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Stem Cell Markers in Cancer: Do They Have Clinical Significance?

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ACADEMIC DISSERTATION

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"They can always hurt you more."
From the House of God, by Samuel Shem.

CONTENTS

ABSTRACT

Background: In cancer, a subpopulation of malignant cells expresses markers of normal stem cells. These cells have the potential of initiating tumor growth and therefore also tumor recurrence. Thus, these cells are called cancer stem cells. A myriad of markers have been applied to identify these cells, but no single marker can be found exclusively in cancer stem cells. In many types of cancer, clinical recurrence and tumor progression are the main causes of mortality, despite intense oncological treatment. It has been proposed that the presence of cancer stem cells causes this resistance to therapy.

Aims: The scope of this thesis is to investigate the role of stem cell markers and genes in the clinical setting. Especially, the aim was to elucidate the clinical significance of stem cell markers as novel prognostic and diagnostic tools in cancer.

Methods: Tumor biopsy material from central nervous system tumors (oligodendroglioma, astrocytoma and glioblatoma), neural crest derived tumors (pheochromocytomas) and oral carcinoma was screened for stem cell markers. Initially, 12 stem cell markers were considered for screening in a test series of gliomas. The markers subsequently applied for expanded tumor analyses (in 305 cases of glioma, 42 cases of pheochromocytoma, and 73 cases of oral carcinoma) were BMI-1, Snail, p16, mdm2, and c-Myc. Data on marker expression was compared with clinical and pathological parameters.

Results: In gliomas, BMI-1 expression was found in nearly all tumors analyzed, but the frequency of BMI-1 expressing cells was highly variable, ranging from 1 to 100%. In oligodendroglioma, BMI-1 expression was identified as a prognostic marker independent of tumor grade and clinical parameters. In pheochromocytoma, Snail expression was shown to distinguish between the metastatic and non-metastatic forms of the tumor. Snail expression was seen only in metastatic tumors, whereas non-metastatic tumors did not commonly express Snail. Finally, in oral squamous cell carcinoma, BMI-1 expression was seen in roughly 80% of tumors, and Snail expression was high or very high in all cases. The lack of BMI-1 expression was associated with early relapse in oral squamous cell carcinoma.

Conclusions: The analysis of stem cell markers can be used as an aid in clinical diagnostics to provide prognostic information in oligodendrogliomas and pheochromocytomas. However, in other types of tumors, such as oral carcinoma, the role of these markers is less clear, because lack of stem cell markers was associated with an aggressive behavior of the tumors. Therefore, the cancer stem cell hypothesis is not clinically relevant in every type of cancer. However, in specific forms of tumors the analysis of stem cell markers is recommendable.

ORIGINAL PUBLICATIONS

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II. Valtteri Häyry, Minna Tanner, Tea Blom, Olli Tynninen, Annariikka Roselli, Miina Ollikainen, Hannu Sariola, Kirmo Wartiovaara and Nina N. Nupponen. Amplification and deletion of the polycomb gene BMI1 in gliomas. **Acta Neuropathologica** *2008;116:97-102*

III. Valtteri Häyry, Kaisa Salmenkivi, Johanna Arola, Päivi Heikkilä, Caj Haglund and Hannu Sariola. High expression of the neural-crest gene Snail confirms and predicts the metastatic potential of pheochromocytoma. **Endocrine-Related Cancer** *2009;16:1211-8.*

IV. Valtteri Häyry, Laura Mäkinen, Timo Atula, Hannu Sariola, Antti Mäkitie, Ilmo Leivo, Harri Keski-Säntti, Johan Lundin, Caj Haglund and Jaana Hagström. BMI-1 expression predicts prognosis in squamous cell carcinoma of the tongue. **British Journal of Cancer***, in press.*

ABBREVIATIONS

BAC	bacterial artificial chromosome
BMI-1	B-lymphoma Moloney murine leukemia virus insertion
CISH	chromogenic in situ hybridization
C-myc	myelocytomatosis viral oncogene homolog
CNS	central nervous system
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
EMT	epithelial mesenchyme transition
ezh2	enhancer of zeste homolog 2
GBM	glioblastoma multiforme
GFAP	glial fibrillary acidic protein
HE	hematoxylin-eosin
Her2	human epidermal growth factor receptor 2
HPV	human papilloma virus
HRP	horseradish perodidase
IHC	immunohistochemistry
ki-67	antigen identified by monoclonal antibody Ki-67
mdm2	murine double minute oncogene
MiB	mitotic index
NOD/SCID	nonobese diabetic, severe combined immunodeficiency
Oct4	octamer-4
OSCC	oral squamous cell carcinoma, oral (tongue) cancer
p16	Cyclin-dependent kinase inhibitor 2A
p53	tumor protein p53
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PNS	peripheral nervous system
PTEN	phosphatase and tensin homolog
Rb	retinoblastoma protein
Sox2	sex determining region Y box 2
WHO	World Health Organization
Wnt	Wingless and Int

INTRODUCTION

Virchow hypothesized cancer to be caused by long-lasting irritation and exposure to external noxious agents (Virchow, 1858). Thus, malignancies would develop after years of irritation and inflammation. This was of course not a plausible explanation in childhood neoplastic diseases, e.g. Wilms' tumor or neuroblastoma. It was therefore proposed, that an embryological remnant, a "seed" was left dormant for a time in the child and it then manifested in the organ as a malignant tumor (Durante 1874; Cohnheim 1885).

During the recent 10 years, new research from altogether different grounds has led us astonishingly close to these ancient theories. Several groups have convincingly shown that most malignant tumors are indeed not cellularly homogenous, but are in fact made up of quite distinctly different cell populations, suggesting that a primitive cell type is capable to differentiate into varied cell types within their original tissue type (Bonnet and Dick 1997; Al-Hajj *et al.* 2003; Hemmati *et al.* 2003; Singh *et al.* 2004). Most importantly, a fraction of the malignant cells in a tumor express the same markers and display similar genetic profiles as embryonic stem cells that are not found in differentiated cells of a healthy adult (Monk and Holding 2001; Ezeh *et al.* 2005).

In brain tumors and pheochromocytoma, no convincing external agents, such as ionizing radiation or carcinogens, have been identified as a cause of the disease (Ohgaki 2009). These malignancies, all originating from the neuroectoderm, provide a beneficial means to investigate stem-cell-related factors in tumor types without known exogenous carcinogenic etiology. In oral squamous cell carcinoma, several external causes have been identified as risk factors (e.g. HPV, tobacco, alcohol). This provides a chance to investigate the relevance of stem-cell-related factors in a life-style-associated carcinoma.

In several forms of cancer, treatment of metastatic disease is quite unsuccessful despite aggressive radio- and chemotherapy. Also, relapse after treatment occurs frequently and management of recurrent disease is often very challenging. These phenomena have been lately attributed to the presence of stem cell like cells in cancer, which reside quiescent in the body, are resistant to cancer treatments and may provide the source of tumor relapse (Zhou *et al.* 2009).

In this thesis, clinical tumor material is used, and the aim is to elucidate the clinical relevance of stem cell like cells in cancer. The presence of stem cell markers in several forms of cancer is investigated. Currently, information based mostly on cell and animal models of cancer stem cells is available, and therefore, these findings should be applied in the clinical settings to clarify the significance of the major theoretical advance in cancer biology made during the past decade.

REVIEW OF THE LITERATURE

1. Cancer stem cell hypothesis

1.1 Theoretical background

During the 21st century, the cancer stem cell hypothesis has gained considerable support in the form of well-performed in vitro cell culture studies, in vivo animal experiments and even to some extent clinical studies (Rosen and Jordan 2009). The hypothesis is composed of two arguments: First, within tumors exist distinct subpopulations of cells that share qualities with normal tissue stem cells (Jordan *et al.* 2006). These characteristics are on one hand the expression of typical stem cell proteins (i.e. stem cell markers) and on the other hand the activity of stem cell genes regulating self-renewal and pluri/multipotency and differentiation. These aberrant stem cell -like cancer cells show a capacity to self renew indefinitely and produce, in this case aberrant progeny, which in turn leads to tumor heterogeneiety.

The second part of the hypothesis concerns the origin of malignancy. Previously it was believed that the accumulation of oncogenic mutations in any cell will eventually lead to malignancy. According to the cancer stem cell hypothesis, these mutations must specifically accumulate in the tissue stem cells that produce new differentiated cells in physiological turnover and tissue repair. Mutations in tissue stem cells eventually lead to malignant growth and invasion.

Third, cancer stem cells are quiescent cells resistant to cytostatic drugs or irradiation and therefore are causing relapse of the tumor after primary therapy (Tanei *et al.* 2009; Vlashi *et al.* 2009). This kind of resistance to cytotoxic treatment displayed by the cancer stem cell phenotype is actually analogous to the function of normal tissue stem cells which can repopulate and regenerate organs damaged by said therapeutic regimes during cancer treatment.

Even though advances in stem cell biology and experimental metastatic models have lent strong experimental support of the hypothesis, it is by no means a proven concept and not even a new one as described above. Indeed, the old propositions by Virchow are surprisingly valid today.

The most intriguing question is why some cancers seem to develop without any clear external influence, i.e. carcinogens. Especially, the brain is very well protected through the blood-brain barrier, meninges and skull. Still, malignant brain tumors arise in both children and adults. Also, the occurrence of malignant tumors in infants is obviously due to some intrinsic mechanism, such as familial or acquired mutation of genes that affect cell differentiation and growth. In the case of many, if not all childhood malignancies, the concept of "embryological remnants" seems plausible. This concept of malignancy arising during embryonic development is described in the "Knudson two hit model" applied in pediatric solid tumors. Working from the incidence rates of retinoblastoma, comparing bilateral and unilateral forms, Knudson calculated that hereditary cases of retinoblastoma require two mutations. While one of these mutations essentially is in the germ-line the other mutation should be somatic. This statistical model could further be applied to non-hereditary forms of retinoblastoma and also in these cases, with no germ-line mutation, the distribution of cases strongly suggested that two mutations were required. Because retinoblastoma presents often very early in childhood, Knudson hypothesized that the tumour develops from fetal retinoblasts that fail to differentiate into postmitotic photoreceptor cells or

neurons, and continued division of the blasts predisposes to malignant transformation. (Knudson 2001). Also in acute lymphoblastic leukaemia (ALL) the pediatric form has been quite convincingly shown to arise *in utero* by several experimental approaches. First, neonatal blood samples available from patients whom later in childhood developed ALL could be shown to contain (pre) leukaemic cells harboring the same fusion genes as found later in the clinically fulminant disease, which however developed with at least one year's latency (Gale *et al.* 1997). Second, concordant leukaemia cases in twins present a clonal phenotype, which necessitates *in utero* spread of the disease from one foetus to another, presumably via the placental circulation. Also in twins with leukaemia, a considerable latency could be observed from birth to disease; evidence that also postnatal events are required to trigger disease (Greaves and Wiemels 2003). One could therefore reason that malignancies in childhood may be caused by mutations in genes of tissue or embryonic stem cells. Also in adults, cancer sometimes seems to present itself with high number of tissue stem-like cells. This has been suggested in the case of Hiroshima and Nagasaki survivors, where women exposed to irradiation in their adolescence developed breast cancer after a delay of 20-30 years, but these tumors displayed mutations typical of irradiation exposure. Therefore, a population of cells bearing these mutations must have survived the interval of several decades (Little and Boice 1999).

1.2 Experimental evidence

Xenografting human tumor cells into laboratory animals has become a golden standard for studying cancer in vivo. This method has been used for a large variety of studies from grafting glioma cells intracranially to transplanting breast carcinoma into mammary fat (Al Hajj *et al.* 2003; Singh *et al.* 2004). Initially, it was observed that in order to produce a tumor xenograft, it was usually necessary to transplant a substantial amount of cancer cells, often in the range of hundreds of thousands of cells. This of course was not consistent with the general concept of aggressive behavior of malignant tumors, and was explained by immunological mechanisms, hostile host environment or innate immunity via the natural killer cells (Kelly *et al.* 2007).

In the meantime, it was shown in stem cell research, how nearly one single stem cell was capable of repopulating a depleted bone marrow; a striking witness to the huge regenerative capacity of stem cells (Bhatia *et al.* 1997). Researchers found that also tumors have a subpopulation of cells bearing the same stem cell epitopes. These cells showed in vitro a very strong growth and self-renewal capacity, in contrast to the other cells in the same tumor. Finally, several research groups experimented with the xenograft tumor model by sorting tumor cells according to the presence or absence of stem cell marker prior to xenografting. It very quickly became clear that only the tumor cells with stem cell characteristics could form a tumor when grafted, often at a very high efficiency, whereas even thousand fold numbers of non-stem cell-like tumor cells could not (table 1).

The above findings strongly suggest that there is a stem-cell-like population in malignant tumors that is responsible for tumor formation and metastasis. I first asked, if it would be possible to detect stem cell gene products in routine clinical tumor biopsies. Second, I wanted to test if the expression of these markers would have any clinical impact on the course of the disease.

