with presence of *TP53* mutations in the subset of recurred gliomas. Twenty-five out of the 28 (89%) recurred gliomas that harboured *TP53* mutation expressed nestin compared with 30 of the 45 (67%) recurred gliomas that did not contain *TP53* mutation ($p = 0.029$). Nestin expression was not associated with *TP53* mutations in the subset of newly diagnosed gliomas, and no significant associations were detected between presence of *EGFR*, *KIT*, *PDGFRA* or *VEGFR2* amplifications and nestin expression.

10. KIT, phosphorylated KIT, SCF, VEGFR2 and HIF-1-alpha expression in endothelial cells of paediatric brain tumors, and in non-malignant fetal and postnatal tissues (IV)

KIT, phosphorylated KIT, SCF and VEGFR-2 expression was studied in 35 juvenile pilocytic astrocytomas and 49 other paediatric brain tumors using immunohistochemistry. KIT and phospho-KIT were moderately or strongly expressed in tumour endothelia of 37% and 35% of pilocytic astrocytomas, respectively, whereas marked SCF and VEGFR-2 expression was uncommon. KIT mRNA was detected in tumour endothelial cells. Tumour endothelial cell KIT expression was strongly ($p < 0.01$) associated with endothelial cell phospho-KIT and SCF expression, and with tumour KIT ($p = 0.0011$) and VEGFR-2 expression ($p = 0.022$). KIT and phospho-KIT were frequently present in the endothelia of ependymomas (six [55%] out of 11 tumors, and five [45%] out of 11 tumors, respectively). Endothelial cell KIT expression was associated with a young age at diagnosis of pilocytic astrocytoma or ependymoma, and it was occasionally present in histologically normal tissue of the foetus and young children.

11. Heterogeneity of EGFR EGFRvIII, KIT and phospho-KIT protein expression in gliomas and glioblastomas (V).

In addition to gene copy number heterogeneity, we studied the gene product (protein) heterogeneity in 15 glioblastomas by examining several tissue blocks from each tumour using immunohistochemistry. EGFR, EGFRvIII, KIT and phospho-KIT expression was frequently heterogeneous, and occurred at least in a part of the tumour in 93%, 33%, 93% and 100% of the tumors, respectively. Five (70%) out of the seven astrocytomas examined in a similar manner for protein expression heterogeneity expressed EGFR in a part of the tumour, and three (43%) out of the seven astrocytomas expressed KIT suggesting substantial heterogeneity in EGFR and KIT expression in astrocytomas.

DISCUSSION

Clinically, gene amplifications have diagnostic and prognostic usefulness, and amplification of genomic regions in cancer cells often represent selection of genes that promote tumour growth (Albertson 2006). *EGFR* gene amplification and EGFR protein overexpression have long been considered as hallmarks of primary glioblastoma (Reifenberger & Collins 2004).

Glioblastomas often display morphological heterogeneity, where tumour areas that show high histological grade of differentiation with well-differentiated cells are commonly found adjacent to poorly differentiated areas (Reifenberger & Collins 2004). Such heterogeneity likely exists also at the molecular level. Amplification of *EGFR* is reported to be present in 40-50% of primary glioblastomas, where its presence is generally associated with a poor patient outcome (Hurtt MR *et al.* 1992, Schlegel J *et al.* 1994, Riemenschneider MJ *et al.* 2010).

The present findings are generally in line with a hypothesis that *EGFR* plays an important role in the molecular pathogenesis of primary glioblastoma. *EGFR* amplification was very frequent and occurred in as many as in 70% of the primary glioblastomas studied (II). In addition to *EGFR* amplification, expression of the truncated form of the EGFR protein (EGFRvIII) was common in glioblastoma (II), whereas other *EGFR* mutations turned out to be infrequent (I). Heterogeneity in *EGFR* amplification was more limited as compared to that of *KIT* amplification in primary glioblastoma (V). EGFR protein was commonly expressed in primary glioblastoma, and the protein was frequently activated (phosphorylated) (II). Expression of phosphorylated EGFR was strongly associated with *EGFR* amplification (II). Taken together, these findings suggest that *EGFR* is of importance for the development of primary glioblastoma. In contrast, in lower grade gliomas (astrocytoma grade II and III) and secondary glioblastomas *EGFR* amplification was relatively rare and occurred in less than 15% of the tumors examined (III), suggesting that *EGFR* is less important in the molecular pathogenesis of low grade gliomas and secondary glioblastomas than of primary glioblastomas, and that the molecular pathogenesis of these tumors differs from that of primary glioblastoma.
We found the rate of *EGFR* amplification may be even higher than previously described in primary glioblastomas. The variation in the reported frequency of *EGFR* amplifications in glioblastoma may in part be explained by tumour heterogeneity, and it likely depends also on the proportion of classical glioblastomas in the series. We found *KIT* amplification, and to a lesser extent *EGFR* amplification, to be heterogeneous in glioblastomas (V).

Genes other than *KIT* or *EGFR* might be variably amplified at specific sites of a single tumour, and their functional activity might also vary within the tumour. More tumour regions with gene amplification may thus be identified when the entire tumour tissue is subjected to analysis as

compared to analysis of a single tumour biopsy. It may not be possible to assess reliably the frequency of gene amplifications from a single tissue biopsy particularly in glioblastomas due to the substantial intratumoral heterogeneity. The degree of intratumoral heterogeneity may vary between genes and their protein expression in a way that is difficult to predict without careful assessment of the entire tumour, which may complicate optimal selection of modern targeted treatments for a glioma patient.

The role of *KIT*, *PDGFRA* and *VEGFR2* in the molecular pathogenesis of primary glioblastomas remains less well understood than that of *EGFR*. The results obtained in this thesis are compatible with a hypothesis that amplification of *KIT*, *PDGFRA* and *VEGFR2* might rather be secondary events in tumour progression than a primary cause of glioma. *KIT*, *PDGFRA* and *VEGFR2* are located adjacent on chromosome 4q, and they were frequently coamplified (II). The amplicon frequently contained *KIT* (III). Although amplification of *KIT*, *PDGFRA* and *VEGFR2* were frequent in primary glioblastoma (in particular amplification of *KIT*, which was found in 47% of the primary glioblastomas studied)(II), KIT protein was infrequently expressed in primary glioblastoma, and *KIT*, *PDGFRA* and *VEGFR2* amplifications were not associated with patient survival. KIT expression was often heterogeneous in primary glioblastoma (V), which is also compatible with the hypothesis that *KIT* amplification is a secondary event. *KIT*, *PDGFRA* and *VEGFR2* amplifications tended to be associated with a high tumour Ki-67 expression and thus with a high cell proliferation rate.

The hypothesis that primary glioblastomas have a different molecular pathogenesis from secondary gliomas and lower grade astrocytomas has recently gained much support following the detection of isocitrate dehydrogenase-1 (*IDH1*) gene mutations (Balss J *et al*. 2008) in gliomas, and characterization of the clinical significance of *IDH1* and its related gene *IDH2* mutations (Yan et *al*. 2009). Presence of *IDH1* and *IDH2* mutations may now became the key criteria when classifying glioblastomas into the primary and the secondary types.

IDH1 and *IDH2* encode critical proteins (R132 and R172, respectively) that function in the NADP+-dependent cellular processes. Mutations of *IDH1* are found in more than 70% of WHO grade II and grade III astrocytomas and oligodendrogliomas, and in secondary glioblastomas that develop from these tumors. Of note, gliomas that lacked *IDH1* mutation often harboured a mutation in the *IDH2* gene. Patients with a tumour with either *IDH1* or *IDH2* mutation had better outcome than patients whose glioma harboured wild-type *IDH* gene (Yan *et al*. 2009) and mutations in *IDH1* occurred in a large fraction of young patients and in most patients with secondary GBMs and were associated with an increase in overall survival (Parsons *et al*. 2008).

Importantly, *IDH1* and *IDH2* mutations are very rare in primary glioblastoma, medulloblastoma, pilocytic astrocytoma and ependymoblastoma, and they were not found in any of the 494 non-CNS tumors investigated (Yan *et al*. 2009). These findings suggest that *IDH1* and *IDH2* mutations are

important in the molecular pathogenesis of WHO grade II and grade III astrocytomas, oligodendrogliomas and secondary glioblastomas. Mutations *IDH1* and *IDH2* may occur early in the molecular pathogenesis that leads to genesis of these tumour types.

A recent large scale study analyzing DNA copy number, gene expression and DNA methylation study provides new insights into the roles of *NF1* and *TP53* in glioblastomas (Cancer Genomic Atlas network 2008). Mutations of *TP53* gene, once thought to predominate in secondary glioblastoma, have been found at a high frequency also from primary glioblastomas, and *NF-1* somatic mutations showed new relevance in sporadic glioblastomas. In addition, A recent genomic analysis of glioblastomas identified four clinically relevant glioblastoma subtypes based on aberrations of *EGFR*, *NF1* and *PDGFRA*/*IDH1* and their gene expression, which resulted in subclassification of glioblastomas into the classical, mesenchymal and proneural subtypes (Verhaak *et al*. 2010). The classical glioblastoma subtype was associated with *EGFR* amplification in as many as 95% of the tumors with no alterations in *TP53*, *NF1*, *PDGFRA* or *IDH1*, whereas the mesenchymal subtype was associated with a high frequency of *NF1* mutations and deletions, and expression of Schwann cell markers such as S100 and microglial markers. The proneural subtype was associated with young age, *PDGFRA* abnormalities, and *IDH1* and *TP53* mutations, which are features of secondary glioblastomas. Moreover, most secondary glioblastomas were classified as proneural. These findings suggest that some of the primary glioblastomas might arise from a clinically silent and undetected lower grade astrocytoma, or perhaps more likely, from a neural progenitor cell that can give rise either to a lower grade astrocytoma or the proneural type of primary glioblastoma. These data may be important in future trials when testing novel treatments for glioblastoma, and they may also aid at developing of multigene predictors for assessing the outcome of patients diagnosed with glioblastoma (Colman *et al*. 2010).