Table 1: Xenograft experiments performed to demonstrate cancer stem cells.*

Cancer stem cells grafted in a NOD/SCID model

		with the marker	without the marker	
clinical diagnosis	**marker**	**tumor formation**	**no tumor**	**reference**
leukemia	CD34+/CD38-	5 000	500 000	Bonnet and Dick 1997
breast cancer	CD44+/CD24low	200	20 000	Al-Hajj et al. 2003
glioma	CD133+	100	100 000	Singh et al. 2004
prostate cancer	CD44+	500	500 000	Wicha et al. 2006

**In several types of cancer, malignant cells were grouped according to the expression of stem cell markers and xenografted into a NOD/SCID mice. The numbers show the number of cells needed to form a tumor and subsequently the number of cells lacking the marker used that did not form a tumor.*

2. Genes and markers in cancer stem cells

2.1 BMI-1

The BMI-1 gene was discovered in 1991 when c-Myc overexpressing mice were found to develop B-cell lymphomas after infection by the Moloney murine leukemia virus. The virus had been inserted into a locus most often named B-lymphoma Mo-MLV insertion region, or BMI-1. Subsequently, this new gene was identified as a developmentally highly conserved region encoding a zinc-finger protein with a nuclear localization, suggesting function as a transcription factor (Goebl 1991; Haupt *et al.* 1991; van Lohuizen *et al.* 1991). From *Drosophila*, a homologue to BMI-1 was identified as Posterior Sex Combs, a gene belonging to the polycomb groups of genes, which are transcription factors, for instance maintaining homeotic gene expression during development (Brunk *et al.* 1991; Martin and Adler 1993). Besides promoting B-cell lymphomas, BMI-1 also induces T-cell lymphomas, both through interactions with c-Myc (Haupt *et al.* 1993; Levy *et al.* 1993). In human, the BMI1 gene is located on the short arm of chromosome 10 (10p13).

As a part of the polycomb complex, BMI-1 has important functions in stem cells, in cellular differentiation and during development (Bunker and Kingston 1994). This is highlighted by the developmental defects seen in BMI-1-deficient mice, where posterior transformation along the anterioposterior axis, brain defects, and perturbed hematopoiesis are seen (van der Lugt *et al.* 1994). In neurogenesis, BMI-1 is vital for the proliferation of cerebellar granule cells and their precursors during development. Also, if BMI-1 is overexpressed in these cells, medulloblastomas, i.e. tumors predominantly found in the cerebellum, will form in mice. In analogy, high expression levels of BMI-1 have been found in human medulloblastoma (Leung *et al.* 2004). In addition, bone development is perturbed in BMI-1-null mice, and the animals display growth retardation, small bones and a decreased amount of osteoblasts possibly due to increased apoptosis and decreased proliferation of these cells, along with a shift from bone towards adiposytic differentiation (Zhang *et al.* 2009).

In cancer and tissue stem cells, BMI-1 has a central function (Lessard and Savegeau 2003). It is essential for the self-renewal of hematopoietic stem cells, explaining the hematological defects in BMI1-null mouse (Park *et al.* 2003). On the other hand, increased expression of BMI-1 in hematopoietic stem cells leads to a shift towards multipotent progenitors and also boosts stem cell self-renewal and bone marrow repopulating capacity (Iwama *et al.* 2004). Also, it is required for the self-renewal of normal neural stem cells, although it does not affect maturation or survival of progenitor cells of the nervous system (Molofsky 2003). BMI-1 can be targeted for instance by microRNA leading to a considerable decrease of the self-renewal of malignant glioma cells, suggesting therapeutical applications in the treatment of this disease (Godlewski *et al.* 2008). Malignant neuroblastoma cells also require BMI-1 for self-renewal (Cui *et al.* 2006) and targeted knock-down of BMI-1 has been shown to reduce the tumorigenic capacity of these cells in a xenograft model. Importantly, BMI-1 influences n-Myc, an oncogene crucial in determining the prognosis of neuroblastoma (Cui *et al.* 2007). BMI-1 can also immortalize normal, non-malignant mammary and nasopharyngeal epithelial stem cells, bypassing senescence and activating telomerase (Dimri *et al.* 2002; Song *et al.* 2006).

In clinicopathological studies, BMI-1 overexpression is seen in several different types of malignancies. These include colorectal cancer (Kim *et al.* 2004), metastatic melanoma (Mihic-Probst *et al.* 2007), ovarian cancer (Zhang et al. 2008), and bladder cancer, where BMI-1 expression is also predictive of poor outcome (Qin *et al.* 2009). BMI-1 gene amplifications are found in mantle cell lymphoma and BMI-1 overexpression or amplification can be linked

to disease progression or outcome (Bea *et al.* 2001). In ductal breast carcinoma, BMI-1 overexpression is linked with lymph node metastasis (Kim *et al.* 2004). Nasopharyngeal carcinoma progression is also predicted by BMI-1 overexpression, in roughly one third of cases (Song *et al.* 2006). Similarly, BMI-1 is linked to the progression of oral carcinogenesis, and expression is seen already in early, precancerous in situ lesions (Kang 2007).

BMI-1 seems to have a key function in hematopoiesis and hematological malignancies. Myelodysplastic syndrome is a group of hematological differentiation disorders characterized by unsuccessful differentiation and maturation of the myelopoietic lineage. The disease often eventually progresses into acute myelogenous leukaemia (AML). It has been proposed that epigenetic mechanisms underlie the failure of blasts to differentiate, and concordant with BMI-1's central role in maintenance of hematopoietic stem cells, BMI-1 expression is a marker for poor prognosis as it predicts rapid disease progression into AML (Mihara *et al.* 2006). In this context, AML can be seen as an immature form with increased stem cell characteristics as compared to myelodysplastic syndrome, which represents a state of perturbed differentiation, but with less immature cells. The capacity of BMI-1 to increase hematopietic stem cell self renewal (as referred to above) suggests that it has a role in maintaining the stem cell state, and this increased expression will inhibit differentiation. This observation is concordant with the increased BMI-1 expression seen in M0 stage AML, which could be described as the most primitive form of AML, void of differentiation (Sawa *et al.* 2005).

2.2 Sox2, Oct4 and Nanog

In their landmark experiments from 2006, Takahashi and Yamanaka went through 24 genes known to be highly expressed in embryonal stem (ES) cells and, using retroviruses, introduced different combinations of these genes into fibroblasts. Narrowing down to four genes, they could produce ES cell-like cells by using the genes Oct4, Sox2, Klf4 and c-Myc. Moreover, fibroblasts transduced with these four genes produced embryoid bodies and, in xenotransplants in nude mice, teratomas. The teratomas showed differentiation into all three embryonic germ layers (Takahashi and Yamanaka 2006). The cells thus created were named induced pluripotent cells. Later, these results were recapitulated by several groups. Human fibroblasts require Oct4, Sox2, Nanog, and LIN28 to regain pluripotency, whereas human neural stem cells can be induced using only Oct4 and Klf4 (Yu *et al.* 2007; Hester *et al.* 2009). However, concerns have been raised on the predisposition of induced pluripotent cells to become malignant, discouraging applications in regenerative therapies (Duinsberg *et al.* 2009). Put together, Oct4 and Sox2 seem to be crucial regulators of the ES cell phenotype, and may be central in maintaining the cancer stem cell phenotype as well.

2.3 Snail

The Snail protein belongs to a group of zinc-finger transcription factors that also includes Slug (Snail2) and Snail3. They are highly conserved during evolution because a multitude of signaling pathways converge on these transcription factors during epithelial-mesenchymal transition, a key element of developmental morphogenesis (Huber *et al.* 2005). As a transcription factor, Snail is active in the cell nucleus and phosphorylated Snail is transported to and from the cytoplasm (Dominguez *et al.* 2003). Cytosolic Snail is rapidly phosphorylated and degraded, suggesting that the analysis of Snail mRNA levels and cytoplasmic Snail expression does not accurately reflect the level of Snail activity in tumor cells (see below; clinical correlations of Snail). An important inhibitor of Snail phoshorylation is Wnt, an extracellularly secreted morphogen promoting neural crest induction (Ko *et al.* 2007; Steventon *et al.* 2009). Snail exerts its action by binding to specific E-box motifs in promoter regions of the E-cadherin, thus inhibiting transcription. Simultanously, occludin expression is

downregulated, leading to the disruption of tight junctions (Kuriama and Mayor 2008). Importantly, Snail also makes cells resistant to pro-apoptotic signals and less dependent of external signaling molecules, thus enabling cells to migrate within the tissue regardless of signals from the microenvironment (Vega *et al.* 2004).

When the neural crest delaminates from the neural tube, its cells gain the properties to migrate and proliferate (Le Douarin *et al.* 1994). The transcription factor Snail is one of the proteins that make the specific molecular signature for developing neural crest cells. Snail regulates epithelial-mesenchymal transition (EMT). Three factors are essential for delamination and migration of the neural crest during development. Snail enables EMT in neuroectodermal cells, but also Oct 9 is required in order to prevent apoptosis of the newly formed neural crest cells. Furthermore, for successful migration, FoxD3 is needed to enable the expression of cell adhesion molecules (Cheung *et al.* 2005). Thus, the neuroectodermal cells making up the neural crest gain a fibroblast-like phenotype with migration capacity. Cell-to-cell junctions, characteristic of epithelium (i.e. tight junctions), are down-regulated. Instead, gap-junctions are promoted. Also, the whole cytoskeleton transforms to a migratory, spindle-like form (Kuriama and Mayor 2008). These developmental phenomena are recapitulated in a multitude of types of cancer (Klymkowsky and Savagner 2009).

Kulesa et al. (2006) have demonstrated the strong influence exerted by the neural crest microenvironment. They used metastatic melanoma cells, based on the fact that melanocytes are of neural crest origin. These malignant cells were transplanted *in ovo* into close proximity of the migrating neural crest. It is worth mentioning that the live chick embryo is a classical experimental model used to study the neural crest (Le Douarin 2004). The transplanted melanoma cells did not form tumors, somewhat surprisingly, in view of the highly malignant nature of metastatic melanoma. Instead, these cells transformed to neural crest cells, migrating along with the normal neural crest into target organs. Furthermore, the transplanted cells underwent transdifferentiation to some extent, incorporating into the branchial arches, dorsal root ganglia and sympathetic ganglia as confirmed by Tuj1 staining (Kulesa *et al.* 2006). These results highlight the peculiar nature of the neural crest microenvironment, and confirm the strong biological similarity between malignant neural cells and migratory neural crest cells.

Snail expression is elevated in several malignancies of epithelial origin, e.g. oral squamous cell carcinoma and breast carcinoma (Blanco *et al.* 2002; Usami *et al.* 2008). In ovarian cancer, high Snail expression correlates with poor prognosis and importantly, its expression is seen in metastases as well as primary tumors, supporting the idea that Snail is involved in metastasis (Blechschmidt *et al.* 2009). In head and neck squamous cell carcinoma, high Snail expression is associated with both cervical and distant metastases (Yang *et al.* 2007). In breast cancer, the impact of Snail expression on prognosis is not straightforward. In clinical breast cancer biopsies, staining for the inactive form of GSK3 (normally inhibited by Wnt) correlates with cytoplasmic Snail expression but also reduced E-cadherin expression (Zhou *et al.* 2004). Also in breast cancer, Snail mRNA levels were significantly lower in patients with a poor outcome, although an association between mRNA levels and immunohistochemically detected Snail could not be demonstrated (Martin *et al.* 2005).

Because Snail is a key regulator of neural crest development, and chromaffin cells of the adrenal medulla are of neural crest origin, I hypothesized that the embryological mechanism of Snail, which provides chromaffin cells the capacity to migrate, might be re-activated in metastatic pheochromocytoma.

3. Malignant tumors with an assumed role of cancer stem cells

3.1 Tumors of the central nervous system

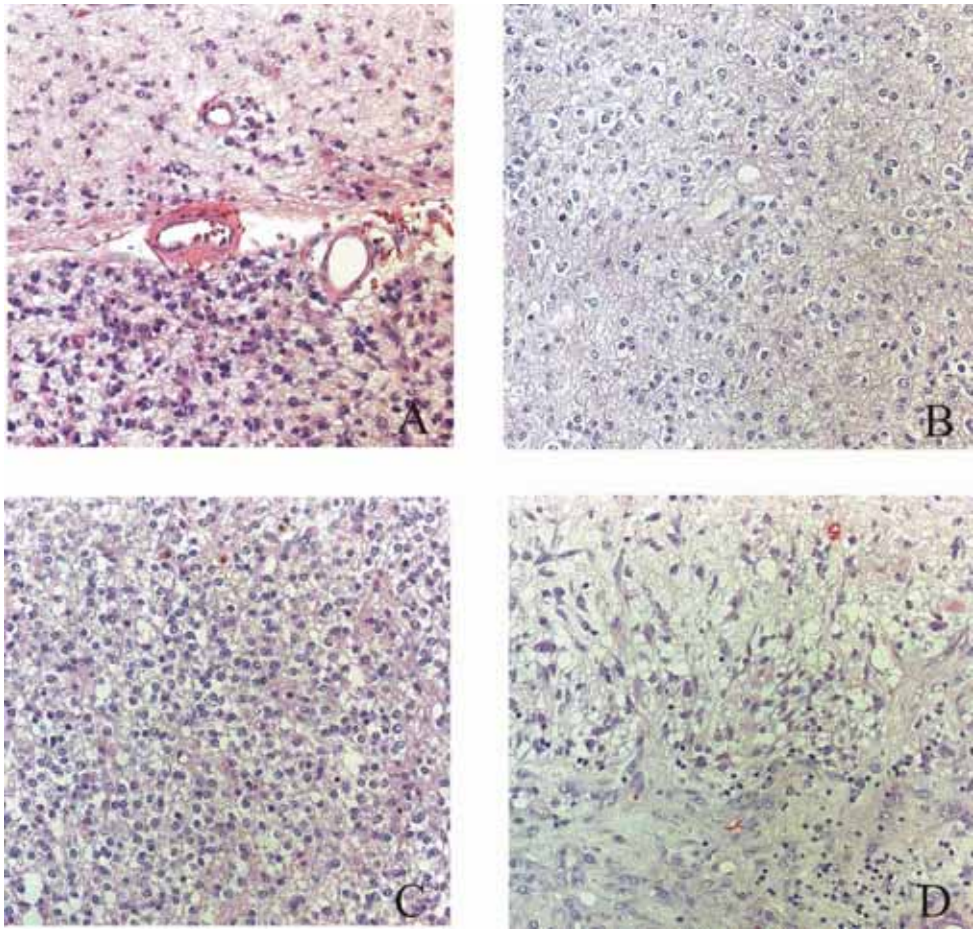
Gliomas are primary brain tumors with a spectrum of disease entities (Figure 1). They may present at any age but the peak incidence is in adults over 40 years of age (Preusser *et al*. 2006). Although divided into different subtypes, gliomas display several similarities in both clinical phenotype and histopathology, most importantly the propensity to progress from low- to high grade tumors (Perry 2003; Ohgaki and Kleihues 2005). The most commonly used classification is the World Health Organization's criteria, which divide gliomas into four grades (I-IV) with distinct entities based on histology, immunohistochemistry, and ultrastructure. These types are astrocytomas, oligodendrogliomas, oligoastrocytomas (a mixed type of tumor with characteristics of both oligodendroglioma and astrocytoma) and glioblastoma (also known as glioblastoma multiforme) (Louis *et al.* 2007). Only grade I tumors (i.e. pilocytic astrocytoma) are potentially curable, whenever surgical resection is possible (Perry 2003), whereas grade II and III tumors possess the potential of eventually progressing to the most malignant type of glioma, grade IV glioma or glioblastoma (Ohgaki 2005). However, pediatric forms of glioma have a better prognosis, and curative treatment can be achieved even in high-grade tumors. Furthermore, progression from low- into high-grade tumors is uncommon in pediatric low-grade gliomas (Bristol 2009).

Symptoms of glioma include seizures, headache, paralysis and nausea. This varies much depending on the localization, as tumors causing increased intracranial pressure due to blockage of CSF flow or mass effect of the tumor may cause typical nausea whereas cortically located tumors act as ectopic epileptic foci. Quite often, gliomas are found during neuroradiological examinations performed primarily for other reasons. Usually, a biopsy is required, although CT/MRI findings are suggestive.

Curative surgical resection of grade II-IV gliomas is not possible. This is due to the predisposition of glioma cells to infiltrate throughout the brain at already a very early phase of the disease (Demuth and Berens 2004). Indeed, even though grade II-IV gliomas most often present in one cerebral hemisphere, in over 50% of cases, tumor cells can be found also in the contralateral hemisphere at the time of treatment (Demuth and Berens 2004). Furthermore, it has been shown that migrating glioma cells are more resistant to apoptosis inducing treatments than stationary glioma cells (Joy 2003). Tumor cells spread along the white matter axons and in the perivascular space of the CNS. Such infiltrating tumor cells cannot be detected radiologically (Perry 2003). Gliomatosis cerebri is a state where infiltrating tumor cells are found in more than two lobes, often bilaterally or even in the spinal cord (Louis *et al*. 2007).

Astrocytomas represent the group of gliomas that are graded I to III according to WHO classification, thus excluding glioblastoma. However, pilocytic astrocytoma is considered benign and thus not discussed here. Grade II tumors are known as diffuse astrocytomas, and

Figure 1: histopathological features of glioma



A) Astrocytoma infiltrating diffusely into the brain. In the lower half, abundant tumor cells. The tissue is divided in the middle by a sulcus. Tumor cells can also be seen in the opposite gyrus, suggesting phenomenal invasive capacity.
B) Oligodendroglioma. Note the "fried egg" appearance of cells.
C) Oligoastrocytoma, with mixed features of both astrocytoma and oligodendroglioma.
D) Glioblastoma. Necrosis, endothelial proliferation, gross atypia and overall a distorted histology is seen. Ergo "multiforme".

grade III tumors as anaplastic astrocytomas. Although grade II astrocytomas often have a slow clinical course with a survival of 5-10 years, they are incurable and subsequently may progress to glioblastoma (Perry 2003). Patient age is a known prognostic factor in astrocytoma (Shafqat *et al.* 1999). Histologically, diffuse astrocytomas display invasion and a relatively low proliferative index, hyperchromatic and elongated nuclei, and gemistocytes (Perry 2003). In addition to the previously mentioned features, anaplasia and angiogenesis can be found in anaplastic astrocytomas. A marker ubiquitously expressed in astrocytomas and discriminating them from oligodendrogliomas is glial fibrillary acidic protein (GFAP), which is also a marker of normal astrocytes (Yung *et al.* 1985). Mutation of the p53 gene (TP53) are considered as a early change in astrocytomas, found in approximately 50% of grade II astrocytomas and more frequently in anaplastic astrocytoma (Watanabe *et al.* 1997).

Oligodendrogliomas are a subtype of gliomas, characterized by a specific histological appearance resembling oligodendrocytes as originally categorized by Bailey and Cushing in 1926. The most typical feature of oligodendrogliomas is the "fried egg" appearance seen in histological samples. Also, a typical pattern of blood vessels can be observed. Tumors are grouped to low- and high grade entities (Louis *et al.* 2007). If the hallmark features of mitoses, nuclear atypia, or endothelial proliferation are seen, the tumor is diagnosed as an anaplastic (grade III) oligodendroglioma. If these features are absent, the diagnosis is low-grade. Besides typical oligodendrogliomas, there exists a mixed form of tumors called oligoastrocytomas. These tumors display histological characteristics of both oligodendroglioma and astrocytoma. Although the histological diagnosis may vary, these tumors are generally considered a distinct clinical entity. Oligodendrogliomas will infiltrate throughout the brain. A commonly used immunohistochermical marker for oligodendrogliomas is Olig2, a differentiation marker of oligodendrocytes. GFAP is not expressed in pure oligodendrocytic tumors but it can be found in astrocytic components of oligoastrocytomas (Mokhtari *et al.* 2005). Besides histology, oligodendrogliomas are diagnosed based on characteristic genetic aberrations found. The most typical is 1p19q loss. Besides being an aberration found on 75% of all oligodendrogliomas, it also incurs a favorable response to therapy (Cairncross *et al.* 1998). In the mixed oligoastrocytic tumors, either p53 or 1p19q can be found. Compared with the incidence of glioblastoma and astrocytoma, oligodendrogliomas are relatively uncommon, contributing from 5 to 20% of all glial tumors, much depending on the diagnostic criteria applied (Van den Bent *et al.* 2008).

Glioblastoma is the most common and also most malignant form of glioma, with a median survival of approximately 12 months after diagnosis, despite treatment (Ohgaki 2009). Glioblastomas are divided, based upon the clinical course of the disease, into primary (de novo) glioblastomas and secondary glioblastomas. Secondary glioblastomas are formed through the transformation of pre-existing astrocytic or oligodendroglial tumors, whereas de novo tumors are thought to arise directly as glioblastoma. This difference is also reflected upon the cytogenetic differenced between these two types of grade IV glioma. The loss of chromosome 10 and amplification of chromosome 7 are characteristic cytogenetic alterations found in glioblastoma (Maher *et al.* 2001). Another significant aberration is the deletion or mutation of the tumor suppressor PTEN, an antagonist of the PI3K pathway. This leads to the uncontrolled proliferation of glioma cells, the reversal of quiescence and also reflects upon survival as a negative prognostic marker and poor responsiveness to therapy (Cully *et al.* 2006 and Phillips *et al.* 2006). Aberrant activation of the epidermal growth factor receptor (EGFR) gene, often via amplification of chromosome 7, but also via direct mutations of the EGFR gene, are typical of glioblastoma. This has also been used as a prognostic marker, although it is potentially

dependant of patient age (Heimberger *et al.* 2005 and Aldape *et al.* 2004). However, EGFR amplifications are also found in grade II-III tumors to a lesser extent (Puputti *et al.* 2006).

Table 2. Common cytogenetic alterations in gliomas

astrocytoma	**oligodendroglioma**	**glioblastoma**
Rb and p53 mutation	deletion of p14ARF and p16INK4a	amplification of chromosome 7
CDK4/6 overexpression	1p and 19q loss	EGFR amplification
KIT and PDGFRA amplifications		mutation or deletion of PTEN
1p and 19q loss		chromosome 10 loss

Phillips *et al.* have identified specific subgroups of glioblastoma using genomic screening of specific gene groups associated with distinct cellular processes. Based on this molecular screening and utilizing clinical data, they divided glioblastomas into three major types: proneural, proliferative and mesenchymal (Phillips *et al.* 2006). Interestingly, they describe a shift from the proneural to the mesenchymal and angiogenic phenotype during tumor progression. Accordingly, patient survival was best in the proneural group. This may represent a new kind of distinction in glioma progression, reflecting the loss of neural differentiation and therefore enhanced stem-cell type functions leads to the advantage of invasion, migration, angiogenesis and growth.

Taken together, there is considerable evidence of the role of stem cell gene involvement in glioma formation. Because there is no known cause for these brain tumors, and because they, in adults, almost invariably progress despite treatment, I found it important to study the role of stem cell genes in these tumors, in order to find new markers of tumor progression and to identify possible novel molecular targets for the development of treatment.

3.2 Neural crest derived tumors

Derived from chromaffin cells of the neural crest, pheochromocytoma is a tumor of the adrenal medulla, known in the sympathetic ganglia as paraganglioma. Paragangliomas can be found anywhere along the chain of sympathetic ganglia, i.e. cervically, in the thorax, abdomen or pelvis. However, the clinical findings as well as prognosis of paraganglioma differ from adrenal pheochromocytoma (Lenders *et al.* 2005). Classical internal medicine guidelines name pheochromocytoma as an uncommon cause of hypertension that must be ruled out, although approximately 20% of pheochromocytomas are asymptomatic and in fact only less than 1% of hypertensive patients in fact are diagnosed with the tumor (Bravo and Gifford 1984; Mannelli *et al.* 1999; Pacak *et al.* 2001).

In 1886, Fränkel reported a case of bilateral adrenal tumors in a young woman. His patient presented with vomiting, headache, rapid fluctuations of the pulse and psychic symptoms. Then, the function of the adrenals was still unknown, but Fränkel speculates that "…abnormal substances can enter the blood…" from the tumor-affected adrenal glands. This was the first published description of

pheochromocytoma; also histological autopsy findings were included (Fränkel 1984 reprint). Later analyses of the original material as well as recent genetic analyses of the relatives of the patient (the patients name was also published in the 1886 article) suggest that she suffered from multiple endocrine neoplasia type 2 (Neumann *et al*. 2007). This case-report illustrates well the classical symptoms of pheochromocytoma, as well as served as evidence of the poor outcome associated with uncontrolled disease. Indeed, patients today with metastatic pheochromocytoma often ultimately succumb to the paracrine and endocrine effects of the sheer tumor burden combined with the uncontrolled, massive secretion of catecholamines (O'Riordan *et al*. 1996; Goldstein *et al*. 1999).

Due to the dramatic increase in radiological examinations performed in modern healthcare, most notably abdominal computer tomography, pheochromocytoma is increasingly diagnosed as an adrenal "incidentaloma" and anywhere between 4 to 11 % of pheochromocytomas are diagnosed in this manner (Mannelli *et al*. 1999; Mantero *et al*. 2000). An important method of diagnosing pheochromocytoma is based on the uncontrolled catecholamine secretion of the tumors. Plasma metabolites, i.e. metanephrines, are routinely measured due to the nearly 99% specificity of detecting pheochromocytoma (Sawka *et al*. 2004). However, the positive predictive value of these tests is limited, and thus they serve as a negative screening measure to rule out disease especially during follow-up for recurrency after surgical treatment.

Pheochromocytoma is most often non-metastatic, and metastatic pheochromocytoma is defined solely by the presence of metastases according to WHO guidelines (DeLellis *et al*. 2004). For instance, locally invasive growth is not constituent of metastatic behaviour. Metastases occur in bone, the liver, lungs and lymph nodes, sometimes decades after initial diagnosis, warranting long follow-up times (Plouin *et al*. 1997). Unfortunately, histology of the primary tumor provides no support in diagnosing the putatively metastatic disease. In 2002, Thompson developed the PASS (Pheochromocytomas of the Adrenal gland Scaled Score) system to distinguish between metastatic and non-metastatic forms (Thompson 2002). A total score of 4 or more (Table 3) is regarded as suggestive of metastatic behavior, although the usefulness of this system has been questioned (Wu *et al*. 2009). Many immunohistological markers have been studied, based on cell proliferation (Ki-67, MIB-1), cell cycle (p53, hTERT), growth factors (c-erbB-2), angiogenesis (VEGF, inhibinβB) and invasive capacity (cox2, heparanase-1) but none of these has proven to be decisive (Salmenkivi *et al*. 2001; Salmenkivi *et al*. 2004).

Table 3: the PASS scoring system*

Feature	Score
Large nests or diffuse growth	2
Central or confluent necrosis	2
High cellularity	2
Cellular monotony	2
Tumor cell spindling	2
Mitotic figures >3 /10 high-power fields	2
Atypical mitotic figures	2
Extension into adipose tissue	2
Vascular invasion	1
Capsular invasion	1
Profound nuclear pleomorphism	1
Nuclear hyperchromasia	1
Total	20

**Histological features scored in estimating metastatic potential. A score of four or more is suggestive of metastatic behavior. Note especially that vascular and capsular invasion only constitute one point each.*

Although pheochromocytoma is a rare tumor and the metastatic form of the disease comprises only apprixamately 10% to 20% of all cases, a reliable marker for metastatic disease is in dire need because of the miserable prognosis patients with the metastatic disease, if the disease progresses undetected until metastases have occurred.