Although *IDH* mutations may be one of the initiating events in the molecular pathogenesis of many secondary glioblastomas and lower grade astrocytomas, the subsequent molecular events that lead to the development of either astrocytic or oligodendrocytic tumors likely require activation of distinct cell signalling pathways. This thesis presents a hypothesis that amplifications of *KIT*, *PDGFRA* and *VEGFR2* are more common in secondary glioblastoma and anaplastic astrocytoma than in astrocytoma, oligoastrocytoma and oligodendroglioma (III). KIT expression is rare in lower grade gliomas, but was it was associated with *KIT* amplification in recurred gliomas (III). The probability of a tumour to acquire amplification of *PDGFRA*, *KIT* and *VEGFR2* may increase with tumour progression, and an amplicon containing these genes may thus be found more frequently at the time of tumour recurrence as compared to the first diagnosis of glioma, and more frequently in higher grade tumors than in lower grade tumors. These data are in line with the general hypothesis that gene amplifications are frequent molecular alterations of later stage tumors (Lengauer *et al.* 1998). Presence of gene amplifications in lower grade diffuse gliomas might suggest that these tumors are more malignant than the mere histopathology implies (Riemenschneider *et al.* 2010).

KIT, *PDGFRA* and *VEGFR2* have all been linked with angiogenesis. VEGFR2 is a key proangiogenic receptor tyrosine kinase (as discussed in 1.4 above) and PDFGR is involved with pericyte recruitment (Pietras *et al.* 2003). SCF may be produced in glioblastoma as a response to hypoxic conditions, possibly by hypoxic perinecrotic glioblastoma cells (Sihto *et al.* 2009). SCF may enhance endothelial cell survival, cell migration and tube formation, and SCF and KIT may thus have a role in tumour angiogenesis (Matsui J *et al*. 2004, Sihto *et al.* 2007, Sun L *et al.* 2004). Endothelial proliferation is a hallmark of glioblastoma, and a number of clinical trials addressing a variety of anti-angiogenic agents are currently underway in the treatment of both newly diagnosed and recurrent glioblastoma (Ahluwalia *et al*. 2010). *KIT, PDGFRA* and *VEGFR2* expression was not significantly associated with the neural stem cell markers nestin and prominin, and they were not associated with *TP53* mutations (III) or p53 expression (II).

Pilocytic astrocytomas lack many of the molecular alterations that are frequently present in adult gliomas such as *IDH1* and *IDH2* mutations (IV, Haapasalo *et al.* 1999, James & Collins 1992, Ohgaki *et al.* 1993 Ransom *et al.* 1992 von Deimling *et al.* 1994, Yan *et al*. 2009). Tandem duplications at 7q34 resulting in a BRAF fusion gene with constitutive BRAF kinase activity are frequent in pilocytic astrocytoma, whereas this alteration does not occur in higher-grade gliomas (Jones DT *et al*. 2008). In line with these findings, we found that pilocytic astrocytomas do not usually have amplifications of *KIT*. We found instead that in pilocytic astrocytomas tumour endothelial cells frequently express KIT and SCF and that endothelial KIT was often phosphorylated (IV). In pilocytic astrocytomas, and possibly in ependymomas, endothelial cell KIT expression was associated with a young age at the time of the diagnosis (IV). Yet, juvenile pilocytic astrocytomas are usually associated with good prognosis (Ohgaki H & Kleihues P 2005a) and are not necrotic, and data from glioblastoma patient series suggest that glioblastomas with moderate to strong KIT expression in the tumour endothelial cells may be associated with favourable outcome as compared to tumors with little KIT expression (Sihto *et al.* 2009). Therefore, other factors, such as the degree of maturation of the vascular system might be associated with endothelial cell KIT expression in young individuals with pilocytic astrocytoma.

Further studies on the topic of this thesis should address the relationships between *EGFR*, *KIT*, *PDGFRA* and *VEGFR2* alterations and the newly discovered *IDH* mutations and *NF1* mutations. Very recently in sonic hedgehog medulloblastomas a phenomena of massive chromosome rearrangements in a one-step, catastrophic event termed chromothripsis has been characterized. Cromothripsis can lead to gene amplifications and, the association of chromothripsis with *TP53* mutations links the phenomena to more complex caryotype form of acute myeloid leukemia and poor survival (Rausch et al 2012). In higher grade gliomas with gene amplifications, it would be interesting to see if all RTK gene amplifications are equal and if more complex caryotypes with poorer outcomes can be identified.

Endothelial cell KIT expression also requires further study, since this might be a potential treatment target. The emerging new molecular classification of astrocytic brain tumors may provide a new basis for future clinical trials, since this may allow improved patient selection for targeted agents, although the marked heterogeneity of glioblastoma, in particular, may turn out to be a difficult obstacle to overcome. Diffusively growing glial tumors are difficult to cure by surgery, they are only moderately sensitive to irradiation and conventional chemotherapy, and targeted therapies have thus far not resulted in impressive results in the treatment of these tumour types. Therefore, further studies on the molecular pathogenesis of gliomas continue to be of critical importance.

CONCLUSIONS

The aims of the present thesis was to study *EGFR* mutations and their association with *EGFR* amplifications in various histological types of human cancer, including brain tumors (I); to investigate the amplifications of *KIT*, *PDGFRA*, *VEGFR2* and *EGFR* genes and their mutations in glioblastoma (II); to investigate amplifications of *KIT*, *PDGFRA*, *VEGFR2* and *EGFR* genes in low grade glioma, anaplastic astrocytoma and their recurrences (III); to evaluate KIT and VEGFR2 expression in endothelial and tumour cells in paediatric brain tumors and to investigate *KIT* gene copy numbers in these tumors (IV); and finally to study the heterogeneity of *EGFR* and *KIT* gene amplification and their protein products in gliomas and glioblastoma (V). We conclude that

1. Apart from the deletion mutant EGFRvIII in glioblastoma, *EGFR* mutations appear rare in human brain tumors. No mutations were found in *EGFR* exons 18, 19 and 21 that encode the kinase part of the EGFR receptor tyrosine kinase in brain tumors. Mutations in *EGFR* resulting in an aberrant kinase domain occur, however, in adenocarcinomas and bronchioloalveolar carcinomas of the lung.

2. *EGFR* gene amplification is common in primary glioblastoma, and was present in 70% of the glioblastomas investigated. EGFR protein is commonly expressed in glioblastoma and it is frequently activated (phosphorylated). Presence of *EGFR* amplification is strongly associated with expression of phosphorylated EGFR. *KIT*, *PDGFRA* and *VEGFR2* genes are also frequently amplified in primary glioblastoma (*KIT* in approximately 50% of the cases, and *PDFRA* and *VEGFR2* in approximately one third of the cases).

3. Amplifications of *KIT*, *PDGFRA*, *VEGFR2* and *EGFR* genes occur occasionally in lower grade gliomas (astrocytomas, oligodendrogliomas, oligoastrocytomas and anaplastic astrocytomas), but generally at a lower frequency than in glioblastoma. *KIT* and *PDGFRA* amplifications are more common in anaplastic astrocytomas than in astrocytomas, oligodendrogliomas and oligoastrocytomas.

KIT amplification is more frequent in recurrent lower grade gliomas and secondary glioblastomas as compared to newly diagnosed lower grade gliomas.

4. Paediatric pilocytic astrocytomas usually neither harbour amplifications of *KIT* nor express the KIT protein. Instead, KIT expression is common in the tumour endothelial cells, and endothelial cell KIT was frequently phosphorylated. Expression of endothelial cell KIT in pilocytic astrocytoma is associated with a young age at the time of the diagnosis. Ependymomas also frequently express KIT in endothelial cells, and endothelial cell KIT expression tends to be associated with young age at the time of the diagnosis.

5. Glioblastomas may show heterogeneity with respect of *EGFR* and *KIT* amplifications. The degree of heterogeneity in tumour *KIT* amplification may be greater than that of *EGFR*, which showed only limited heterogeneity between several tissue samples analyzed from one tumour. Expression KIT and phospho-KIT proteins were also markedly heterogeneous in glioblastomas, and heterogeneous expression of KIT and EGFR proteins was found also in astrocytomas and anaplastic astrocytomas.

Taken together, these findings support an important role for *EGFR* in the molecular pathogenesis of many primary glioblastomas. The importance of *KIT*, *PDGFRA* and *VEGFR2* amplifications in glioma molecular pathogenesis seems less evident, and these alterations might often be secondary events related to glioma progression. The role of *KIT* may be multifunctional, and one of the roles may be associated with glioma angiogenesis. None of these receptor tyrosine kinases has thus far turned out to be a viable therapeutic target in clinical trials.

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REFERENCES

Ahluwalia MS, Gladson CL. (2010) Progress on antiangiogenic therapy for patients with malignant glioma. J Oncol. 2010;2010:689018. Epub 2010 Apr 6.

Albertson DG. (2006) Gene amplification in cancer. Trends Genet 22(8): 447-455.

Al-Nedawi K, Meehan B, Micallef J, Lhotak V, May L, Guha A & Rak J. (2008) Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. Nat Cell Biol May;10(5):619-24.

Antonyak MA, Moscatello DK & Wong AJ. (1998) Constitutive activation of c-Jun N-terminal kinase by a mutant epidermal growth factor receptor. J Biol Chem 273(5): 2817-2822.

Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G & Isner JM. (1997) Isolation of putative progenitor endothelial cells for angiogenesis. Science 275(5302): 964-967.

Audero E, Cascone I, Zanon I, Previtali SC, Piva R, Schiffer D & Bussolino F. (2001) Expression of angiopoietin-1 in human glioblastomas regulates tumor-induced angiogenesis: in vivo and in vitro studies. Arterioscler Thromb Vasc Biol 21(4): 536-541.

Balss J, Meyer J, Mueller W, Korshunov A, Hartmann C & von Deimling A (2008). Analysis of the IDH1 codon 132 mutation in brain tumors. Acta Neuropathol 116: 597–602.

Behin A, Hoang-Xuan K, Carpentier AF & Delattre JY. (2003) Primary brain tumours in adults. Lancet 361(9354): 323-331.

Bene MC, Bernier M, Casasnovas RO, Castoldi G, Knapp W, Lanza F, Ludwig WD, Matutes E, Orfao A, Sperling C & van't Veer MB. (1998) The reliability and specificity of c-kit for the diagnosis of acute myeloid leukemias and undifferentiated leukemias. The European Group for the Immunological Classification of Leukemias (EGIL). Blood 92(2): 596-599.

Berdel WE, de Vos S, Maurer J, Oberberg D, von Marschall Z, Schroeder JK, Li J, Ludwig WD, Kreuser ED & Thiel E. (1992) Recombinant human stem cell factor stimulates growth of a human glioblastoma cell line expressing c-kit protooncogene. Cancer Res 52(12): 3498-3502.

Bergers G & Benjamin LE. (2003) Tumorigenesis and the angiogenic switch. Nat Rev Cancer 3(6): 401-410.

Bergsagel DJ, Finegold MJ, Butel JS, Kupsky WJ & Garcea RL. (1992) DNA sequences similar to those of simian virus 40 in ependymomas and choroid plexus tumors of childhood. N Engl J Med 326(15): 988-993.

Berkman RA, Merrill MJ, Reinhold WC, Monacci WT, Saxena A, Clark WC, Robertson JT, Ali IU & Oldfield EH. (1993) Expression of the vascular permeability factor/vascular endothelial growth factor gene in central nervous system neoplasms. J Clin Invest 91(1): 153-159.

Bertolini F, Shaked Y, Mancuso P & Kerbel RS. (2006) The multifaceted circulating endothelial cell in cancer: towards marker and target identification. Nat Rev Cancer 6(11): 835-845.

Bhargava R, Gerald WL, Li AR, Pan Q, Lal P, Ladanyi M & Chen B. (2005) EGFR gene amplification in breast cancer: correlation with epidermal growth factor receptor mRNA and protein expression and HER-2 status and absence of EGFR-activating mutations. Mod Pathol 18(8): 1027-1033.

Bijlsma EK, Voesten AM, Bijleveld EH, Troost D, Westerveld A, Merel P, Thomas G & Hulsebos TJ. (1995) Molecular analysis of genetic changes in ependymomas. Genes Chromosomes Cancer 13(4): 272-277.

Blume-Jensen P & Hunter T. (2001) Oncogenic kinase signalling. Nature 411(6835): 355-365.

Blume-Jensen P, Janknecht R & Hunter T. (1998) The kit receptor promotes cell survival via activation of PI 3-kinase and subsequent Akt-mediated phosphorylation of Bad on Ser136. Curr Biol 8(13): 779-782.

Bondy ML, Scheurer ME, Malmer B, Barnholtz-Sloan JS, Davis FG, Il'yasova D, Kruchko C, McCarthy BJ, Rajaraman P, Schwartzbaum JA, Sadetzki S, Schlehofer B, Tihan T, Wiemels JL, Wrensch M & Buffler PA. (2008) Brain Tumor Epidemiology Consortium. Brain tumor epidemiology: consensus from the Brain Tumor Epidemiology Consortium.Cancer. 113(7 Suppl):1953-68.

Boonstra J, Rijken P, Humbel B, Cremers F, Verkleij A & van Bergen en Henegouwen, P. (1995) The epidermal growth factor. Cell Biol Int 19(5): 413-430.

Bredel M, Bredel C, Juric D, Harsh GR, Vogel H, Recht LD & Sikic BI. (2005) High-resolution genome-wide mapping of genetic alterations in human glial brain tumors. Cancer Res 65(10): 4088-4096.

Calabrese C, Poppleton H, Kocak M, Hogg TL, Fuller C, Hamner B, Oh EY, Gaber MW, Finklestein D, Allen M, Frank A, Bayazitov IT, Zakharenko SS, Gajjar A, Davidoff A & Gilbertson RJ. (2007) A perivascular niche for brain tumor stem cells. Cancer Cell 11(1): 69-82.

Cancer Genome Atlas Research Network. (2008) Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature. 2008 455(7216):1061-8.

Capdeville R, Buchdunger E, Zimmermann J & Matter A. (2002) Glivec (STI571, imatinib), a rationally developed, targeted anticancer drug. Nat Rev Drug Discov 1(7): 493-502.

Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D & Keshert E. (1998) Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature 394(6692):485-90.

Carmen J, Magnus T, Cassiani-Ingoni R, Sherman L, Rao MS & Mattson MP. (2007) Revisiting the astrocyte-oligodendrocyte relationship in the adult CNS. Prog Neurobiol 82(3):151-62.

Carroll RS, Zhang J, Bello L, Melnick MB, Maruyama T & McL Black P. (1999) KDR activation in astrocytic neoplasms. Cancer 86(7): 1335-1341.

Cavaliere R, Lopes MB & Schiff D. (2005) Low-grade gliomas: an update on pathology and therapy. Lancet Neurol 4(11): 760-770.

Cavaliere R, Wen PY & Schiff D. (2007) Novel therapies for malignant gliomas. Neurol Clin 25(4): 1141-71, x.

Centeno RS, Lee AA, Winter J & Barba D. (1986) Supratentorial ependymomas. Neuroimaging and clinicopathological correlation. J Neurosurg 64(2): 209-215.

Chen R, Nishimura MC, Bumbaca SM, Kharbanda S, Forrest WF, Kasman IM, Greve JM, Soriano RH, Gilmour LL, Rivers CS, Modrusan Z, Nacu S, Guerrero S, Edgar KA, Wallin JJ, Lamszus K, Westphal M, Heim S, James CD, VandenBerg SR, Costello JF, Moorefield S, Cowdrey CJ, Prados M & Phillips HS. (2010) A hierarchy of self-renewing tumor-initiating cell types in glioblastoma. Cancer Cell 17(4):362-75.

Chernova OB, Chernov MV, Ishizaka Y, Agarwal ML & Stark GR. (1998) MYC abrogates p53-mediated cell cycle arrest in N-(phosphonacetyl)-L-aspartate-treated cells, permitting CAD gene amplification. Mol Cell Biol 18(1): 536-545.

Chu CT, Everiss KD, Wikstrand CJ, Batra SK, Kung HJ & Bigner DD. (1997) Receptor dimerization is not a factor in the signalling activity of a transforming variant epidermal growth factor receptor (EGFRvIII). Biochem J 324 (Pt 3)(Pt 3): 855-861.

Citri A & Yarden Y. (2006) EGF-ERBB signalling: towards the systems level. Nat Rev Mol Cell Biol 7(7): 505-516.

Cockman ME, Masson N, Mole DR, Jaakkola P, Chang GW, Clifford SC, Maher ER, Pugh CW, Ratcliffe PJ & Maxwell PH. (2000) Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. J Biol Chem 275(33): 25733-25741.

Colin C, Baeza N, Bartoli C, Fina F, Eudes N, Nanni I, Martin PM, Ouafik L & Figarella-Branger D. (2006) Identification of genes differentially expressed in glioblastoma versus pilocytic astrocytoma using Suppression Subtractive Hybridization. Oncogene 25(19): 2818-2826.

Collins VP. (2004) Brain tumours: classification and genes. J Neurol Neurosurg Psychiatry 75 Suppl 2: ii2-11.

Colman H, Zhang L, Sulman EP, McDonald JM, Shooshtari NL, Rivera A, Popoff S, Nutt CL, Louis DN, Cairncross JG, Gilbert MR, Phillips HS, Mehta MP, Chakravarti A, Pelloski CE, Bhat K, Feuerstein BG, Jenkins RB, Aldape K. (2010) Neuro-Oncol 12(1):49-57.

Conejo-Garcia JR, Buckanovich RJ, Benencia F, Courreges MC, Rubin SC, Carroll RG & Coukos G. (2005) Vascular leukocytes contribute to tumor vascularization. Blood 105(2): 679-681.

Coquelle A, Pipiras E, Toledo F, Buttin G & Debatisse M. (1997) Expression of fragile sites triggers intrachromosomal mammalian gene amplification and sets boundaries to early amplicons. Cell 89(2): 215-225.

Corless CL, Fletcher JA & Heinrich MC. (2004) Biology of gastrointestinal stromal tumors. J Clin Oncol 22(18): 3813-3825.

Dang L, White DW, Gross S, Bennett BD, Bittinger MA, Driggers EM, Fantin VR, Jang HG, Jin S, Keenan MC, Marks KM, Prins RM, Ward PS, Yen KE, Liau LM, Rabinowitz JD, Cantley LC, Thompson CB, Vander Heiden MG & Su SM. (2009) Cancer-associated IDH1 mutations produce 2-hydroxyglutarate Nature 462(7274):739-44.

De Palma M, Venneri MA, Galli R, Sergi Sergi L, Politi LS, Sampaolesi M & Naldini L. (2005) Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. Cancer Cell 8(3): 211-226.

DeAngelis LM. (2001) Brain tumors. N Engl J Med 344(2): 114-123.

Deltour I, Christoffer J, Anssi A, Feychting M, Klaeboe L & Schüz J. (2009) Time Trends in Brain Tumor Incidence Rates in Denmark, Finland, Norway, and Sweden, 1974–2003. J Natl Cancer Inst 101(24):1721-4.

Dematteo RP, Ballman KV, Antonescu CR, Maki RG, Pisters PW, Demetri GD, Blackstein ME, Blanke CD, von Mehren M, Brennan MF, Patel S, McCarter MD, Polikoff JA, Tan BR & Owzar K; American College of Surgeons Oncology Group (ACOSOG) Intergroup Adjuvant GIST Study Team. (2009) Adjuvant imatinib mesylate after resection of localised, primary gastrointestinal stromal tumour: a randomised, double-blind, placebo-controlled trial. Lancet 373(9669):1097-104.

Dentelli P, Rosso A, Balsamo A, Colmenares Benedetto S, Zeoli A, Pegoraro M, Camussi G, Pegoraro L & Brizzi MF. (2007) C-KIT, by interacting with the membrane-bound ligand, recruits endothelial progenitor cells to inflamed endothelium. Blood 109(10): 4264-4271.

Desjardins A, Quinn JA, Vredenburgh JJ, Sathornsumetee S, Friedman AH, Herndon JE, McLendon RE, Provenzale JM, Rich JN, Sampson JH, Gururangan S, Dowell JM, Salvado A, Friedman HS & Reardon DA. (2007) Phase II study of imatinib mesylate and hydroxyurea for recurrent grade III malignant gliomas. J Neurooncol 83(1): 53-60.

Dhall G. Medulloblastoma. (2009) J Child Neurol 24(11):1418-30.

Dresemann G. (2005) Imatinib and hydroxyurea in pretreated progressive glioblastoma multiforme: a patient series. Ann Oncol 16(10): 1702-1708.

Dresemann G, Weller M, Rosenthal MA, Wedding U, Wagner W, Engel E et al (2010) Imatinib in combination with hydroxyurea versus hydroxyurea alone as oral therapy in patients with progressive pretreated glioblastoma resistant to standard dose temozolomide. J Neurooncol 96:393-402.

Druker BJ, Guilhot F, O'Brien SG, Gathmann I, Kantarjian H, Gattermann N, Deininger MW, Silver RT, Goldman JM, Stone RM, Cervantes F, Hochhaus A, Powell BL, Gabrilove JL, Rousselot P, Reiffers J, Cornelissen JJ, Hughes T, Agis H, Fischer T, Verhoef G, Shepherd J, Saglio G, Gratwohl A, Nielsen JL, Radich JP, Simonsson B, Taylor K, Baccarani M, So C, Letvak L, Larson RA & IRIS Investigators. (2006) Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. N Engl J Med 355(23): 2408-2417.