3.3 Oral squamous cell carcinoma

Oral squamous cell carcinoma (OSCC) of the tongue represents a typical cancer of epithelial origin. Characteristic of carcinomas, it is often preceded by (pre)malignant lesions, such as leukoplakia, and carcinoma *in situ*. The incidence rises with age, and the disease is more frequent in males. Incidence rates are rising in Scandinavia, also in the young populace where oral cancer is relatively rare (Annertz *et al*. 2002). The typical high incidence rates in old patients can be explained by prolonged exposure to carcinogens. Well characterized risk factors are tobacco and alcohol. In other types of oral squamous cell cancer human papilloma virus infection is a recognized risk factor, but in cancer of the tongue it may represent a minor etiological role (Liang *et al*. 2008, Andrews *et al*. 2009).

Prognosis is good only in cases where surgical resection is possible, and the outcome of metastatic disease is generally poor (Zhen *et al*. 2004). Unfortunately, occult metastases and subsequent poor outcome are often seen even in small, low-stage tumors (Keski-Säntti *et al*. 2007). Histopathologically, the depth of tumor invasion is regarded as an important sign of higher risk in clinically non-metastatic cases (Alkuresishi *et al*. 2008).

Several genetic aberrations and molecular markers have been identified in oral squamous cell carcinoma. The expression profile of cytokeratins varies between aggressive, metastatic disease and less malignant forms of disease (Silveira *et al*. 2007). The epidermal growth factor receptor (EGFR) gene is amplified in over 50% of cases of oral squamous cell carcinoma of the tongue, and amplification correlates with high EGFR protein expression, although the prognostic value of this is somewhat unclear (Ryott *et al*. 2009). Cell to cell adhesion molecules disrupted in EMT are found to be significantly downregulated in metastatic oral squamous cell carcinoma, suggesting EMT involvement in metastases (Tanaka *et al*. 2003). However, the details of EMT in clinical disease are not exactly characterized, and expression of Snail, known to downregulate E-cadherin, has been reported not to correlate with lymph node metastasis or decreased E-cadherin expression (Franz *et al*. 2009). The proliferation marker Ki-67, thus proliferation, is increased in recurrent, aggressive low-stage tumors (Wangsa *et al*. 2008). The tumor suppressor p53 is mutated in approximately 50% of advanced-stage tongue carcinoma, although the prognostic significance of p53 immunohistochemiscal staining does not correlate with outcome or the genetic status of the p53 gene (Atula *et al*. 1996; Fourati *et al*. 2009). Recently, the altered expression of micro-RNA has been found in OSCC, suggesting even strong prognostic significance (Li *et al*. 2009). Also, a small (1-2%) subpopulation of CD133-positive cells has been identified in tongue cancer derived cell lines, and these CD133-positive cells show an increased resistance to chemotherapy as well as in vivo tumorigenic potential, suggesting cancer stem cell involvement in this disease (Zhang *et al*. 2009).

4. Stem cells and malignancy

Stem cell transplantation has been used in humans already for over three decades in the form of hematopoietic stem cell transplantation in the treatment of blood malignancies (Leiper 1999). However, the use of other kinds of stem cells has not been possible, simply because the isolation of somatic and embryonal cells was achieved only recently. Hematopoietic stem cells have a differentiation capacity confined to hematopoiesis. Therefore, stem cells from which other types of tissue have been unavailable until of late. Another type of a stem cell that promises novel clinical applications is the mesenchymal stem cell (Sensebé and Bourin 2009). The use of stem cells as regenerative therapy is a rapidly growing field of medicine, with clinical applications emerging and new treatments made available to patients all the time (Lunde *et al*. 2007; Mesimäki *et al*. 2009). However, based on the long-term follow-up data available from bone marrow transplant registries, it has been speculated that transplanted stem cells might bear with them the risk of secondary malignancies (Avital *et al.* 2007). A report based on relatively few patients have indeed confirmed that at least head and neck tumors may arise from donor-derived cells (Janin *et al.* 2009). This observation is probably not universal, and other studies could not demonstrate the same phenomenon (Worthley *et al.* 2009). Still, donor derived tumor associated stromal myofibroblasts were found in gastrointestinal tumors by Worthley *et al.*, suggesting the involvement of transplanted stem cells in the progression or development of the tumors, as described in experimental settings (Karnoub *et al.* 2007). Indeed, the capacity of mesenchymal stem cells from healthy donors to undergo spontaneous malignant transformation has been reported, and this process is probably due to the expansion *ex vivo* of these cells in cell culture (Rubio *et al.* 2005 and Tolar *et al*. 2007). A very interesting finding is that the c-Myc and p16 pathways and Ink4a/Arf and Rb loci, which are known to be central for the malignant potential of tumor-derived stem cells, are also disrupted in transformed mesenchymal stem cells, suggesting a stem cell origin of cancer (Rubio *et al*. 2008). Therefore it seems critical, in future clinical applications of stem cells, to carefully consider the risk of malignancy in comparison with the benefit gained from regenerative or reconstructive stem cell treatments.

AIMS OF THE STUDY

First, I wanted to investigate whether stem cell gene products are expressed in clinical tumor biopsies and if this would reflect upon the clinical course of the disease.

Second, I wanted to investigate if such findings would provide additional information on the pathological diagnostics of the cancer.

Third, I wanted to investigate if the expression pattern of stem cell related genes would support or contradict the stem cell hypothesis in cancer.

MATERIALS AND METHODS

A detailed description is found in the original publications. This is a summary describing the central features of the clinical material and laboratory, statistical and histopathological methods used. For each method, the publication in which it has been used is given as follows:

Paper I: BMI-1 protein expression in gliomas. Stem cell protein BMI-1 is an independent marker for poor prognosis in oligodendroglial tumours. Neurobiology and Applied Neuropathology *2008:34:555-63*
Paper II: BMI-1 gene analysis in gliomas. Amplification and deletion of the polycomb gene BMI1 in gliomas. Acta Neuropathologica *2008:116:97-102*
Paper III: Snail expression in pheochromocytoma.
High expression of the neural-crest gene Snail confirms and predicts the metastatic potential of pheochromocytoma. Endocrine-Related Cancer, *2009; 16:1211-8*
Paper IV:BMI, Snail and c-Myc expression in oral squamous cell carcinoma.
BMI-1 expression predicts prognosis in squamous cell carcinoma of the tongue. British Journal of Cancer, *in press*

1. Patient material (I, II, III & IV)

Gliomas

In total 305 samples of primary gliomas were collected from adult patients treated at the Departments of Neurosurgery at the University Hospitals of Helsinki and Tampere, Finland, and University Hospital Münster, Germany during 1980-2006. Histopathological diagnoses based on the WHO criteria were blindly reviewed by experienced neuropathologists. All patients received a gross total or partial resection of the tumor, in most cases followed by postoperative radiation therapy. Survival times were collected for all patients and were calculated from the time of diagnosis to primary endpoint. It was defined as death caused by brain tumor. For CISH, samples of 100 tumors were used, all of which were from the Department of Pathology, Helsinki University Central Hospital. Of these, 43 were primary (included in the above material) and 57 were recurrent tumors. For the patients, clinical data was obtained from the hospital records and Statistics Finland.

Pheochromocytomas

Samples from pheochromocytomas of both adrenal (n=48) and extra-adrenal (n=2) origin were collected. The 50 surgical samples were from 42 individuals treated at the Department of Surgery, Helsinki University Central Hospital (HUCH) during 1985 to 2008. The material, including clinical records, was collected retrospectively during the year 2009. Data was obtained from the HUCH records and from the Population Registry of Finland and Statistics Finland. The clinical diagnosis was non-metastatic pheochromocytoma in 32 patients and metastatic pheochromocytoma in 10 patients including two patients with paraganglioma. From 4 individuals with a metastatic disease, tissue from both the primary tumor and metastases was available. Four patients developed a recurrence of the disease, and samples of the recurrent tumor were included in the material. All samples underwent routine histopathological diagnostics during the time of treatment. Furthermore, all samples were re-evaluated during the study by pathologists specialized in endocrine pathology.

Oral squamous cell carcinoma

Histological samples and clinical data from seventy-three patients were collected (36 males and 37 females, median age 59 years, range 23-95 years). Of the tumors, 35 (48%) had been clinically classified as T1 and 38 (52%) as T2. All patients had undergone resection of the primary tumor. In 31 patients there had been no further treatment primarily. 42 patients underwent elective neck treatment (neck dissection: n=9, neck dissection + radiotherapy: n=32, radiotherapy: n=1). All patients were treated with curative intent. The dates and causes of death were provided by Statistics Finland. The original histological sections of each patient were re-assessed and tumor grade and depth of invasion were determined by a single experienced head and neck pathologist.

2. Immunohistochemistry (I, II, III & IV)

Epitope retrieval for BMI-1, p16, mdm2 was performed by autoclaving samples at 120°C for 2 minutes in a 10 mM sodium citrate buffer (pH 6.0) equipment. For Snail, epitope retrieval was performed by pretreatment in a microwave oven for 7 min in a 10 mM sodium citrate buffer (pH 6.0) . For E-cadherin, pretreatment was done in a microwave oven in Tris-EDTA buffer (pH 9.0).

The mouse monoclonal BMI-1 antibody (1:750 dilution, clone 1.T.21, catalogue # ab14389, AbCam, Cambridge, U.K.) was incubated for 60 min at 4°C. Binding of the primary antibody was detected with a Powervision + Poly-HRP histostaining kit (Immunovision Technologies & Co, CA). Stainings with the mouse monoclonal antibodies for p16 (1:150 dilution, clone 16P07, catalogue # MS-1064, Neomarkers, LabVison Corp., Fremont, CA) and mdm2 (1:50 dilution, clone 1B10, NCL-MDM2, Novocastra, VisionBiosystems, Newcastle, U.K) were performed using the UltraVision IHC detection kit (LabVision), according to the manufacturer's instructions. The rabbit polyclonal Snail antibody (1:600 dilution, catalogue # ab17732, AbCam plc. Cambridge, UK) was incubated for 60 min at 21°C. Binding of the primary antibody was detected with a Powervision + Poly HRP histostaining kit (Immunovision Technologies & Co, CA). Mouse monoclonal c-Myc antibody (diluted 1:400, catalogue # 9E10, Santa Cruz, CA) was incubated for one hour at 21 °C, and binding was detected with the Dako REAL EnVision/HRP detection system. The mouse monoclonal E-cadherin antibody (1:200 dilution, catalogue # 13-13700, Invitrogen Corp, Camarillo, CA) was incubated for 30 minutes at 21°C and detected by Envision (K5007, DAKO, Glostrup, Denmark). The E-cadherin staining protocol is also used in routine diagnostics at the Helsinki University Central Hospital. Ki-67 stainings were scored according to routine diagnostic criteria (Tynninen *et al*. 1999).

3. Scoring of immunostainings (I, II, III & IV)

For analyses of gliomas, tissue arrays were used. Immunostainings were grouped for BMI-1 expression according to the percentage of BMI-1-positive tumor cell nuclei in the tissue array samples: 0 = no staining, 1 = low (less than 20% of positive nuclei), 2 = intermediate (20%-70% of positive nuclei), and 3 = high (more than 70% of positive nuclei). Neurons and stromal cells were ignored.

Snail immunostainings were scored from whole biopsy slides. Expression was scored according to the percentage of immunoreactive tumor cell nuclei. This semiquantitative scale was the same as the one used for gliomas.

In OSCC, tissue arrays were also used. The percentage of positive tumor cells was evaluated. No positivity was graded as 0, up to 30% positive cells as 1 (very low), 30-50% as 2 (low), and 51-80 % as 3 (moderate) and over 80% as 4 (high).

All scoring were first blindly by myself and another scientist, who was a pathologist specialized in the type of tumor analyzed. A consensus score was applied in joint re-evaluation, if the scoring did not match.

4. Chromogenic in situ hybridisation (II)

A digoxigen-labelled BAC-probe for *BMI1* (clone ID RP11-232K21, Invitrogen Ltd, Paisley, UK) was applied to the slides, sections were denatured, and hybridization was performed overnight at 37°C. In order to correct for chromosomal aneuploidy, a probe for the centromere of chromosome 10 was applied in adjacent sections of all the samples analysed with *BMI1* CISH. CISH signals were evaluated by counting at least 30 nuclei per sample. Euploidy for *BMI1* was defined as a ratio of 1:1 between *BMI1* and centromere 10 signal numbers. Increased copy number (gain) was defined as three or more *BMI1* signals per nucleus in at least 15 % of nuclei and a ratio of 1.5:1 or higher between *BMI1* and centromere 10. Deletions of *BMI1* were defined as cases where at least 30% of the nuclei in a sample showed only a single signal for *BMI1* when predominantly two signals per nucleus were present in the corresponding sample hybridized with the probe for centromere 10. If only one signal per nucleus could be seen for both of the probes used, this was interpreted as a loss of the whole chromosome 10.

5. Tissue microarray (I, II & IV)

For glioma samples, tissue microarray blocks were constructed using a 0.6 mm diameter core biopsy needle. From each gross tumor sample, the neuropathologist identified a morphologically representative tumor area including the diagnostic features of the tumor type to be used in microarray construction.

For OSSC samples, 3 different areas in two sets from normal Haematoxyllin-Eosin (HE) blocks from each patient were detached with a 1 mm punch. The first area was selected close to the surface epithelium, the next in the middle of the tumor and the last at the invading front.

6. Sequencing (II)

Genomic DNA was extracted from 51 formalin-fixed paraffin-embedded glioma samples using standard methods. The whole coding sequence of BMI-1 consisting of exons 1-9 were PCR amplified. Bidirectional sequencing of the PCR products was performed using BigDye3 termination chemistry (Applied Biosystems) and an ABI 3100 Genetic Analyzer (Applied Biosystems) according to the instructions provided by the manufacturer.