Ekstrand AJ, James CD, Cavenee WK, Seliger B, Pettersson RF & Collins VP. (1991) Genes for epidermal growth factor receptor, transforming growth factor alpha, and epidermal growth factor and their expression in human gliomas in vivo. Cancer Res 51(8): 2164-2172.

Ekstrand AJ, Longo N, Hamid ML, Olson JJ, Liu L, Collins VP & James CD. (1994) Functional characterization of an EGF receptor with a truncated extracellular domain expressed in glioblastomas with EGFR gene amplification. Oncogene 9(8): 2313-2320.

Ekstrand AJ, Sugawa N, James CD & Collins VP. (1992) Amplified and rearranged epidermal growth factor receptor genes in human glioblastomas reveal deletions of sequences encoding portions of the N- and/or C-terminal tails. Proc Natl Acad Sci U S A 89(10): 4309-4313.

Entz-Werle N, Gaub MP, Lavaux T, Marcellin L, Metzger N, Marec-Berard P, Schmitt C, Brugiere L, Kalifa C, Tabone MD, Pacquement H, Gentet P, Lutz P, Oudet P & Babin A. (2007) KIT gene in pediatric osteosarcomas: could it be a new therapeutic target? Int J Cancer 120(11): 2510-2516.

Erlandsson A, Larsson J & Forsberg-Nilsson K. (2004) Stem cell factor is a chemoattractant and a survival factor for CNS stem cells. Exp Cell Res 301(2): 201-210.

Escribano L, Ocqueteau M, Almeida J, Orfao A & San Miguel JF. (1998) Expression of the c-kit (CD117) molecule in normal and malignant hematopoiesis. Leuk Lymphoma 30(5-6): 459-466.

Farrell CJ & Plotkin SR. Genetic causes of brain tumors: neurofibromatosis, tuberous sclerosis, von Hippel-Lindau, and other syndromes. (2007) Neurol Clin 25:925-946.

Feero WG, Guttmacher AE & Collins FS. Genomic medicine – an updated primer. (2010) N Engl J Med 362:2001-2011.

Fenstermaker RA & Ciesielski MJ. (2000) Deletion and tandem duplication of exons 2 - 7 in the epidermal growth factor receptor gene of a human malignant glioma. Oncogene 19(39):4542-8.

Fischer I, de la Cruz C, Rivera AL, Aldape K (2008) Utility of chromogenic in situ hybridization (CISH) for detection of EGFR amplification in glioblastoma: comparison with fluorescence in situ hybridization (FISH). Diagn Mol Pathol 17:227-230.

Fisher JL, Schwartzbaum JA, Wrensch M & Wiemels JL. Epidemiology of brain tumors. (2007) Neurol Clin 25:867-890.

Fisher PG, Tihan T, Goldthwaite PT, Wharam MD, Carson BS, Weingart JD, Repka MX, Cohen KJ & Burger PC. Outcome analysis of childhood low-grade astrocytomas. (2008) Pediatr Blood Cancer 51(2):245-50.

Franceschi E, Cavallo G, Lonardi S, Magrini E, Tosoni A, Grosso D et al (2007) Gefitinib in patients with progressive high-grade gliomas: a multicentre phase II study by Gruppo Italiano Cooperativo di Neuro-Oncologia (GICNO). Br J Cancer 96:1047-1051.

Frederick L, Wang XY, Eley G & James CD. (2000) Diversity and frequency of epidermal growth factor receptor mutations in human glioblastomas. Cancer Res 60(5): 1383-1387.

Fritsche-Polanz R, Fritz M, Huber A, Sotlar K, Sperr WR, Mannhalter C, Födinger M & Valent P. High frequency of concomitant mastocytosis in patients with acute myeloid leukemia exhibiting the transforming KIT mutation D816V. (2010) Mol Oncol. 2010 Apr 24. [Epub ahead of print]

Gajjar AJ, Stewart CF, Ellison DW, Curran T, Phillips P, Goodman S, Packer R, Kun LE, Boyett JM & Gilbertson RJ. A phase I pharmacokinetic trial of sonic hedgehog antagonist GDC-0449 in pediatric patients with recurrent or refractory medulloblastoma: a pediatric Brain Tumor Consortium study (PBTC 25). (2010) J Clin Oncol 28 (18S):959s.

Galli R, Binda E, Orfanelli U, Cipelletti B, Gritti A, De Vitis S, Fiocco R, Foroni C, Dimeco F & Vescovi A. (2004) Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. Cancer Res 64(19): 7011-7021.

Galli R, Gritti A & Vescovi AL. (2008) Adult neural stem cells. Methods Mol Biol 438: 67-84.

Gan HK, Kaye AH & Luwor RB. The EGFRvIII variant in glioblastoma multiforme. (2009) J Clin Neurosci 16(6):748-54.

Gesundheit B, Klement G, Senger C, Kerbel R, Kieran M, Baruchel S & Becker L. (2003) Differences in vasculature between pilocytic and anaplastic astrocytomas of childhood. Med Pediatr Oncol 41(6): 516-526.

Gilbertson RJ. (2004) Medulloblastoma: signalling a change in treatment. Lancet Oncol 5(4): 209-218.

Glover TW, Arlt MF, Casper AM & Durkin SG. (2005) Mechanisms of common fragile site instability. Hum Mol Genet 14 Spec No. 2: R197-205.

Grassot J, Gouy M, Perriere G & Mouchiroud G. (2006) Origin and molecular evolution of receptor tyrosine kinases with immunoglobulin-like domains. Mol Biol Evol 23(6): 1232-1241.

Grunewald M, Avraham I, Dor Y, Bachar-Lustig E, Itin A, Jung S, Chimenti S, Landsman L, Abramovitch R & Keshet E. (2006) VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. Cell 124(1): 175-189.

Guillaudeau A, Durand K, Pommepuy I, Robert S, Chaunavel A, Lacorre S et al (2009) Determination of EGFR status in gliomas: usefulness of immunohistochemistry and fluorescent in situ hybridization. Appl Immunohistochem Mol Morphol 17:220-226.

Haapasalo H, Sallinen S, Sallinen P, Helen P, Jaaskelainen J, Salmi TT, Paetau A, Paljarvi L, Visakorpi T & Kalimo H. (1999) Clinicopathological correlation of cell proliferation, apoptosis and p53 in cerebellar pilocytic astrocytomas. Neuropathol Appl Neurobiol 25(2): 134-142.

Hanahan D. (1997) Signaling vascular morphogenesis and maintenance. Science 277(5322): 48-50.

Hanahan D & Weinberg RA. (2000) The hallmarks of cancer. Cell 100(1): 57-70.

Hanawa M, Suzuki S, Dobashi Y, Yamane T, Kono K, Enomoto N & Ooi A. (2006) EGFR protein overexpression and gene amplification in squamous cell carcinomas of the esophagus. Int J Cancer 118(5): 1173-1180.

Handolias D, Hamilton AL, Salemi R, Tan A, Moodie K, Kerr L, Dobrovic A & McArthur GA. Clinical responses observed with imatinib or sorafenib in melanoma patients expressing mutations in KIT. (2010) Br J Cancer 102(8):1219-23.

Harada K, Nishizaki T, Ozaki S, Kubota H, Ito H, Sasaki K (1998) Intratumoral cytogenetic heterogeneity detected by comparative genomic hybridization and laser scanning cytometry in human gliomas. Cancer Res 58:4694-4700.

Hashimoto K, Matsumura I, Tsujimura T, Kim DK, Ogihara H, Ikeda H, Ueda S, Mizuki M, Sugahara H, Shibayama H, Kitamura Y & Kanakura Y. (2003) Necessity of tyrosine 719 and phosphatidylinositol 3'-kinase-mediated signal pathway in constitutive activation and oncogenic potential of c-kit receptor tyrosine kinase with the Asp814Val mutation. Blood 101(3): 1094-1102.

Hatva E, Kaipainen A, Mentula P, Jaaskelainen J, Paetau A, Haltia M & Alitalo K. (1995) Expression of endothelial cell-specific receptor tyrosine kinases and growth factors in human brain tumors. Am J Pathol 146(2): 368-378.

Hawkins E, Heifetz SA, Giller R & Cushing B. (1997) The prepubertal testis (prenatal and postnatal): its relationship to intratubular germ cell neoplasia: a combined Pediatric Oncology Group and Children's Cancer Study Group. Hum Pathol 28(4):404-10.

Heidaran MA, Pierce JH, Jensen RA, Matsui T & Aaronson SA. (1990) Chimeric alpha- and beta-platelet-derived growth factor (PDGF) receptors define three immunoglobulin-like domains of the alpha-PDGF receptor that determine PDGF-AA binding specificity. J Biol Chem 265(31): 18741-18744.

Heinrich MC, Corless CL, Demetri GD, Blanke CD, von Mehren M, Joensuu H, McGreevey LS, Chen CJ, Van den Abbeele AD, Druker BJ, Kiese B, Eisenberg B, Roberts PJ, Singer S, Fletcher CD, Silberman S, Dimitrijevic S & Fletcher JA. (2003) Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. J Clin Oncol 21(23):4342-9.

Heinrich MC, Corless CL, Duensing A, McGreevey L, Chen CJ, Joseph N, Singer S, Griffith DJ, Haley A, Town A, Demetri GD, Fletcher CD & Fletcher JA. (2003) PDGFRA activating mutations in gastrointestinal stromal tumors. Science 299(5607): 708-710.

Heldin CH & Westermark B. (1999) Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev 79(4): 1283-1316.

Hirota S, Isozaki K, Moriyama Y, Hashimoto K, Nishida T, Ishiguro S, Kawano K, Hanada M, Kurata A, Takeda M, Muhammad Tunio G, Matsuzawa Y, Kanakura Y, Shinomura Y & Kitamura Y. (1998) Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. Science 279(5350): 577-580.

Holtkamp N, Ziegenhagen N, Malzer E, Hartmann C, Giese A & von Deimling A. (2007) Characterization of the amplicon on chromosomal segment 4q12 in glioblastoma multiforme. Neuro Oncol 9(3): 291-297.

Hornick JL & Fletcher CD. (2002) Immunohistochemical staining for KIT (CD117) in soft tissue sarcomas is very limited in distribution. Am J Clin Pathol 117(2): 188-193.

Huang HS, Nagane M, Klingbeil CK, Lin H, Nishikawa R, Ji XD, Huang CM, Gill GN, Wiley HS & Cavenee WK. (1997) The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling. J Biol Chem 272(5):2927-35.

Hubbard SR & Till JH. (2000) Protein tyrosine kinase structure and function. Annu Rev Biochem 69: 373-398.

Hurtt MR, Moossy J, Donovan-Peluso M & Locker J. (1992) Amplification of epidermal growth factor receptor gene in gliomas: histopathology and prognosis. J Neuropathol Exp Neurol 51(1):84-90.

Hui AB, Lo KW, Yin XL, Poon WS & Ng HK. (2001) Detection of multiple gene amplifications in glioblastoma multiforme using array-based comparative genomic hybridization. Lab Invest 81(5): 717-723.

Huizinga JD, Thuneberg L, Kluppel M, Malysz J, Mikkelsen HB & Bernstein A. (1995) W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. Nature 373(6512): 347-349.

Huse JT & Holland EC. (2010) Targeting brain cancer: advances in the molecular pathology of malignant glioma and medulloblastoma. Nat Rev Cancer 10(5):319-31.

Janeway KA, Liegl B, Harlow A, Le C, Perez-Atayde A, Kozakewich H, Corless CL, Heinrich MC & Fletcher JA. (2007) Pediatric KIT wild-type and platelet-derived growth factor receptor alpha-wild-type gastrointestinal stromal tumors share KIT activation but not mechanisms of genetic progression with adult gastrointestinal stromal tumors. Cancer Res 67(19): 9084-9088.

Jiang BH & Liu LZ. (2008) PI3K/PTEN signaling in tumorigenesis and angiogenesis. Biochim Biophys Acta 1784(1): 150-158.

Jin K, Mao XO, Sun Y, Xie L & Greenberg DA. (2002) Stem cell factor stimulates neurogenesis in vitro and in vivo. J Clin Invest 110(3): 311-319.

Joensuu H. (2006) Gastrointestinal stromal tumor (GIST). Ann Oncol 17 Suppl 10:x280-6.

Joensuu H, Fletcher C, Dimitrijevic S, Silberman S, Roberts P & Demetri G. (2002) Management of malignant gastrointestinal stromal tumours. Lancet Oncol 3(11): 655-664.

Jogi A, Ora I, Nilsson H, Lindeheim A, Makino Y, Poellinger L, Axelson H & Pahlman S. (2002) Hypoxia alters gene expression in human neuroblastoma cells toward an immature and neural crest-like phenotype. Proc Natl Acad Sci U S A 99(10): 7021-7026.

Jones AV & Cross NC. (2004) Oncogenic derivatives of platelet-derived growth factor receptors. Cell Mol Life Sci 61(23): 2912-2923.

Jones C, Rodriguez-Pinilla M, Lambros M, Bax D, Messahel B, Vujanic GM, Reis-Filho JS & Pritchard-Jones K. (2007) c-KIT overexpression, without gene amplification and mutation, in paediatric renal tumours. J Clin Pathol 60(11): 1226-1231.

Jones DT, Ichimura K, Liu L, Pearson DM, Plant K & Collins VP. (2006) Genomic analysis of pilocytic astrocytomas at 0.97 Mb resolution shows an increasing tendency toward chromosomal copy number change with age. J Neuropathol Exp Neurol 65(11): 1049-1058.

Jones DT, Kocialkowski S, Liu L, Pearson DM, Bäcklund LM, Ichimura K, Collins VP. (2008) Tandem duplication producing a novel oncogenic braf fusion gene defines the majority of pilocytic astrocytomas. Cancer Res. 68(21):8673-8677.

Jung V, Romeike BF, Henn W, Feiden W, Moringlane JR, Zang KD et a. (1999) Evidence of focal genetic microheterogeneity in glioblastoma multiforme by area-specific CGH on microdissected tumor cells. J Neuropathol Exp Neurol 58:993-999.

Kanakura Y, Ikeda H, Kitayama H, Sugahara H & Furitsu T. (1993) Expression, function and activation of the proto-oncogene c-kit product in human leukemia cells. Leuk Lymphoma 10(1-2): 35-41.

Kato N, Honma K, Hojo H, Sasou S, Matsuzaki O & Motoyama T. (2005) KIT expression in normal and neoplastic renal tissues: immunohistochemical and molecular genetic analysis. Pathol Int 55(8): 479-483.

Kawagishi J, Kumabe T, Yoshimoto T & Yamamoto T. (1995) Structure, organization, and transcription units of the human alpha-platelet-derived growth factor receptor gene, PDGFRA. Genomics 30(2): 224-232.

Keller G, Hartmann A, Mueller J & Hofler H. (2001) Denaturing high pressure liquid chromatography (DHPLC) for the analysis of somatic p53 mutations. Lab Invest 81(12): 1735-1737.

Kemmer K, Corless CL, Fletcher JA, McGreevey L, Haley A, Griffith D, Cummings OW, Wait C, Town A &Heinrich MC. (2004) KIT mutations are common in testicular seminomas. Am J Pathol 164(1):305-13.

Kerbel R & Folkman J. (2002) Clinical translation of angiogenesis inhibitors. Nat Rev Cancer 2(10): 727-739.

Kindblom LG, Remotti HE, Aldenborg F & Meis-Kindblom JM. (1998) Gastrointestinal pacemaker cell tumor (GIPACT): gastrointestinal stromal tumors show phenotypic characteristics of the interstitial cells of Cajal. Am J Pathol 152(5): 1259-1269.

King N & Carroll SB. (2001) A receptor tyrosine kinase from choanoflagellates: molecular insights into early animal evolution. Proc Natl Acad Sci U S A 98(26): 15032-15037.

Kleihues P, Cavenee WK. Pathology and genetics of tumours of the nervous system. Lyon: IARC Press; 2000

Kleihues P, Louis DN, Scheithauer BW, Rorke LB, Reifenberger G, Burger PC & Cavenee WK. (2002) The WHO classification of tumors of the nervous system. J Neuropathol Exp Neurol 61(3): 215-25; discussion 226-9.

Knuutila S. (2004) Cytogenetics and molecular pathology in cancer diagnostics. Ann Med 36(3): 162-171.

Kostich M, English J, Madison V, Gheyas F, Wang L, Qiu P, Greene J & Laz TM. (2002) Human members of the eukaryotic protein kinase family. Genome Biol 3(9): RESEARCH0043.

Kramar F, Zemanova Z, Michalova K, Babicka L, Ransdorfova S, Hrabal P et al (2007) Cytogenetic analyses in 81 patients with brain gliomas: correlation with clinical outcome and morphological data. Neurooncol 84:201-211.

Larouche V, Huang A, Bartels U & Bouffet E. (2007) Tumors of the central nervous system in the first year of life. Pediatr Blood Cancer 49(7 Suppl): 1074-1082.

Layfield LJ, Willmore C, Tripp S, Jones C, Jensen RL (2006) Epidermal growth factor receptor gene amplification and protein expression in glioblastoma multiforme: prognostic significance and relationship to other prognostic factors. Appl Immunohistochem Mol Morphol 14:91-96.

Lednicky JA, Garcea RL, Bergsagel DJ & Butel JS. (1995) Natural simian virus 40 strains are present in human choroid plexus and ependymoma tumors. Virology 212(2): 710-717.

Lengauer C, Kinzler KW & Vogelstein B. (1998) Genetic instabilities in human cancers. Nature 1998 396(6712):643-9.

Leung SY, Chan AS, Wong MP, Yuen ST, Cheung N & Chung LP. (1997) Expression of vascular endothelial growth factor and its receptors in pilocytic astrocytoma. Am J Surg Pathol 21(8): 941-950.

Levison SW, Chuang C, Abramson BJ & Goldman JE. (1993) The migrational patterns and developmental fates of glial precursors in the rat subventricular zone are temporally regulated. Development 119(3): 611-622.

Linnekin D. (1999) Early signaling pathways activated by c-Kit in hematopoietic cells. Int J Biochem Cell Biol 31(10): 1053-1074.

Litz J & Krystal GW. (2006) Imatinib inhibits c-Kit-induced hypoxia-inducible factor-1alpha activity and vascular endothelial growth factor expression in small cell lung cancer cells. Mol Cancer Ther 5(6): 1415-1422.

Lohela M, Bry M, Tammela T & Alitalo K. (2009) VEGFs and receptors involved in angiogenesis versus lymphangiogenesis. Curr Opin Cell Biol 21(2):154-65

Looijenga LH, de Leeuw H, van Oorschot M, van Gurp RJ, Stoop H, Gillis AJ, de Gouveia Brazao CA, Weber RF, Kirkels WJ, van Dijk T, von Lindern M, Valk P, Lajos G, Olah E, Nesland JM, Fosså SD & Oosterhuis JW. (2003) Stem cell factor receptor (c-KIT) codon 816 mutations predict development of bilateral testicular germ-cell tumors. Cancer Res 63(22):7674-8.

Lopez-Gines C, Cerda-Nicolas M, Gil-Benso R, Pellin A, Lopez-Guerrero JA, Callaghan R et al (2005) Association of chromosome 7, chromosome 10 and EGFR gene amplification in glioblastoma multiforme. Clin Neuropathol 24:209-218.

Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, Scheithauer BW & Kleihues P. (2007) The 2007 WHO Classification of Tumours of the Central Nervous System. Acta Neuropathol (Berl) 114(2): 97-109.

Maeda H, Yamagata A, Nishikawa S, Yoshinaga K, Kobayashi S, Nishi K & Nishikawa S. (1992) Requirement of c-kit for development of intestinal pacemaker system. Development 116(2): 369-375.

Maher EA, Furnari FB, Bachoo RM, Rowitch DH, Louis DN, Cavenee WK et al (2001) Malignant glioma: genetics and biology of a grave matter. Genes Dev 15:1311-1333.

Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN & Yancopoulos GD. (1997) Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 277(5322):55-60.

Mathew P, Look T, Luo X, Ashmun R, Nash M, Gajjar A, Walter A, Kun L & Heideman RL. (1996) DNA index of glial tumors in children. Correlation with tumor grade and prognosis. Cancer 78(4): 881-886.

Matsui J, Wakabayashi T, Asada M, Yoshimatsu K & Okada M. (2004) Stem cell factor/c-kit signaling promotes the survival, migration, and capillary tube formation of human umbilical vein endothelial cells. J Biol Chem 279(18): 18600-18607.

Matsui T, Heidaran M, Miki T, Popescu N, La Rochelle W, Kraus M, Pierce J & Aaronson S. (1989) Isolation of a novel receptor cDNA establishes the existence of two PDGF receptor genes. Science 243(4892): 800-804.

McClintock B. (1942) The Fusion of Broken Ends of Chromosomes Following Nuclear Fusion. Proc Natl Acad Sci U S A 28(11): 458-463.