7. Ethical considerations (I, II, III & IV)

Papers I&II: The project was been approved by the Ethical Committee of Helsinki University Central Hospital, Hospital District of Helsinki and Uusimaa (diary number 217/E9/06), and all work has been done in accordance with the Helsinki declaration.

paper III: The project was been approved by the Ethical Committee of Helsinki University Central Hospital, Hospital District of Helsinki and Uusimaa (diary number 226/E6/06), and all work has been done in accordance with the Helsinki declaration.

paper IV: The project was been approved by the Ethical Committee of Helsinki University Central Hospital, Hospital District of Helsinki and Uusimaa (diary number 166/E9/07), and all work has been done in accordance with the Helsinki declaration.

8. Statistical analysis (I, II, III & IV)

For categorical, non-ordered variables, cross-tabulations were analyzed using the chi-square test or, when the chi-square test could not be used, the Fisher's exact test was chosen. All p-values are two-sided. For the statistical analyses, patient age was divided into categories. For survival analysis, an event was defined as death caused by glioma in papers I & II. In paper IV, an event was

defined as a clinical and confirmed recurrence of disease. Follow-up time was calculated from the date of diagnosis until event, and patients still alive or deceased from non-tumor related causes were censored on the last date of follow-up. Kaplan-Meier curves were plotted from survival data and the log-rank test was applied to compare outcome between patient categories. To control for confounding factors, a multivariate Cox regression model was used. SPSS version 12.0.1 software (SPSS Inc., IL, USA) was used for all statistical analyses.

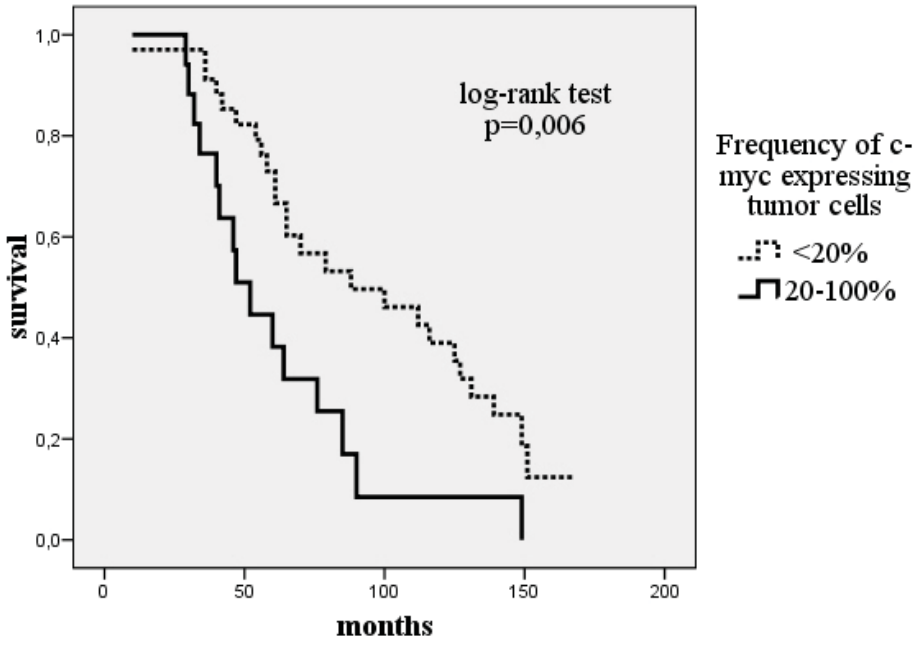
RESULTS

1. Stem cell markers in glioma

1.1 Preface

The expression pattern of several genes expressed by neural stem cells and during CNS development was screened first in a small series of specimen from gliomas. The genes products initially screened are summarized in table 2. The immunoreactivity for ezh2, and Sox2 antibodies was weak and variable and the staining did not correlate with any one of the tested clinical parameters. The staining for Oct4 protein, a very early embryonic stem cell transcription factor, was negative in all samples. c-Myc expression was analyzed in 49 cases of grades II and II astrocytoma (Häyry *et al*., unpublished). The frequency of c-Myc expressing tumor cells showed a correlation with the proliferative index of the tumors in astrocytoma (Fisher's exact test p=0,002). In this group, c-Myc immunostaining also correlated with patient survival (p<0.006) (figure 2). These markers were not studied any further because similar results had already been published by other researchers (Faria *et al*. 2008).

Figure 2: Kaplan-Meier graph of c-Myc expression in astrocytoma



In this initial screen, results suggesting promising, novel clinical relevance were only obtained for BMI-1. Therefore, the study was expanded in regard to BMI-1 and its downstream targets p16 and mdm2. To gain statistical power, the tumor material was expanded to 305 specimens.

Table 4: genes selected for screening in 2005.*

gene considered for screening	immunohistochemical application available	putatively relevant clinical correlations
Sox2	Sox2	Sox2
ezh2	ezh2	ezh2
LGL		
BMI-1	BMI-1	BMI-1
CD 133		
Nestin	Nestin	
Oct 4	Oct 4	
Nanog		
Ret	Ret	
c-Myc	c-Myc	c-Myc
p16	p16	p16
mdm2	mdm2	mdm2

* In the first column from left to right, genes selected for screening are listed. In the middle column, the genes for which a successful immunohistochemical protocol could be produced are listed. Finally, in the last column, genes with relevant correlates between protein expression ad clinical parameters are listed.

1.2 BMI-1 protein expression

The protein expression of BMI-1 was assessed in 305 cases of gliomas (WHO grades 2-4) with tissue arrays. In this series, all WHO grade 2 and 3 oligodendrogliomas and oligoastrocytomas were BM-1-positive and likewise all WHO grades 2 and 3 astrocytomas. Nearly all glioblastomas also expressed BMI-1, although with a lower overall frequency, as shown in Table 2 and Figure 1 of the paper I. Although expressed in the majority of grade 2-4 gliomas, BMI-1 immunoreactivity did not correlate with tumor grade neither in oligodendroglial tumors nor in grade 2 and 3 astrocytomas when analyzed separately.

1.3 p16 and mdm2 expression

In all tumor types, a correlation between cytoplasmic and nuclear p16 expression was found (p<0,001, Fisher's exact two-sided test). Interestingly, high BMI-1 expression was predominantly found in samples with both cytoplasmic and nuclear p16 expression. The BMI-1 expression correlated with the pattern of both cytoplasmic (p=0,038) and nuclear p16 expression (p=0,009) in astrocytomas and in glioblastoma. In oligodendroglioma and oligoastrocytoma, only cytoplasmic p16 expression correlated with BMI-1 expression (p=0,033).

In relation to patient survival, only nuclear expression of p16 provided prognostic significance in the univariate survival analysis of astrocytomas and glioblastomas (Log-rank test for the equality of survival distributions for cytoplasmic expression of p16, p=0,053 and nuclear expression of p16, p=0,032). Neither cytoplasmic nor nuclear p16 expression showed prognostic relevance in oligodendrogliomas and oligoastrocytomas. A high Ki-67 proliferative index, on the other hand, was found to correlate with the absence of both cytoplasmic and nuclear p16 (p=0,020 and p=0,005, respectively) in astrocytomas including glioblastoma. No such association was found in oligodendroglial tumors.

Mdm2 was expressed found in all oligodendroglial and astrocytic tumors, but the level of mdm2 expression, as determined

by immunostaining intensity, did not correlate with BMI-1 expression. mdm2 expression correlated neither with tumor grade, proliferative index nor with the expression of p16.

1.4 Expression of BMI-1 correlates with survival in oligodendroglial tumors

To reveal the relevance of BM-1 expression for the clinical outcome, we estimated patient survival from first diagnosis to the primary endpoint of death by cancer with the Kaplan-Meier method. BMI-1 expressing tumor cells were quantified, and tumors were divided according to the overall frequency of BMI-1 positive tumor cells into three groups (<20% positive, 20-70% positive and >70% positive). In oligodendroglial tumors (WHO grades 2 and 3 oligodendroglioma and oligoastrocytoma), the high frequency of BMI-1 expressing cells strongly associated with poor outcome (log-rank test P = 0.007) (Figure 2 of Paper I). The median survival time was 191 ±SE27 months (15.9 years) if the expression of BMI-1 was low (13 cases) while in tumors of intermediate BMI-1 expression (32 cases), the median survival of the patients was less, 151 ±SE11 months (12.6 years). The lowest survival time was in tumors with high BMI-1 expression (17 cases): the median survival being only 68 ±SE14 months (5.7 years).

1.5 BMI-1 is an independent marker for poor prognosis

A multivariate Cox regression analysis was used to compare prognostic factors in oligodendroglioma and oligoastrocytoma and the independent nature of BMI-1 expression as a prognostic marker. BMI-1 expression, adjusted for proliferation index as well as WHO grade, age at onset, gender, and presence of astrocytic features, remains significantly associated with patient survival (P = 0.043) (Table 4 of Paper I).

1.6 Clinical correlates of BMI-1 expression in astrocytomas and glioblastoma

As for astrocytomas of grade 2 and 3, BMI-1 expression did not correlate with patient age at onset, gender, proliferative index or histological grade. Neither was there any statistically significant difference in survival between BMI-1 immunoreactivity groups in low-grade and anaplastic astrocytomas. Although not statistically significant in this study, an interesting trend, not unlike the result obtained for oligodendroglial tumors, was seen when looking at BMI-1 protein expression and patient survival separately in grade 2 & 3 astrocytomas (unpublished result). Frequent BMI-1 expression seemed to coincide with poor outcome, but, as stated, this was not significant in statistical analyses.

In glioblastomas, on the other hand, strong BMI-1 expression correlated with high proliferative activity (142 cases; P = 0.006), but BMI-1 expression did not correlate with patient survival (log-rank test, P = 0.688) in a total of 151 cases of glioblastoma.

1.7 Genetic status of BMI-1

Having demonstrated that BMI-1 is quite heterogenously expressed in gliomas regardless of grade, and that this observation has a clinical significance, I analyzed what the underlying reason might be. From the literature, it is well known that over- and underexpression of a gene in cancer may be due to a mutation or chromosomal aneuploidy. Thus, I investigated next if the BMI1 gene is amplified, deleted or mutated in gliomas.

1.8 BMI1 gene copy number changes are frequent in glioma

In CISH analysis, increased copy numbers (gains) of *BMI1* were found in 30% of oligodendrogliomas, 50% of oligoastrocytomas, 24% of grade II astrocytomas and 47% of anaplastic astrocytomas. In glioblastomas, increased copy numbers of *BMI1* were found in 47%. The BMI-1 gain was a dominant feature despite deletions of the whole chromosome 10 could be seen in all tumors studied, with the exception of low-grade astrocytomas.

Deletions of *BMI1* were found in all types of tumors, too, and especially in oligodendrogliomas (15%) and

oligoastrocytomas (13%) (Table 1 of paper II). In glioblastoma, deletions were less frequent (7%). When comparing primary and recurrent tumors of the same diagnosis, the distribution of *BMI1* copy number aberrations was strikingly similar (Table 1 of Paper II).

1.9 BMI1 gene mutations were not found

To reveal whether BMI1 sequence mutations occur in gliomas, 51 samples were randomly selected so that all different *BMI1* copy number variations were represented (i.e. deletion, gain etc.). For twenty seven of these cases, CISH data could not be obtained due to possible degradation of the tissue in paraffin-embedded, formalin fixed samples. Only one point mutation (P198S) was detected in our screen.

1.10 BMI1 gene aberrations in relation to clinical and pathological parameters

Whether the BMI1 copy number changes would associate with histopathological parameters, we compared the mitotic index of the tumor cells with the occurrence of *BMI1* deletions and gains in 49 cases (Table 2 of Paper II). A high proliferative index associated with *BMI1* deletion, whereas cases with *BMI1* gain often had a low proliferative index. This inverse correlation was statistically significant (Spearman correlation, p=0,002). Interestingly, deletions of *BMI1* and chromosome 10 were both associated with poor outcome in grade II-IV astrocytomas and glioblastomas (Log-rank test, p=0,039, n=30) (Figure 2 of Paper II) while no association between *BMI1* aneuploidy and poor survival could be confirmed for oligodendrogliomas and oligoastrocytomas.

2 Stem cell markers in pheochromocytoma

After thoroughly screening our material of CNS tumors for expression of the chosen stem cell markers, we asked ourselves whether similar phenomena might exist in the peripheral nervous system (PNS), most importantly neural crest derived tumor diseases. Development of the neural crest distuinguishes the peripheral nervous system organs both due to gene expression as well as anatomy. Several options were considered. Out of these, neuroblastoma and pheochromocytoma were the most interesting malignant diseases of the PNS. A considerably large series of clinical samples was only available for the latter.

2.1 Some CNS stem cell markers are expressed in pheochromocytoma

First, the following markers: BMI-1, p16, c-Myc, ezh2 and mdm2 were screened. Some of these were initially promising, but after further investigations, no significant clinically relevant pattern of expression was found (Häyry *et al*., unpublished).

2.2 Snail is differentially expressed in pheochromocytoma

We hypothesized that due to the well-documented involvement of Snail (a zinc-finger transcription factor) during the development and the migration of the adrenal glands and the paraganglionic system from the neural crest, Snail may be linked with tumor formation. In particular, reactivation of this embryological mechanism may connect with the metastatic potential (migration) of the primary tumor.

To test our hypothesis, we initially stained a set of eleven pheochromocytomas, both metastatic and non-metastatic. One of these was a tumor originating in the adrenals gland, which showed invasive growth through the capsule and concurrent distant metastases in the spinal column confirming the malignancy. Within this biopsy, some of the normal adrenal medulla was present, with the normal and cancerous tissue histologically distinguishable from each other. This sample was stained for Snail protein expression. Whilst the flanking normal medullary adrenal tissue was negative, the tumor tissue stained positively (Figure 2). Such an internal control proved that metastatic pheochromocytoma tissue does express Snail and that expression is absent in normal chromaffin tissue. Subsequently we expanded the study to cover 50 pheochromocytomas of both metastatic and non-metastatic types.

Figure 3: Snail expression in a case of pheochromocytoma.*

* The case of adrenal gland and outgrowth of the tumor tissue. The central picture demonstrates the over all anatomy (100x magnification). Inset to the left demonstrates lack of staining in the adrenal medulla and inset to the right high expression of the Snail protein in the outgrowth (counterstaining HE, 400x magnification).

2.3 Snail expression is high in metastatic pheochromocytomas

In all four controls of normal adrenal medulla, no Snail expression was detected. Likewise, in a majority of non-metastatic pheochromocytomas, no Snail protein expression was seen. However, in a few of the non-metastatic tumors, Snail-positive tumor cells were observed at low frequency. In contrast, all primary tumors with known metastases showed high Snail expression, and in most of these, the majority, if not all tumor cell nuclei were immunoreactive. For the group of tumors classified as borderline tumors based on the histopathological criteria, the overall frequency of Snail-positive tumor cells was higher than in the group of non-metastatic tumors but less than in metastatic tumors. In the borderline tumors, Snail expression was especially seen in the tumors with invasive growth through the adrenal capsule. In the tissue samples from which tissue from both the primary tumor and its metastases was available, high Snail expression was seen in both the primary tumor and the metastasis. The observed difference in Snail expression in non-metastatic versus metastatic tumors was statistically highly significant (Fisher's exact test $p<0{,}001$). Also, Snail protein expression correlated statistically significantly with invasive growth seen in the histopathological material (Fisher's exact test $p<0{,}001$). Snail expression in different subtypes of pheochromocytoma is summarized in Table 2 of Paper III.

2.4 Snail protein expression does not correlate with E-cadherin expression

Immunostaining for E-cadherin was performed on all samples, because its expression is negatively regulated by Snail in many cell types (Battle *et al.* 2000; Cano *et al.* 2000; Poser *et al.* 2001). We found 28% of metastatic and 21% of non-metastatic tumors express E-cadherin. In these, usually only few immunopositive cells could be found. Thus, E-cadherin expression did not correlate with clinical behavior of the tumors. Furthermore, no statistical correlation between E-cadherin and Snail expression could be demonstrated. Finally, E-cadherin expression did not correlate with invasive or metastatic growth (Häyry *et al*., unpublished).

3 Stem cell markers in OSCC

Finally, since I found several of the chosen stem-cell genes to be expressed and clinically relevant in both CNS and PNS tumors, I further expanded my hypothesis to epithelial malignancies, i.e. carcinoma. A series (n=73) of well-characterized, clinically and histologically homogenous tumors was available in the form of OSCC. Particularly beneficial to this material was the high variance in aggressiveness between seemingly identical cases, typical of this type of carcinoma.

3.1 Bmi-1, c-Myc, and Snail are expressed in OSCC

Protein expression of Bmi-1 and c-Myc was found in a majority of tumors and Snail expression in all of OSCC tumors. Bmi-1 expression varied considerably between individual cases, from no expression to a very high frequency of immunoreactive cells. The frequency of c-Myc expressing cells was often low; in less than 50% of tumor cells.

3.2 Bmi-1 protein expression is a prognostic marker

An inverse correlation between Bmi-1 protein expression and recurrence (Log-Rank test p=0,005) was detected. Complete absence of Bmi-1 protein in the tumor cells of a sample was associated with a high risk of recurrence. However, it was difficult to assess overall or disease-related survival because during the follow-up time, only three patients died of OSCC. Still it should be pointed out that all three of the tumor-related deaths occurred in Bmi-1-negative cases. The mean disease free time for Bmi-1 negative cases was 53 months (95% CI 29-77 months), whereas patients with tumors with a high frequency of Bmi-1 expressing cells had a mean disease-free time of 112 months (95% CI 97-126 months).

To control for confounding factors, a multivariate analysis was performed. The depth of invasion, tumor size, margin of surgical resection and T-classification were included, along with the Bmi-1 protein expression score. In a Cox regression model, Bmi-1 expression remained the only independent covariate (p=0.012). The other factors were not statistically significant. The hazard ratio for absent Bmi-1 expression vs. high Bmi-1 expression was 5.2 (95% CI 1.5-18.3). The expression of c-Myc, Snail and Ki-67 did not correlate with tumor recurrence.

3.3 Clinical and histopathological correlates in OSCC

The protein expression levels of all the markers (BMI-1, Snail, c-Myc) were examined for correlations with clinical and histopathological parameters. These included: degree of histological differentiation, tumor size, TNM classification, depth of invasion, margin of resection. Snail expression was significantly lower in well differentiated tumors, whereas in poorly differentiated tumors Snail expression was high (Fisher's exact test p=0.007). Snail protein expression was also found to correlate with depth of invasion. In cases with the highest Snail score (>80% positive tumor cells), the depth of invasion was greater (χ2-test, p=0.037).

DISCUSSION

1. Discussion of results

1.1 High BMI-1 protein expression is an independent prognostic marker in oligodendroglioma

The results of the BMI-1 expression in gliomas demonstrate that a high frequency of BMI-1-positive tumor cells is associated with poor prognosis in both low- and high-grade oligodendrogliomas and oligoastrocytomas. Cox's multivariate regression analysis showed that the prognostic value of BMI-1 expression is independent of histological grade, proliferative index, and other known prognostic factors. Patients with a high frequency of BMI-1-positive cells had a shorter overall survival than patients with intermediate or low frequency (5.7 vs. 12.6 vs. 15.9 years, respectively). This difference in prognosis became evident after approximately 50 months follow-up, when the survival of patients with a low fraction of BMI-1 positive cells distinctly diverged from the 'high-BMI' group. Therefore, BMI-1 expression might be most useful in screening out cases, who are most in need of intense follow-up to detect recurrence.

This result suggests that, because BMI-1 is expressed in neural stem cells, that cancer stem cell -like cells might be present in low grade gliomas. My results do not however provide evidence to the origin of these cells, nor do they show if these cells also express other (neural) stem cell markers in addition to BMI-1. Despite this, the frequency of the BMI-1-expressing cells is directly proportionate to the aggressive behavior of a tumor. Especially in glioma, where typically low-grade tumors remain clinically dormant for years, slowly disseminating cancerous cells throughout the brain may later suddenly burst into fulminant high-grade disease. My current results suggest that the progression of the disease is highly associated with BMI-1 expressing cells. It has not been accurately explained by which means this progression is triggered. It would be tempting to speculate that this may be due to the polycomb activity of BMI-1 (Rajasekhar and Begemann 2007). During development, the polycomb protein complex 2 simultaneously suppresses and activates the transcription of large families of genes (Rajasekhar and Begemann 2007, Spivakov and Fisher 2007). This is orchestrated in a highly complex manner, whilst often during development the same genes may be either activated or shut down by the same polycomb complex through intricate epigenetic mechanisms. Thus aberrant BMI-1 activity in a tumor might cause broad and profound changes in the already abnormal gene activity of cancer cells.

1.2 BMI-1 expression in astrocytoma

An interesting finding was that BMI-1 and p16 protein expression are associated in astrocytomas. This may suggest that in these tumors, BMI-1 is not necessarily functioning as a suppressor of p16, but rather that other mechanisms, e.g. mutations in the *p16Ink4a* gene, render this tumor suppressor inactive (Sherr 2001). Also, a recent study demonstrates that astrocytes and neural stem cells of BMI-1-deficient mouse show diminished capacity to form tumors independently of $p16^{Ink4a}$ in a xenograft model (Bruggeman *et al.* 2007).

In astrocytic tumors, especially in glioblastoma, we did not find any statistically significant association between BMI-1 expression and patient survival. As is also demonstrated in the above mentioned tumor model, BMI-1-deficient, transformed cells are predominantly biased to a glial lineage. In particular, glioblastomas derived from

BMI-1-deficient astrocytes show distinct glial differentiation. In tissue array, we found considerable heterogeneity in the expression of BMI-1 in glioblastoma. A possible explanation would be that in glioblastoma, characterized by severe chromosomal aberrations, the function of epigenetic regulators of self-renewal and differentiation genes is distorted. Subsequently these factors neither are required for self-renewal nor can any longer inhibit the growth of tumor cells by inducing differentiation (Ohgaki 2005; Kotliarov *et al.* 2006).

1.3 BMI1 aneuploidy in glioma

After elucidating the role of BMI-1 protein expression in glioma, I naturally set forth to investigate the underlying reasons. Often in cancer genes, such as p53, Rb, Her-2 and VEGFR, are mutated or their copy number is altered. Tumor suppressor genes are often deleted or mutated and thus inactive, whereas genes providing a growth advantage to the cancer are usually amplified, thus providing excessive transcripts and increased protein expression. In our panel of tumors, roughly two thirds of the tumors showed no copy number alterations of BMI1 as detected with the CISH method. The minimal commonly deleted region has more often been narrowed at 10p14-p15, and recent reports, using quantitative single strand conformation polymorphism analysis and array-CGH have suggested 10p13 and BMI1 as a novel deleted region in malignant gliomas (Simon *et al.* 2003; Song *et al.* 2006). I detected *BMI1* deletions in high a percentage of tumors, especially in oligodendrogliomas and oligoastrocytomas. Furthermore, *BMI1* deletions were shown to be associated with poor outcome by univariate analysis.

1.4 Aneuploidy does not explain the prognostic significance of BMI-1 protein expression

Seventy-nine tumors were analyzed both for BMI-1 protein expression and using CISH. We did not observe any correlation to prognosis in regard to the *BMI1* gene copy numbers in oligodendroglial tumors. Also, the BMI-1 protein expression did not correlate with the observed genetic status of *BMI1* deletion or gain. Still, one should note that no high-level copy number increases (over 5 copies per cell) of *BMI1* were not found even in high-grade gliomas. This suggests that the frequency of BMI-1 expressing cells is regulated by other mechanisms than gene copy number alterations.

BMI1 deletions were associated with a poor prognosis in glioblastoma and astrocytoma. In accordance, it has been proposed that glioblastoma and high-grade astrocytoma are no longer dependent of cancer stem cells (Clement *et al.* 2007). This would also explain the result that BMI-1 expression did not correlate with survival of glioblastoma patients. Rather, the highly aggressive glioblastoma may have evolved to the stage where angiogenesis and proliferation dominate. In other words, one may consider all tumor cells capable of self-renewal and metastasis without cancer stem cell properties. Further it can be speculated that BMI-1 sustains a phenotype of neural differentiation, actually inhibiting glioblastoma formation in astrocytoma. Alternatively, the high frequency of *BMI1* loss in glioblastoma may reflect the vulnerability of the *BMI-1* gene locus. It should be considered that loss of important tumor-suppressor genes distal of BMI1 on 10p, might infer glioblastoma and high-grade astrocytoma with a stronger growth advantage or angiogenic potential than the effects of concomitant *BMI1* haploinsufficiency (Kimmelmann *et al.* 1996).

1.5 Snail expressing cells are frequent in metastatic pheochromocytoma

There are no distinct histological features that would distinguish between metastatic and non-metastatic pheochromocytomas. The malignant behavior of pheochromocytomas can currently only be defined by the appearance of metastases. Thus, patients are followed up for a long time, monitoring catecholamine levels and symptoms to identify recurrence. It would be of utmost value to the clinician to be able to predict, at the time of surgery, whether future recurrencies and/or metastases are to be expected.

Snail protein expression may help in detecting the potentially relapsing and metastasizing pheochromocytomas. The high frequency of Snail expressing cells in primary pheochromocytomas predicts metastatic potential, and lack of Snail expression is a characteristic of non-metastatic tumors. This may provide valuable guidelines for which individuals should be followed up meticulously (high Snail expression in the primary tumor) and which patients do not necessitate intense follow-up (no Snail protein expression in the primary tumor).

It is particularly important to understand that none of the Snail-negative tumors metastasized during the follow-up of 99 months median. However, a few cases of both metastatic and non-metastatic pheochromocytoma showed a low frequency of Snail-positive cells. Intense follow-up is also warranted for these patients.

Snail suppresses E-cadherin expression in carcinoma cells that also undergo EMT. (Battle *et al.* 2000; Cano *et al.* 2000). Thus, high Snail expression incurs a migratory phenotype to various cancer cell types. These cells are prone to metastasize. It is unclear what role Snail plays in the metastatic pheochromocytomas. It would be plausible that Snail expression enhances migratory properties of these cells as well. However, in pheochromocytomas, Snail expression was not associated with the lack of E-cadherin expression, like in carcinomas (Poser *et al.* 2001). This may be that other members of the cadherin family being the prominent in adrenal chromaffin cells. In this study, only antibodies against E-cadherin (E refers to epithelial). Therefore, further analyses of the cadherins expression in pheochromocytoma and experiments with pheochromocytoma cell lines, are needed to illuminate the mechanisms of Snail function in pheochromocytoma. It is not excluded, that the migratory properties of neural crest stem cells are reactivated in neural crest derived cancers.

1.6 Lack of BMI-1 expression predicts recurrence in OSCC

In this study the expression of the polycomb protein Bmi-1, c-Myc, and Snail was studied in oral tongue carcinoma (OSCC) patients in relation to clinical outcome. These markers have been shown to be prognostic and clinically relevant in neural/neural crest derived tumors in the previous publications of this dissertation (Papers I and III). Somewhat surprisingly, a statistically significant correlation was found between the lack of BMI-1 expression and poor prognosis of OSCC patients. Bmi-1 overexpression has been earlier reported in a small series (N=10) of patients with oral dysplastic and carcinoma tissue (Kang *et al.* 2007). In my study, the material was considerably larger (N=73).