McGuire CS, Sainani KL & Fisher PG. (2009) Both location and age predict survival in ependymoma: a SEER study. Pediatr Blood Cancer 52(1):65-9.

McIntyre A, Summersgill B, Grygalewicz B, Gillis AJ, Stoop J, van Gurp RJ, Dennis N, Fisher C, Huddart R, Cooper C, Clark J, Oosterhuis JW, Looijenga LH & Shipley J. (2005) Amplification and overexpression of the KIT gene is

associated with progression in the seminoma subtype of testicular germ cell tumors of adolescents and adults. Cancer Res 65(18): 8085-8089.

Meiniel A. (2007) The secretory ependymal cells of the subcommissural organ: which role in hydrocephalus? Int J Biochem Cell Biol 39(3):463-8.

Mellinghoff IK, Wang MY, Vivanco I, Haas-Kogan DA, Zhu S, Dia EQ et al (2005) Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. N Engl J Med 353:2012-2024.

Metzger AK, Sheffield VC, Duyk G, Daneshvar L, Edwards MS & Cogen PH. (1991) Identification of a germ-line mutation in the p53 gene in a patient with an intracranial ependymoma. Proc Natl Acad Sci U S A 88(17): 7825-7829.

Miettinen M & Lasota J. (2005) KIT (CD117): a review on expression in normal and neoplastic tissues, and mutations and their clinicopathologic correlation. Appl Immunohistochem Mol Morphol 13(3): 205-220.

Miettinen M, Sarlomo-Rikala M & Lasota J. (2000) KIT expression in angiosarcomas and fetal endothelial cells: lack of mutations of exon 11 and exon 17 of C-kit. Mod Pathol 13(5): 536-541.

Miliaras D, Karasavvidou F, Papanikolaou A & Sioutopoulou D. (2004) KIT expression in fetal, normal adult, and neoplastic renal tissues. J Clin Pathol 57(5): 463-466.

Mitsudomi T & Yatabe Y. (2010) Epidermal growth factor receptor in relation to tumor development: EGFR gene and cancer. FEBS J 277(2):301-8.

Miyanaga T, Hirato J, Nakazato Y (2008) Amplification of the epidermal growth factor receptor gene in glioblastoma: an analysis of the relationship between genotype and phenotype by CISH method. Neuropathology 28:116-126.

Moscatello DK, Holgado-Madruga M, Godwin AK, Ramirez G, Gunn G, Zoltick PW, Biegel JA, Hayes RL, Wong AJ. (1995) Frequent expression of a mutant epidermal growth factor receptor in multiple human tumors. Cancer Res 55(23):5536-9.

Muleris M, Almeida A, Dutrillaux AM, Pruchon E, Vega F, Delattre JY, Poisson M, Malfoy B & Dutrillaux B. (1994) Oncogene amplification in human gliomas: a molecular cytogenetic analysis. Oncogene 9(9): 2717-2722.

Murnane JP & Sabatier L. (2004) Chromosome rearrangements resulting from telomere dysfunction and their role in cancer. Bioessays 26(11): 1164-1174.

Myllykangas S & Knuutila S. (2006) Manifestation, mechanisms and mysteries of gene amplifications. Cancer Lett 232(1): 79-89.

Nicholson RI, Gee JM & Harper ME. (2001) EGFR and cancer prognosis. Eur J Cancer 37 Suppl 4: S9-15.

Necesalová E, Vranová V, Kuglík P, Cejpek P, Jarosová M, Pesáková M et al (2007) Incidence of the main genetic markers in glioblastoma multiforme is independent of tumor topology. Neoplasma 54:212-218.

Ohgaki H, Eibl RH, Schwab M, Reichel MB, Mariani L, Gehring M, Petersen I, Holl T, Wiestler OD & Kleihues P. (1993) Mutations of the p53 tumor suppressor gene in neoplasms of the human nervous system. Mol Carcinog 8(2): 74-80.

Ohgaki H & Kleihues P. (2005a) Epidemiology and etiology of gliomas. Acta Neuropathol 109(1): 93-108.

Ohgaki H & Kleihues P. (2005b) Population-based studies on incidence, survival rates, and genetic alterations in astrocytic and oligodendroglial gliomas. J Neuropathol Exp Neurol 64(6): 479-489.

Ohgaki H & Kleihues P. (2007) Genetic pathways to primary and secondary glioblastoma. Am J Pathol 170(5): 1445-1453.

Ohgaki H & Kleihues P. (2009) Genetic alterations and signaling pathways in the evolution of gliomas. Cancer Sci 100(12):2235-41.

Ohh M, Park CW, Ivan M, Hoffman MA, Kim TY, Huang LE, Pavletich N, Chau V & Kaelin WG. (2000) Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. Nat Cell Biol 2(7): 423-427.

Okamoto I, Kenyon LC, Emlet DR, Mori T, Sasaki J, Hirosako S, Ichikawa Y, Kishi H, Godwin AK, Yoshioka M, Suga M, Matsumoto M, Wong AJ. (2003) Expression of constitutively activated EGFRvIII in non-small cell lung cancer. Cancer Sci 94(1):50-6.

Okazaki T, Ebihara S, Asada M, Kanda A, Sasaki H & Yamaya M. (2006) Granulocyte colony-stimulating factor promotes tumor angiogenesis via increasing circulating endothelial progenitor cells and Gr1+CD11b+ cells in cancer animal models. Int Immunol 18(1): 1-9.

Olapade-Olaopa EO, Moscatello DK, MacKay EH, Horsburgh T, Sandhu DP, Terry TR, Wong AJ & Habib FK. (2000) Evidence for the differential expression of a variant EGF receptor protein in human prostate cancer. Br J Cancer 82(1):186-94.

Oliner J, Min H, Leal J, Yu D, Rao S, You E, Tang X, Kim H, Meyer S, Han SJ, Hawkins N, Rosenfeld R, Davy E, Graham K, Jacobsen F, Stevenson S, Ho J, Chen Q, Hartmann T, Michaels M, Kelley M, Li L, Sitney K, Martin F, Sun JR, Zhang N, Lu J, Estrada J, Kumar R, Coxon A, Kaufman S, Pretorius J, Scully S, Cattley R, Payton M, Coats S, Nguyen L, Desilva B, Ndifor A, Hayward I, Radinsky R, Boone T & Kendall R. (2004) Suppression of angiogenesis and tumor growth by selective inhibition of angiopoietin-2. Cancer Cell 6(5): 507-516.

Olson JD, Riedel E & DeAngelis LM. (2000a) Long-term outcome of low-grade oligodendroglioma and mixed glioma. Neurology 54(7): 1442-1448.

Oppenheim JS, Strauss RC, Mormino J, Sachdev VP & Rothman AS. (1994) Ependymomas of the third ventricle. Neurosurgery 34(2): 350-2; discussion 352-3.

Papapetropoulos A, Garcia-Cardena G, Dengler TJ, Maisonpierre PC, Yancopoulos GD & Sessa WC. (1999) Direct actions of angiopoietin-1 on human endothelium: evidence for network stabilization, cell survival, and interaction with other angiogenic growth factors. Lab Invest 79(2): 213-223.

Parikka M, Kainulainen T, Tasanen K, Bruckner-Tuderman L & Salo T. (2001) Altered expression of collagen XVII in ameloblastomas and basal cell carcinomas. J Oral Pathol Med 30(10): 589-595.

Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Siu IM, Gallia GL, Olivi A, McLendon R, Rasheed BA, Keir S, Nikolskaya T, Nikolsky Y, Busam DA, Tekleab H, Diaz LA Jr, Hartigan J, Smith DR, Strausberg RL, Marie SK, Shinjo SM, Yan H, Riggins GJ, Bigner DD, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE & Kinzler KW. (2008) An integrated genomic analysis of human glioblastoma multiforme. Science 321: 1807–12.

Partanen J, Armstrong E, Mäkelä TP, Korhonen J, Sandberg M, Renkonen R, Knuutila S, Huebner K & Alitalo K. (1992) A novel endothelial cell surface receptor tyrosine kinase with extracellular epidermal growth factor homology domains. Mol Cell Biol12 (4): 1698–707.

Paulson TG, Almasan A, Brody LL & Wahl GM. (1998) Gene amplification in a p53-deficient cell line requires cell cycle progression under conditions that generate DNA breakage. Mol Cell Biol 18(5): 3089-3100.

Pawson T & Scott JD. (2005) Protein phosphorylation in signaling--50 years and counting. Trends Biochem Sci 30(6): 286-290.

Pedersen MW, Pedersen N, Damstrup L, Villingshoj M, Sonder SU, Rieneck K, Bovin LF, Spang-Thomsen M & Poulsen HS. (2005) Analysis of the epidermal growth factor receptor specific transcriptome: effect of receptor expression level and an activating mutation. J Cell Biochem 96(2): 412-427.

Piccirillo SG & Vescovi AL. (2007) Brain tumour stem cells: possibilities of new therapeutic strategies. Expert Opin Biol Ther 7(8): 1129-1135.

Pietras K, Sjöblom T, Rubin K, Heldin CH, Ostman A. (2003) PDGF receptors as cancer drug targets. Cancer Cell 3(5):439-43.

Pipiras E, Coquelle A, Bieth A & Debatisse M. (1998) Interstitial deletions and intrachromosomal amplification initiated from a double-strand break targeted to a mammalian chromosome. EMBO J 17(1): 325-333.

Plate KH, Breier G & Risau W. (1994a) Molecular mechanisms of developmental and tumor angiogenesis. Brain Pathol 4(3): 207-218.

Plate KH, Breier G, Weich HA, Mennel HD & Risau W. (1994b) Vascular endothelial growth factor and glioma angiogenesis: coordinate induction of VEGF receptors, distribution of VEGF protein and possible in vivo regulatory mechanisms. Int J Cancer 59(4): 520-529.

Plate KH & Mennel HD. (1995) Vascular morphology and angiogenesis in glial tumors. Exp Toxicol Pathol 47(2-3): 89-94.

Plate KH & Risau W. (1995) Angiogenesis in malignant gliomas. Glia 15(3): 339-347.

Poppleton H & Gilbertson RJ. (2007) Stem cells of ependymoma. Br J Cancer 96(1): 6-10.

Pouyssegur J, Dayan F & Mazure NM. (2006) Hypoxia signalling in cancer and approaches to enforce tumour regression. Nature 441(7092): 437-443.

Purhonen S, Palm J, Rossi D, Kaskenpää N, Rajantie I, Ylä-Herttuala S, Alitalo K, Weissman IL & Salven P. (2008) Bone marrow-derived circulating endothelial precursors do not contribute to vascular endothelium and are not needed for tumor growth. Proc Natl Acad Sci U S A. 105(18):6620-5.