1.7 Function of BMI-1 in OSCC

Several plausible explanations can be found for the result presented above. First, the roles of polycomb proteins are highly varied and depend on the composition of the polycomb repressive complex that Bmi-1 is a part of. The target genes and thus cellular functions such as migration, senescence and proliferation vary considerably (Spivakov and Fisher 2007). Furthermore, Bmi-1 expression has been identified as a prognostic factor only in certain types of cancer. As shown earlier in my communications (Paper I), Bmi-1 expression is prognostic in oligodendroglial tumors, where its expression is abundant, whereas in the much more aggressive high-grade astrocytomas and glioblastomas, Bmi-1 expression is frequently very low, and does not correlate with prognosis. The apparent difference between the role of Bmi-1 in reported nasopharyngeal carcinoma patients (Song *et al.* 2006) and our material of OSSC may also be explained by the fact that the nasopharyngeal carcinoma studied was less differentiated than the tongue carcinoma I studied, based on keratinisation.

1.8 c-Myc in OSCC

c-Myc is presumed to take part in early oral carcinogenesis (Freier *et al.* 2003) and C-Myc mRNA downregulation has been shown to correlate with poor prognosis and progression of the disease (Vora *et al.* 2007). I did not study c-Myc mRNA levels. Therefore, my study merely shows that c-Myc protein expression is varied in OSSC and protein expression levels seem to have no value as a prognostic marker. However, it would be valuable to analyze the relation between c-Myc protein and mRNA levels, because of possible post-tracriptional events regulating c-Myc function in OSCC.

1.9 Snail in OSCC

We found Snail expression in all of our samples, possibly because all samples studied were from invasive carcinomas and none was microinvasive nor *in situ*. Snail expression correlated with the invasion depth in our material suggesting a role in the invasiveness of OSCC and EMT activity. This is interesting because tumor thickness is known to predict both metastasis and local recurrence of tongue carcinoma and oesophageal squamous cell carcinoma (Po Wing Yuen 2002). Snail expression has been linked with lymph node metastases in another type of squamous cell carcinoma (Usami *et al.* 2008) but I could not detect this phenomenon in my study.

2. General discussion

Here I attempt to bring together my observations and place them in the context of literature.

The historical background of the stem cell hypothesis in cancer has already been referred to in the Introduction. Here I wish to consider the following points:

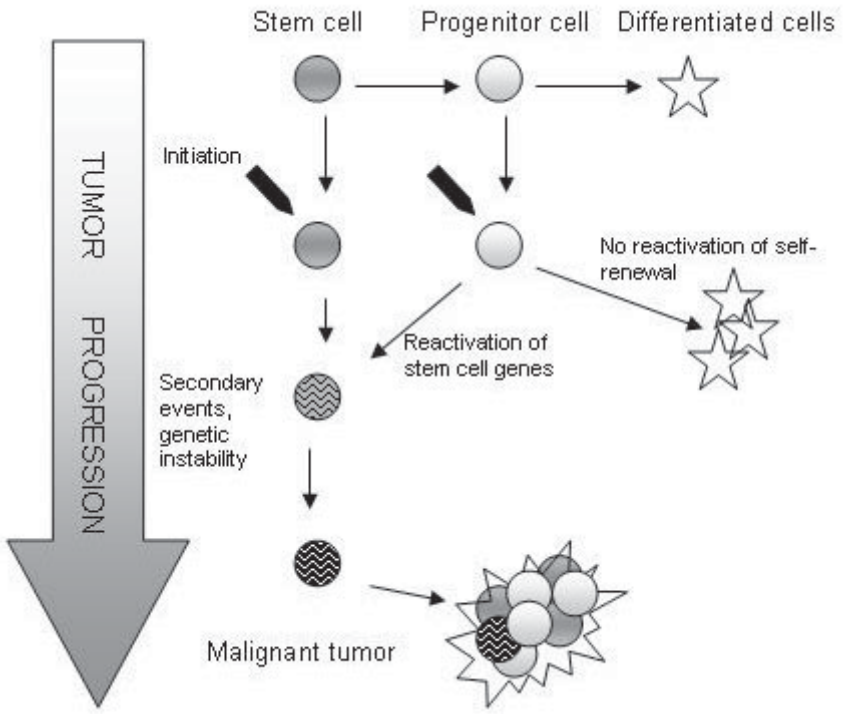
Do my results support or contradict the stem cell hypothesis?

Do my results provide new diagnostic and prognostic parameters in addition to conventional histolopathology in the studied tumors?

2.1 Paradigm of cancer stem cells

The paradigm of cancer stem cell hypothesis may be simplified as below (figure 4).

Figure 4: Stem cell hypothesis in cancer.*



* Events leading to cancer initiation happen randomly, and therefore may target a mature, differentiated cell or a progenitor cell or a tissue stem cell (small black arrow). However, only if a cell capable of self-renewal is affected, do malignant tumors develop. This can be achieved in two ways, either via direct mutations in a stem cell, or the re-activation of self-renewal in a more differentiated cell or progenitor cell (this is seen on the left). If self-renewal is not activated in the pre-cancerous cell, despite further mutations, no tumor will form because eventually, terminal differentiation will occur without the production of new malign cells (this is seen on the upper-right area).

2.2 Do my results support of conflict the stem-cell hypothesis in cancer?

2.2.1 Multiple different ways for a cell to make up a tumor

My results show an interesting finding not consistent with the *in vivo* results on cancer stem cells obtained primarily from xenotransplantation experiments. I found BMI-1 expression in glioma to vary considerably. While some highly malignant, aggressive glioblastomas showed nearly no BMI-1 positive cells, others were nearly 100% immunopositive, when quantifying the frequency of BMI-1 immunoreactive tumor cell nuclei. Furthermore, even low-grade, rather indolent low-grade oligodendrogliomas did show BMI-1 expression ranging from appriximately 10% to 90%. It is noteworthy that I found the most aggressive, potentially most lethal oligodendral tumors to contain over 70% BMI-1 immunoreactive tumor cells. Therefore it is in blatant conflict with the experimental evidence of brain tumors containing roughly 0,5-10% stem cells. Therefore it is worth considering whether BMI-1 in fact is a marker for tumor stem cells or merely an important gene for both tumor cells and stem cells alike.

Clearly, in the xenograft experimets, there is a different experimental setting, and a different marker for glioma stem cells is used (CD133) (Hemmati *et al*. 2003, Singh *et al*. 2004). Also, in these studies, mainly high-grade glioblastoma cells were used. In the light of my results, this may in fact be the type of brain tumor where the stem cell component is least relevant. Only in glioblastoma, did we find cases with a very low frequency of BMI-1 positive cells. However, these were equally aggressive as clinically identical cases, where BMI-1 positive cells were abundant.

In fact, the xenograft model has been criticized. For instance, when transplanting mouse malignancies to histocompatible mice (not NOD-SCID), the frequency of tumor initiating cells was found to be much higher, estimated to be over 10% (Kelly *et al*. 2007).

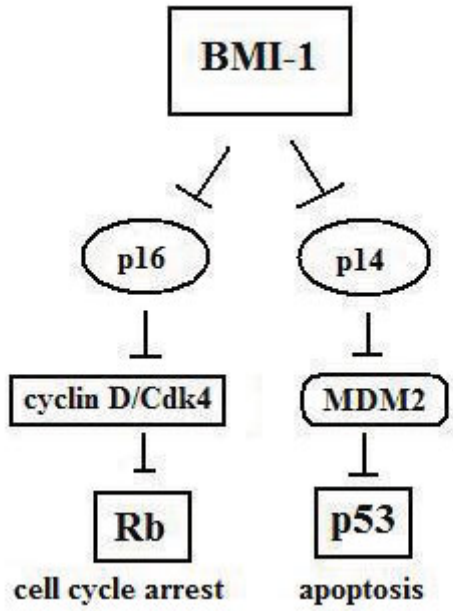
Despite this inconsistency with preclinical experiments, or maybe especially because I used a clinical material of actual patients, one should carefully consider the true nature of the stem cells in human, clinically manifest cancer. It may well be that the frequency and relevance of tumor stem cells is transient. In low grade tumors, the amount of stem cells is prognostic over a time span of several years. On the other hand, in high grade tumors progression is nearly unavoidable.

Expression of stem cell genes in glioma is frequent. The studied material of brain tumors covers the whole spectrum of gliomas, from different subtypes (based on differentiation) of low grade tumors to anaplastic, high grade tumors and glioblastoma multiforme. It is worth emphasis that childhood tumors are not covered (including pilocytic astrocytoma and medulloblastoma). Also, both primary and secondary glioblastoma are included, representing two quite different "roads" to the same clinicopathological phenotype, all expressing BMI-1 (Oghaki 2005).

2.2.2 Possible functions of BMI-1 in regard to the stem cell hypothesis

BMI-1 is essential for the self-renewal of tissue stem cells in many organs. It is essential for brain (cerebellum) development (Leung *et al*. 2004). These effects are mediated by repressing the Ink4a locus encoding the p16Ink4a/p14Arf proteins, which in turn regulate Rb phosphorylation and p53 function. The net effect is inhibiting cell cycle arrest and allowing cell cycle progression. When suppressing the Ink/Arf locus, BMI-1 is a part of a larger protein complex, the polycomb repressive complex 1. Thus, the specific activity of the protein complex may vary, and is also regulated by interaction with the polycomb repressive complex 2. The main pathways are shown below (Figure 5).

Figure 5: Established pathways of BMI-1 regulating the cell cycle.*



* BMI-1 inhibits both p16 and p14. Because p16 inhibits the cyclin/Cdk complex, which in turn phosphorylates and inactivates Rb, inhibition of p16 leads to Rb inactivation and thus cell-cycle progression. On the other hand, the inhibition of p14, which should inhibit mdm2, leads to mdm2 being able to block the tumor-suppressor p53.

Interestingly, progression from low to high grade oligodendroglioma is associated with Rb and p53 dysregulation. This is in 90% of cases preceded by 1pq19 loss (Wen and Kesari 2008). This is difficult to understand because the BMI1 gene is located on chromosome 10, instead of chromosomes 1 or 19. Still, as seen in Figure 4, BMI-1 regulates both Rb and p53.

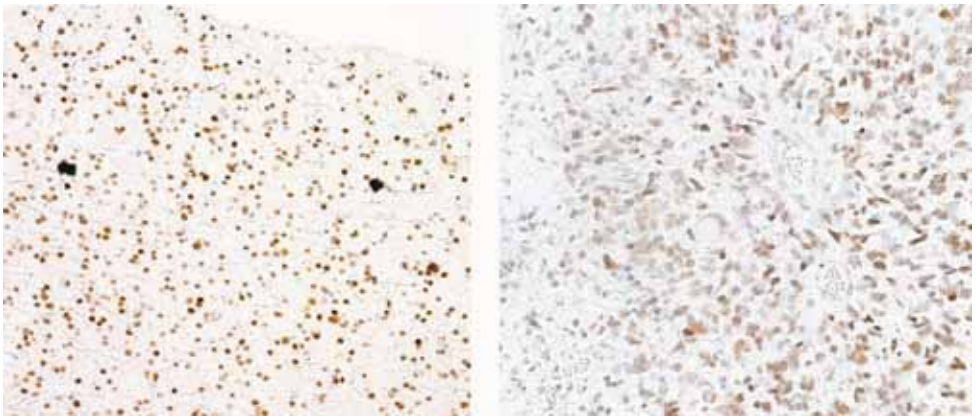
Because BMI-1 is functionally essential for neural stem cells, I chose it as a marker for stem cells in the brain and in brain tumors. Another option would have been CD133, a surface epitope found in both on hematopoietic, embryonic and neural stem cells alike. However, it was when my studies were undertaken, and still very much is now, quite unknown what the function of CD133 is. Therefore I chose not to include it in my study. Lately, CD133 expression has been shown not to be prognostic in glioblastoma, although a combination of CD133 expression and Ki67 expression was shown to be prognostic in a Cox regression model (Pallini *et al.* 2008). On the other hand, p16 is known to be dysfunctional in glioma (Miettinen *et al.* 1999; Kirla *et al.* 2000). It was thus logical to analyze p16 and mdm2 expression, because they are downstream targets of BMI-1.

I found a positive correlation between BMI-1 and p16 protein expression in both oligoastrocytic tumors as well as astrocytoma and glioblastoma. Homozygous deletions of p16 have often been described as a key event in the progression of glioma/glioblastoma. Up to 75% of glioblastomas are reported to harbor deletions of p16 (Solomon *et al.* 2008). What this review article does not address, is the role of BMI-1. It is intriguing that p16 dysfunction is so common in high grade glioma, given that a key regulator of this gene, i.e. BMI-1 is prognostic already in low grade disease. It is commonly believed that "all" low grade

brain tumors will eventually progress into glioblastoma. The question, unanswered so far, remains: how does altered BMI-1 function precede deletions of the genetic loci it directly binds to through polycomb action (i.e. Rb/p53 dysregulation)? The precise nature of polycomb function is unknown, but it has been demonstrated that they act via direct methylation, acetylation etc influence on DNA (Spivakov and Fisher 2007), suggesting that epigenetic factors may be involved.

Although the findings above are supportive of the stem cell hypothesis in glioma, some of my results may also be considered contradictory. The most important such finding is that some samples of high-grade glioma only very few cells were expressing BMI-1. It is interesting to hypothesize that these few cells would be the actual cancer stem cells of the glioblastomas, although my results do not provide evidence for this concept. Further, the overall frequency of BMI-1 positive cells in glioblastomas was lower than in grade 2-3 tumors. The reason is probably that glioblastoma (grade 4) in very chaotic in gene expression, and a shift from quiescence genes and self-renewal-genes and differentiation genes to angiogenesis, proliferation and invasion has occurred (Figure 6). Therefore, these results are, in the end, not necessarily contradictory.