Przygodzki RM, Hubbs AE, Zhao FQ & O'Leary TJ. (2002) Primary mediastinal seminomas: evidence of single and multiple KIT mutations. Lab Invest 82(10):1369-75.

Rao SK, Edwards J, Joshi AD, Siu IM & Riggins GJ. (2010) A survey of glioblastoma genomic amplifications and deletions. J Neurooncol. 96(2):169-79.

Rapley EA, Hockley S, Warren W, Johnson L, Huddart R, Crockford G, Forman D, Leahy MG, Oliver DT, Tucker K, Friedlander M, Phillips KA, Hogg D, Jewett MA, Lohynska R, Daugaard G, Richard S, Heidenreich A, Geczi L, Bodrogi I, Olah E, Ormiston WJ, Daly PA, Looijenga LH, Guilford P, Aass N, Fossa SD, Heimdal K, Tjulandin SA, Liubchenko L, Stoll H, Weber W, Einhorn L, Weber BL, McMaster M, Greene MH, Bishop DT, Easton D & Stratton MR. (2004) Somatic mutations of KIT in familial testicular germ cell tumours. Br J Cancer 90(12): 2397-2401.

Reardon DA, Egorin MJ, Quinn JA, Rich JN, Gururangan S, Vredenburgh JJ, Desjardins A, Sathornsumetee S, Provenzale JM, Herndon JE,2nd, Dowell JM, Badruddoja MA, McLendon RE, Lagattuta TF, Kicielinski KP, Dresemann G, Sampson JH, Friedman AH, Salvado AJ & Friedman HS. (2005) Phase II study of imatinib mesylate plus hydroxyurea in adults with recurrent glioblastoma multiforme. J Clin Oncol 23(36): 9359-9368.

Reber L, Da Silva CA & Frossard N. (2006) Stem cell factor and its receptor c-Kit as targets for inflammatory diseases. Eur J Pharmacol 533(1-3): 327-340.

Reifenberger G & Collins VP. (2004) Pathology and molecular genetics of astrocytic gliomas. J Mol Med 82(10): 656-670.

Ren ZP, Olofsson T, Qu M, Hesselager G, Soussi T, Kalimo H et al (2007) Molecular genetic analysis of p53 intratumoral heterogeneity in human astrocytic brain tumors. J Neuropathol Exp Neurol 66:944-954.

Reni M, Gatta G, Mazza E & Vecht C. (2007) Ependymoma. Crit Rev Oncol Hematol 63(1): 81-89.

Rausch T, Jones DT, Zapatka M, Stütz AM, Zichner T, Weischenfeldt J, Jäger N, Remke M, Shih D, Northcott PA, Pfaff E, Tica J, Wang Q, Massimi L, Witt H, Bender S, Pleier S, Cin H, Hawkins C, Beck C, von Deimling A, Hans V, Brors B, Eils R, Scheurlen W, Blake J, Benes V, Kulozik AE, Witt O, Martin D, Zhang C, Porat R, Merino DM, Wasserman J, Jabado N, Fontebasso A, Bullinger L, Rücker FG, Döhner K, Döhner H, Koster J, Molenaar JJ, Versteeg R, Kool M, Tabori U, Malkin D, Korshunov A, Taylor MD, Lichter P, Pfister SM, Korbel JO. (2012) Genome Sequencing of Pediatric Medulloblastoma Links Catastrophic DNA Rearrangements with TP53 Mutations. Cell. 2012 Jan 20;148(1-2):59-71.

Rich JN, Reardon DA, Peery T, Dowell JM, Quinn JA, Penne KL, Wikstrand CJ, Van Duyn LB, Dancey JE, McLendon RE, Kao JC, Stenzel TT, Ahmed Rasheed BK, Tourt-Uhlig SE, Herndon JE,2nd, Vredenburgh JJ, Sampson JH, Friedman AH, Bigner DD & Friedman HS. (2004) Phase II trial of gefitinib in recurrent glioblastoma. J Clin Oncol 22(1): 133-142.

Riemenschneider MJ, Jeuken JW, Wesseling P, Reifenberger G. (2010) Molecular diagnostics of gliomas: state of the art. Acta Neuropathol Aug 17. [Epub ahead of print]

Rickert CH, Probst-Cousin S & Gullotta F. (1997) Primary intracranial neoplasms of infancy and early childhood. Childs Nerv Syst 13(10):507-13.

Roberts PJ & Der CJ. (2007) Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. Oncogene 26(22): 3291-3310.

Robinson DR, Wu YM & Lin SF. (2000) The protein tyrosine kinase family of the human genome. Oncogene 19(49): 5548-5557.

Rorive S, Maris C, Debeir O, Sandras F, Vidaud M, Bieche I, Salmon I & Decaestecker C. (2006) Exploring the distinctive biological characteristics of pilocytic and low-grade diffuse astrocytomas using microarray gene expression profiles. J Neuropathol Exp Neurol 65(8): 794-807.

Roskoski R,Jr. (2005a) Signaling by Kit protein-tyrosine kinase--the stem cell factor receptor. Biochem Biophys Res Commun 337(1): 1-13.

Roskoski R,Jr. (2005b) Structure and regulation of Kit protein-tyrosine kinase--the stem cell factor receptor. Biochem Biophys Res Commun 338(3): 1307-1315.

Ross JS & Fletcher JA. (1998) The HER-2/neu Oncogene in Breast Cancer: Prognostic Factor, Predictive Factor, and Target for Therapy. Oncologist 3(4): 237-252.

Ruano Y, Mollejo M, Ribalta T, Fiaño C, Camacho FI, Gómez E et al (2006) Identification of novel candidate target genes in amplicons of Glioblastoma multiforme tumors detected by expression and CGH microarray profiling. Mol Cancer 5:39.

Saharinen P, Bry M & Alitalo K. (2010) How do angiopoietins Tie in with vascular endothelial growth factors? Curr Opin Hematol 17(3):198-205.

Sakuma Y, Sakurai S, Oguni S, Hironaka M & Saito K. (2003) Alterations of the c-kit gene in testicular germ cell tumors. Cancer Sci 94(6):486-91.

Sakuma Y, Sakurai S, Oguni S, Satoh M, Hironaka M & Saito K. (2004) c-kit gene mutations in intrac-ranial germinomas. Cancer Sci 95(9):716-20.

Samoto K, Ikezaki K, Ono M, Shono T, Kohno K, Kuwano M & Fukui M. (1995) Expression of vascular endothelial growth factor and its possible relation with neovascularization in human brain tumors. Cancer Res 55(5): 1189-1193.

Sanoudou D, Tingby O, Ferguson-Smith MA, Collins VP & Coleman N. (2000) Analysis of pilocytic astrocytoma by comparative genomic hybridization. Br J Cancer 82(6): 1218-1222.

Sarlomo-Rikala M, Kovatich AJ, Barusevicius A & Miettinen M. (1998) CD117: a sensitive marker for gastrointestinal stromal tumors that is more specific than CD34. Mod Pathol 11(8): 728-734.

Sato K, Schauble B, Kleihues P & Ohgaki H. (1996) Infrequent alterations of the p15, p16, CDK4 and cyclin D1 genes in non-astrocytic human brain tumors. Int J Cancer 66(3): 305-308.

Savelyeva L & Schwab M. (2001) Amplification of oncogenes revisited: from expression profiling to clinical application. Cancer Lett 167(2): 115-123.

Schiffer D, Chio A, Giordana MT, Migheli A, Palma L, Pollo B, Soffietti R & Tribolo A. (1991) Histologic prognostic factors in ependymoma. Childs Nerv Syst 7(4): 177-182.

Schlegel J, Stumm G, Brändle K, Merdes A, Mechtersheimer G, Hynes NE & Kiessling M. (1994) Am-plification and differential expression of members of the erbB-gene family in human glioblastoma. J Neurooncol 22(3):201-7.

Schwartz S, Heinecke A, Zimmermann M, Creutzig U, Schoch C, Harbott J, Fonatsch C, Loffler H, Buchner T, Ludwig WD & Thiel E. (1999) Expression of the C-kit receptor (CD117) is a feature of almost all subtypes of de novo acute myeloblastic leukemia (AML), including cytogenetically good-risk AML, and lacks prognostic significance. Leuk Lymphoma 34(1-2): 85-94.

Semenza GL. (2003) Targeting HIF-1 for cancer therapy. Nat Rev Cancer 3(10): 721-732.

Shweiki D, Itin A, Soffer D & Keshet E. (1992) Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359(6398): 843-845.

Sihto H, Sarlomo-Rikala M, Tynninen O, Tanner M, Andersson LC, Franssila K, Nupponen NN & Joensuu H. (2005) KIT and platelet-derived growth factor receptor alpha tyrosine kinase gene mutations and KIT amplifications in human solid tumors. J Clin Oncol 23(1): 49-57.

Sihto H, Tynninen O, Butzow R, Saarialho-Kere U & Joensuu H. (2007) Endothelial cell KIT expression in human tumours. J Pathol 211(4): 481-488.

Sihto H, Tynninen O, Halonen M, Puputti M, Karjalainen-Lindsberg ML, Kukko H, Joensuu H. (2009) Tumour microvessel endothelial cell KIT and stem cell factor expression in human solid tumours. Histopathology 55(5):544-53.

Slamon DJ & Press MF. (2009) Alterations in the TOP2A and HER2 genes: association with adjuvant anthracycline sensitivity in human breast cancers. J Natl Cancer Inst 101(9):615-8.

Smeitink J. Metabolism, gliomas, and IDH1. (2010) N Engl J Med 25;362(12):1144-5

Smithey BE, Pappo AS & Hill DA. (2002) C-kit expression in pediatric solid tumors: a comparative immunohistochemical study. Am J Surg Pathol 26(4): 486-492.

Snuderl M, Fazlollahi L, Le LP, Nitta M, Zhelyazkova BH, Davidson CJ, Akhavanfard S, Cahill DP, Aldape KD, BetenskyRA, Louis DN, Iafrate AJ. (2011) Mosaic amplification of multiple receptor tyrosine kinase genes in glioblastoma. Cancer Cell. 20(6):810-7.

Sok JC, Coppelli FM, Thomas SM, Lango MN, Xi S, Hunt JL, Freilino ML, Graner MW, Wikstrand CJ, Bigner DD, Gooding WE, Furnari FB & Grandis JR. (2006) Mutant epidermal growth factor receptor (EGFRvIII) contributes to head and neck cancer growth and resistance to EGFR targeting. Clin Cancer Res 12(17):5064-73.