Figure 6: The difference between glioblastoma and astrocytoma.*



* On the left hand panel, a typical low-grade astrocytoma (BMI-1 stain) is shown. Note the relatively high level of organization of the tissue the homogenous texture. On the right hand panel, a typical glioblastoma multiforme is shown. Chaotic and disrupted histology is evident. Necrotic areas, endothelial proliferation, atypical cells and frequent mitotic figures are prominent (BMI-1 stain). Even the gross histology of these tumors suggests that in glioblastoma, several processes are active simultaneously suggesting that the stem-cell component has been suppressed.

2.2.3 Cancer stem cell hypothesis in pheochromocytoma

Pheochromocytoma cell lines have been used for *in vitro* studies because they are capable of neuronal differentiation (Eaton and Duplan 2004). However, no data are available on the existence of tumor stem cells in human pheochromocytoma. Subsequently, neither does the literature provide any data on the possible clinical impact of cancer stem cells in pheochromocytoma.

I found several of the genes studied to be expressed in samples of both malignant and benign pheochromocytomas. However, no clinically relevant pattern of BMI-1 and c-Myc expression could be found. Importantly, still, the genes that promote the primitive stem cell phenotype are expressed in virtually all studied samples suggesting some kind of involvement. Experiments performed *in vitro* support this idea, because c-Myc has been shown to inhibit the differentiation of pheochromocytoma cells (Vaqué *et al*. 2008).

Snail is known to downregulate cadherins. In many carcinomas this is the way by which Snail induces an invasive and metastatic phenotype. However, it has not been shown why Snail is dysregulated in other cancers than carcinoma such as pheochromocytoma. Moreover, I could not demonstrate any correlation between E-cadherin expression and Snail expression. This suggests that the action of Snail is mediated via other mechanisms. Although little evidence is available, it is relevant to hypothesize that Snail is re-activated in chromaffin tissue stem cells and thus a process that mimics neural crest migration is activated, leading to metastases. Thus the role of stem cells in pheochromocytoma progression cannot be verified in my study and need further biochemical and experimental models.

2.2.4 Cancer stem cell hypothesis in oral carcinoma

In oral squamous cell carcinoma, BMI-1 expression varied from entirely negative samples to samples with abundant BMI-1 expression. Further, the prognostic impact of BMI-1 expression was entirely the opposite as initially hypothesized. BMI-1 loss correlated with relapse. This does to some extent contradict the cancer stem cell hypothesis. Interestingly, in nasopharyngeal carcinoma, BMI-1 expression is indeed a marker of poorer prognosis (Song *et al*. 2006). However, like already hypothesized about glioblastoma (where also BMI-1 expression is occasionally very low), loss of BMI-1 may reflect the advancement of the tumor to a stage where angiogenesis, invasion and proliferation dominate over stem cell activity. This may thus be the case in OSCC as well.

Of the other markers studied, we found abundant expression of both Snail and c-Myc, but the prognostic relevance of these markers was not statistically significant. Therefore, although stem cell genes are expressed in oral squamous cell carcinoma, the clinical significance of this is not clear in the light of my results, and therefore also the cancer stem cell theory does not gain support from this study.

2.3 Do my results provide additional prognostic and diagnostic means in cancer?

The "malignancy" of tumor, reflecting its metastatic potential and poor prognosis are predominantly based on histological criteria. These vary from tumor to tumor and are not necessarily sufficient to predict the prognosis. This is seen for example in gliomas and pheochromocytomas, two tumors studied in detail in this Dissertation. Therefore, additional means to classify the tumor and predict prognosis are clearly warranted. Such additional diagnostic criteria are often referred to as "tumor markers".

Here other investigators have considered two different means: (a) further assessment of the pre- or perioperative biopsy histology and (b) assessment of blood or serum to detect and monitor the progression of the disease, like the detection of the PSA in prostatic tumors. Only the first option will be considered here.

The means to cure cancer are surgery, chemotherapy and radiation therapy, often in combination. Solid tumors may be treated by surgery. Traditionally surgeons aim to a

radical result; complete removal of the tumor. In some, but certainly not all cases radical surgical extirpation can be performed with tolerable hardship to the patient. However, particularly upon operations of the CNS, radical removal of the tumor may result in irrectable trauma and severe handicap of the patient (Ilveskoski *et al*. 1996; Ilveskoski *et al*. 1997). Although the current principles of treating glioma underscore extensive surgery, it may be argued that removal of viable brain tissue inevitably leads to loss of function and predisposes to a considerable decrease in quality of life. Thus if a pre- or perioperative assessment of the tumor would with reasonably accuracy reflect the prognosis, it would help the surgeon to decide the extent of radicality.

Generally speaking, surprisingly little evidence on the clinical use of tumor markers exists. Albeit particular markers in certain diseases have been extremely rigorously characterized, e.g. Her2 in breast cancer, and found useful in deciding the postoperative therapy, there are not that many of these. Seemingly, new histological markers are used as an aid in clinical practice regionally without international or even national consensus. This suggests prospective multicenter studies to reach international acceptance.

Whatever the case, some key concepts should be first clarified: can markers be used to estimate the outcome of a patient independent of a therapy (i.e. prognostic value) or the effectiveness of a certain type of therapy (i.e. predictive value). Examples of predictive value are mutated K-ras in colorectal cancer and and prediction of response to Cetuximab/panitumumab therapy, the hormone receptor expression in breast cancer and prediction to endocrine therapy and the marker for mitotic index Ki-67, to mention a few (Oldenhuis *et al*. 2008). Furthermore, prognostic markers can be divided into markers that reflect the disease-free time of patients treated with curative intent or markers that reflect the survival time (progression free time) of patients with metastatic or otherwise incurable disease.

The accuracy of tumor markers in predicting the prognosis may also be compared to registration requirements of new anti-cancer drugs. In clinical Phase 3 trials surprisingly short survival benefits, compared to standard/best before therapy, are accepted for registration by Regulatory Agencies. If far longer survival benefits could be obtained by accurate prognostic markers, this would mean a change in clinical practise, particularly in the delicate desision making between small resection/large resection and, possibly, in the selection of postoperative therapy.

2.3.1 Glioma

Despite this wide range in protein expression, the absolute frequency of BMI-1 positive cells was found to be a prognostic marker independent of histological tumor grade or proliferative index. In my material, BMI-1 in gliomas is a prognostic marker which effectively gives information on the progression free time of patients with oligodendroglioma. It is well-established that low-grade gliomas are always spread throughout the brain and albeit not metastatic, often incurable and usually, in adults, evolve (progress) into aggressive glioblastomas during follow-up. Obviously, preserving as much healthy brain tissue as possible would often be desirable when it comes to the treatment of low-grade brain tumors to preserve the patient's functional capacity.

I am aware, though, that the current treatment guidelines for all types of glioma recommend maximal surgical resection as the first-line treatment (Vives *et al*. 1999). However, because the tumor tissue dies in fact infiltrate througout the brain in microscopic proportions, perhaps only gross removal of the tumor could be applied. Also, excess radiotherapy is highly crippling (Nieder *et al*. 2008). If, through the use of BMI-1 expression as a marker, one could simply, from a stereotactic core biopsy ascertain that the patient possibly has a high chance of surviving many years without tumor progression (=low BMI-1 score) unnecessary aggressive treatment and consequent adverse effects on quality-of-life and function could be avoided.

2.3.2 Oral squamous cell carcinoma

BMI-1 expression in OSCC is interestingly rather different compared with the above discussed neural tumors. This is possibly because oral squamous cell carcinoma is altogether a different type of malignancy; a typical carcinoma of epithelial origin. Also, whereas in tumors arising from neural tissue, cell turnover is generally low, in epithelial tissue and carcinoma proliferation is very high. In our material of OSCC, the patient material is based on curative treatment of low-grade tumors. Surprisingly, lack of BMI-1 expression singles out cases with a high risk of recurrence, both local and/or distant. In these patients, therefore, radical neck dissection on several levels, and intense follow-up may be validated early on, whereas the vast majority of patients, i.e. the BMI-1 positive group, could and should be considered fully cured.

2.3.3 Pheochromocytoma

In pheochromocytoma, the question of predictive or prognostic value of markers is quite dissimilar to the above discussed cases of BMI-1. The main problem in pheochromocytoma is not the prognosis or even the treatment, but rather the initial diagnosis. The vast majority of pheochromocytomas are bening, and require no further treatment after surgical excision, but the diagnostic means to early-on single out malignant cases are lacking. Therefore, one could even consider Snail expression as a prognostic marker. Because none of the cases with absent Snail expression developed metastases, the existence of Snail expression is a prerequisite for intense follow-up and radical surgery versus less aggressive treatment.

2.4 Assessment of clinical value

In order to systematically evaluate prognostic markers, Hayes proposed a "Tumor Marker Utility Grading System" (TMUGS) (Hayes *et al.* 1996). In this a semiquantitative scale for estimating the utility of a marker in the clinical setting is used. Also, a five-tier scale is applied to judge the level of evidence.

TMUGS is designed to evaluate the clinical utility of tumor markers and to establish an investigational agenda for the evaluation of new tumor. Hayes states that the knowledge of tumor marker data should contribute to a decision in practice that results in a more favorable clinical outcome for the patient, including increased overall survival, increased disease-free survival, improvement in quality of life, or reduction in cost of care. Semiquantitative utility scales were thus developed for each end point. The only markers recommended by these authors for use in routine clinical practice are those that are assigned utility scores of "++" or "+++" on a 6-point scale (ranging from 0 to +++) in the categories relative to more favorable clinical outcomes. Furthermore, each utility score assignment should be supported by documentation of the level of evidence used to evaluate the marker. Hayes grades the level of evidence as follows: I (high-powered, prospective, controlled study), II (marker is determined in relationship to prospective therapeutic trial), III (large but retrospective studies), IV (small retrospective studies), V (small pilot study).

Below, I have evaluated my results according to the TMUGS (Table 5).

Table 5: My findings assessed according to TMUGS

marker	disease	utility	level of evidence	conclusion
BMI-1	glioma	++	III	Consider clinical use, prospective studies still needed.[1]
Snail	pheochromocytoma	+++	IV	Likely to provide high additional value in clinical use.[2]
BMI-1	oral squam. cell ca.	+	III	Marker may be of value as addition to other parameters.[3]
Oct4	glioma	0	III	Not expressed in studied cases.
Sox2	glioma	0	III	Does not correlate with clinical parameters.
c-Myc	glioma	+	III	Limited additional value in selected cases.
BMI-1	pheochromocytoma	0	IV	Does not correlate with clinical parameters.
Snail	oral squam. cell ca.	+/-	III	Very limited evidence of clinical correlates.
c-Myc	oral squam. cell ca.	0	III	No known correlation with clinical parameters.
mdm2	glioma	+/-	III	Limited additional value in selected cases.

Footnotes:
(1) BMI-1 in glioma: Provides considerable prognostic value in oligodendroglial tumors independent of other, previously known parameters. Current data, although retrospective, warrants immediate clinical use if careful monitoring is available. To fully evaluate the usefulness of BMI-1 as a prognostic marker in oligodendroglial tumors, prospective trials, including pre- or perioperative BMI-1 histology combined with optimal treatment regimes according to the BMI-1 status of each individual tumor and intense follow-up are required.

(2) Snail in pheochromocytoma: Because Snail expression is observed exclusively in metastatic pheochromocytomas, it delivers for the first time an immunohistochemical marker for metastatic disease. Because no other such marker is known, immediate incorporation into clinical practice is suggested. However, this with the following caveat: because PET, imaging and biochemistry (catecholamine metabolites) are standard practice already, Snail immunohistochemistry use should be added to these, not omitting the other diagnostic tools since Snail expression detects metastatic disease, but not the anatomical location of these metastases. In the future, the diagnostic use of Snail immunohistochmistry should be confirmed from larger trials with extended follow-up.

(3) BMI-1 in OSCC: There are other prognostic factors in OSCC and the use of BMI-1 compared to these has not been evaluated. Also, in the current study all patients received curative treatment in the beginning of the retrospective follow-up but the treatment was heterogenous and curative treatment was only defined according to clinical and histological criteria including imaging/ultrasound. Furthermore, the follow-up time was quite short. Importantly, because of the short follow-up, BMI-1 immunohistochemistry could not be reliably evaluated with regard to patient survival, and only a correlation with relapse, not survival, was seen.

SYNOPSIS

The combined results of this thesis provide evidence both supporting and contradicting the stem cell hypothesis of cancer from a clinical viewpoint. Taken together, the involvement of stem-cell gene products in all the studied malignancies is unequivocally demonstrated. However, the possible effect of cancer stem cells on patient survival and clinical outcome is not obvious in all of the different types of diseased studied. Rather, my data confirms what often is obvious to any clinician: the course of disease and behavior a malignancy in human patients is very complex and often no single factor can be said to dominate the process of cancer as may be the case *in vitro*.

Still, several novel prognostic markers were found. Importantly, these were unrelated to conventional markers used in histopathology (e.g. nestin, Ki-67, E-cadherin, growth factor receptors). In the view of my results as well as the available literature, stem cell based markers in these tumors seem to be more accurate in predicting relapse than previously used markers, which are based on differentiated cancer cells and probe altogether different processes such as proliferation, tumor vasculature, enzymatic activity but do not attempt to assess the presence of self-renewing stem cells. My view is that in the future, pathologist and clinicians should focus on the assessment of the stem cell component and the expression of genes involved in stem cell function in tumors in order to provide better prognostic and predictive information than conventional histopathology.

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