Speicher MR & Carter NP. (2005) The new cytogenetics: blurring the boundaries with molecular biology. Nat Rev Genet 6(10): 782-792.

Steiner HH, Karcher S, Mueller MM, Nalbantis E, Kunze S & Herold-Mende C. (2004) Autocrine pathways of the vascular endothelial growth factor (VEGF) in glioblastoma multiforme: clinical relevance of radiation-induced increase of VEGF levels. J Neurooncol 66(1-2): 129-138.

Stephens PJ, McBride DJ, Lin ML, Varela I, Pleasance ED, Simpson JT, Stebbings LA, Leroy C, Edkins S, Mudie LJ, Greenman CD, Jia M, Latimer C, Teague JW, Lau KW, Burton J, Quail MA, Swerdlow H, Churcher C, Natrajan R, Sieuwerts AM, Martens JW, Silver DP, Langerød A, Russnes HE, Foekens JA, Reis-Filho JS, van 't Veer L, Richardson AL, Børresen-Dale AL, Campbell PJ, Futreal PA & Stratton MR. (2009) Complex landscapes of somatic rearrangement in human breast cancer genomes. Nature 462(7276):1005-10.

Sun L, Hui AM, Su Q, Vortmeyer A, Kotliarov Y, Pastorino S, Passaniti A, Menon J, Walling J, Bailey R, Rosenblum M, Mikkelsen T & Fine HA. (2006) Neuronal and glioma-derived stem cell factor induces angiogenesis within the brain. Cancer Cell 9(4): 287-300.

Surawicz TS, Davis F, Freels S, Laws ER Jr & Menck HR. (1998) Brain tumor survival: results from the National Cancer Data Base. J Neurooncol 40(2):151-60.

Suzuki S, Dobashi Y, Sakurai H, Nishikawa K, Hanawa M & Ooi A. (2005) Protein overexpression and gene amplification of epidermal growth factor receptor in nonsmall cell lung carcinomas. An immunohistochemical and fluorescence in situ hybridization study. Cancer 103(6): 1265-1273.

Suzuki SO & Iwaki T. (2000) Amplification and overexpression of mdm2 gene in ependymomas. Mod Pathol 13(5): 548-553.

Tabone S, Theou N, Wozniak A, Saffroy R, Deville L, Julie C, Callard P, Lavergne-Slove A, Debiec-Rychter M, Lemoine A & Emile JF. (2005) KIT overexpression and amplification in gastrointestinal stromal tumors (GISTs). Biochim Biophys Acta 1741(1-2): 165-172.

Takano S, Yoshii Y, Kondo S, Suzuki H, Maruno T, Shirai S & Nose T. (1996) Concentration of vascular endothelial growth factor in the serum and tumor tissue of brain tumor patients. Cancer Res 56(9): 2185-2190.

Takeuchi H, Kubota T, Sato K & Arishima H. (2004) Ultrastructure of capillary endothelium in pilocytic astrocytomas. Brain Tumor Pathol 21(1): 23-26.

Taylor MD, Poppleton H, Fuller C, Su X, Liu Y, Jensen P, Magdaleno S, Dalton J, Calabrese C, Board J, Macdonald T, Rutka J, Guha A, Gajjar A, Curran T & Gilbertson RJ. (2005) Radial glia cells are candidate stem cells of ependymoma. Cancer Cell 8(4):323-35.

Terada T. (2010) Low incidence of KIT gene mutations and no PDGFRA gene mutations in primary cutaneous melanoma: an immunohistochemical and molecular genetic study of Japanese cases. Int J Clin Oncol Apr 28. [Epub ahead of print].

Tian Q, Frierson HF Jr, Krystal GW & Moskaluk CA. (1999) Activating c-kit gene mutations in human germ cell tumors. Am J Pathol 154(6):1643-7.

Tomlinson FH, Scheithauer BW, Hayostek CJ, Parisi JE, Meyer FB, Shaw EG, Weiland TL, Katzmann JA & Jack CR,Jr. (1994a) The significance of atypia and histologic malignancy in pilocytic astrocytoma of the cerebellum: a clinicopathologic and flow cytometric study. J Child Neurol 9(3): 301-310.

Torihashi S, Nishi K, Tokutomi Y, Nishi T, Ward S & Sanders KM. (1999) Blockade of kit signaling induces transdifferentiation of interstitial cells of cajal to a smooth muscle phenotype. Gastroenterology 117(1): 140-148.

Tsuura Y, Hiraki H, Watanabe K, Igarashi S, Shimamura K, Fukuda T, Suzuki T & Seito T. (1994) Preferential localization of c-kit product in tissue mast cells, basal cells of skin, epithelial cells of breast, small cell lung carcinoma and seminoma/dysgerminoma in human: immunohistochemical study on formalin-fixed, paraffin-embedded tissues. Virchows Arch 424(2): 135-141.

Uhm JH, Ballman KV, Wu W, Giannini C, Krauss JC, Buckner JC et al (2010) Phase II evaluation of gefitinib in patients with newly diagnosed grade 4 astrocytoma: Mayo/North Central Cancer Treatment Group study N0074. Int J Radiat Oncol Biol Phys May 24 [Epub ahead of print].

Ueda S, Mizuki M, Ikeda H, Tsujimura T, Matsumura I, Nakano K, Daino H, Honda Zi Z, Sonoyama J, Shibayama H, Sugahara H, Machii T & Kanakura Y. (2002) Critical roles of c-Kit tyrosine residues 567 and 719 in stem cell factor-induced chemotaxis: contribution of src family kinase and PI3-kinase on calcium mobilization and cell migration. Blood 99(9): 3342-3349.

Valverde LR, Matutes E, Farahat N, Heffernan A, Owusu-Ankomah K, Morilla R & Catovsky D. (1996) C-kit receptor (CD117) expression in acute leukemia. Ann Hematol 72(1): 11-15.

Veikkola T, Karkkainen M, Claesson-Welsh L & Alitalo K. (2000) Regulation of angiogenesis via vascular endothelial growth factor receptors. Cancer Res 60(2): 203-212.

Venneri MA, De Palma M, Ponzoni M, Pucci F, Scielzo C, Zonari E, Mazzieri R, Doglioni C & Naldini L. (2007) Identification of proangiogenic TIE2-expressing monocytes (TEMs) in human peripheral blood and cancer. Blood 109(12): 5276-5285.

Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, Miller CR, Ding L, Golub T, Mesirov JP, Alexe G, Lawrence M, O'Kelly M, Tamayo P, Weir BA, Gabriel S, Winckler W, Gupta S, Jakkula L, Feiler HS, Hodgson JG, James CD, Sarkaria JN, Brennan C, Kahn A, Spellman PT, Wilson RK, Speed TP, Gray JW, Meyerson M, Getz G, Perou CM & Hayes DN; Cancer Genome Atlas Research Network.(2010) Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. Cancer Cell 17(1):98-110.

Vescovi AL, Galli R & Reynolds BA. (2006) Brain tumour stem cells. Nat Rev Cancer 6(6): 425-436.

Vogt N, Lefevre SH, Apiou F, Dutrillaux AM, Cor A, Leuraud P, Poupon MF, Dutrillaux B, Debatisse M & Malfoy B. (2004) Molecular structure of double-minute chromosomes bearing amplified copies of the epidermal growth factor receptor gene in gliomas. Proc Natl Acad Sci U S A 101(31): 11368-11373.

von Deimling A, Louis DN, Schramm J & Wiestler OD. (1994) Astrocytic gliomas: characterization on a molecular genetic basis. Recent Results Cancer Res 135: 33-42.

Wahl GM, Robert de Saint Vincent,B. & DeRose ML. (1984) Effect of chromosomal position on amplification of transfected genes in animal cells. Nature 307(5951): 516-520.

Waskow C, Terszowski G, Costa C, Gassmann M & Rodewald HR. (2004) Rescue of lethal c-KitW/W mice by erythropoietin. Blood 104(6):1688-.

Watanabe T, Nobusawa S, Kleihues P & Ohgaki H. (2009) IDH1 Mutations are early events in the development of astrocytomas and oligodendrogliomas. Am J Pathol 174: 1149–53.

Wechsler-Reya R & Scott MP. (2001) The developmental biology of brain tumors. Annu Rev Neurosci 24: 385-428.

Wemmert S, Romeike BF, Ketter R, Steudel WI, Zang KD & Urbschat S. (2006) Intratumoral genetic heterogeneity in pilocytic astrocytomas revealed by CGH-analysis of microdissected tumor cells and FISH on tumor tissue sections. Int J Oncol 28(2): 353-360.

Wen PY & Kesari S. (2008) Malignant gliomas in adults. N Engl J Med 359(5):492-507.

Wong AJ, Ruppert JM, Bigner SH, Grzeschik CH, Humphrey PA, Bigner DS & Vogelstein B. (1992) Structural alterations of the epidermal growth factor receptor gene in human gliomas. Proc Natl Acad Sci U S A 89(7): 2965-2969.

Wong ML, Kaye AH & Hovens CM. (2007) Targeting malignant glioma survival signalling to improve clinical outcomes. J Clin Neurosci 14(4): 301-308.

Xiao W & Oefner PJ. (2001) Denaturing high-performance liquid chromatography: A review. Hum Mutat 17(6): 439-474.

Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA, Yuan W, Kos I, Batinic-Haberle I, Jones S, Riggins GJ, Friedman H, Friedman A, Reardon D, Herndon J, Kinzler KW, Velculescu VE, Vogelstein B & Bigner DD. (2009) IDH1 and IDH2 mutations in gliomas. N Engl J Med 360: 765–73.

Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ & Holash J. (2000) Vascular-specific growth factors and blood vessel formation. Nature 407(6801): 242-248.

Yock TI, Yeap BY, Ebb D, MacDonald SM, Pulsifer MB, Marcus KC, Tarbell N. (2010) A phase II trial of proton radiotherapy for medulloblastoma: preliminary results. J Clin Oncol 28 (18S):960s.

Zandi R, Larsen AB, Andersen P, Stockhausen MT & Poulsen HS. (2007) Mechanisms for oncogenic activation of the epidermal growth factor receptor. Cell Signal 19(10): 2013-2023.

Zhao S, Lin Y, Xu W, Jiang W, Zha Z, Wang P, Yu W, Li Z, Gong L, Peng Y, Ding J, Lei Q, Guan KL & Xiong Y. (2009) Glioma-derived mutations in IDH1 dominantly inhibit IDH1 catalytic activity and induce HIF-1alpha. Science 324: 261–5.

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