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MARJU KASE

Glioblastoma multiforme: possibilities to improve treatment efficacy

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Glioblastoma multiforme: possibilities to improve treatment efficacy

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Paper I:	participation in experimental work, data collection, data analysis and writing the manuscript.
Papers II–IV:	participation in study design, data collection, data analysis and writing the manuscript.

ABBREVATIONS

CDKN	CDK-dependent kinase inhibitor
CI	Confidence interval
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CSC	Cancer stem cell
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DSB	Double-strand brakes
EBRT	External beam radiation therapy
EGFR	Epidermal growth factor receptor
EORTC	European Organisation for Research and Treatment of Cancer
FACS	Fluorescence activated cell scanning
GBM	Glioblastoma multiforme
GTV	Gross tumor volume
H&E	Hematoxylin-eosin
HR	Hazard Ratio
ICAM-1	Intercellular adhesion molecule 1
IDH1	Isocitrate dehydrogenase 1
IHC	Immunohistochemistry
KPS	Karnofsky performance Status
LOH	Loss of heterozygosity
MRI	Magnetic Resonance Imaging
PARP-1	Poly(ADP-ribose) polymerase 1
PDGFRA	Platelet-derived growth factor receptor alpha
PDGF-alpha	Platelet-derived growth factor alpha
PDGFR	PDGF receptor
PTEN	Phosphatase and tensin homolog
PTV	Planned target volume
shRNA	Short hairpin RNA
RT	Radiation therapy
RTOG	Radiation Therapy Oncology Group
SD	Standard deviation
SSB	Single-strand brakes
TIMP-4	Tissue inhibitor of metalloproteinase-4
TMZ	Temozolomide
VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor 2
WHO	World Health Organization

1. LITERATURE OVERVIEW

1.1. INCIDENCE

Glioblastoma multiforme (GBM) is the most feared type of primary central nervous system (CNS) cancer, not only for poor prognosis, but also because of the direct influence on the quality of life and cognitive function. GBM accounts for approximately 20–45% of all malignant primary CNS tumors and 82% of high grade (WHO grades III and IV) gliomas [1–3].

Overall, about 27,700 new CNS primary cancers are diagnosed every year in Europe, with an annual incidence rate of 4.8 per 100000 person-years for astrocytic tumors (including GBM) [4]. Nevertheless, for malignant tumors, the incidence rates are highest for GBM, being approximately 2–3 cases per 100000 person-years in most European and North-American countries [5,3]. The incidence rates of GBM in Estonia are 1.8 cases per 100000 person-years in females and 2.2 cases per 100000 person-years in males [6].

The incidence of GBM increases with age, being more common in older adults and uncommon in children. The average age at diagnosis of GBM is 64 years of age and, for reasons yet unclear, it has demonstrated a clear male predilection, being about 1.57 times more common in males than females [3].

1.2. SURVIVAL

Despite aggressive and combined therapy, median survival of GBM patients is still unsatisfactory, extending only up to 15 months [7]. However, few patients with GBM (3–5%) survive for more than 3 years and are referred to as long-term survivors [8]. The relative survival estimates for glioblastoma are quite low, since less than 5% of patients survive five years post diagnosis [3,9]. GBM survival estimates are somewhat higher for the small number of patients who are diagnosed under the age of 20 [3]. Nevertheless, survival times of GBM patients have remained largely unchanged for more than 50 years, pointing toward the urgent need for more effective treatment choices [10].

1.3. CLASSIFICATION AND DESCRIPTION

The World Health Organization (WHO) classification system groups gliomas into 4 histological grades defined by increasing degrees of undifferentiation, anaplasia, and aggressiveness [11,12]. Grade I tumors are biologically benign and total removal leads to recovery. Grade II tumors are low-grade malignancies that may follow long clinical courses, but early diffuse infiltration of the surrounding brain renders them often not totally resectable. Grade III tumors exhibit aggressive behaviour characterized by increased anaplasia and mitosis over grade II tumors and due to this biological pattern, these tumors progress rather quickly. Grade IV tumors, also known as GBM, exhibit more advanced

features of malignancy, including vascular proliferation and necrosis and are often refractory to radiotherapy or chemotherapy [13,14]. Figure 1 illustrates characteristic histological features of GBM (abundant angiogenesis, glomeruloid vascular proliferations and necrosis).

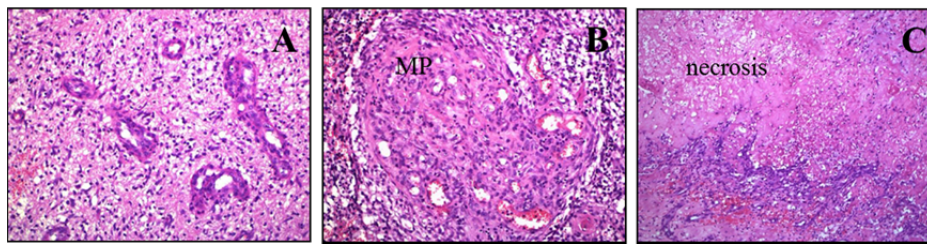

Figure 1. Histological characteristics of GBM
Histological hallmarks of GBM are: abundant angiogenesis (A, x40), microvascular proliferations (MP) (B, x40), and necrosis (C, x10).

GBM is the most malignant of all astrocytic tumors. GBM is defined by the hall-mark features, such as uncontrolled cellular proliferation, diffuse infiltration, propensity for necrosis, robust angiogenesis, intense resistance to apoptosis, and genomic instability [15]. GBM presents with significant intratumoral heterogeneity on the cytopathological, transcriptional, and genomic levels. This complexity, combined with a putative cancer stem cell (CSC) subpopulation and an incomplete (epi)genetic lesions driving GBM pathogenesis, make this cancer one of the most difficult to understand and to treat [15].

On the basis of clinical presentation, GBMs have been further subdivided into the primary or secondary GBM subtypes [13]. Primary GBMs account for the great majority of GBM cases in older patients, while secondary GBMs are quite rare and tend to occur in patients below the age of 45 year. Primary GBM presents in an acute de novo manner with no evidence of a prior symptoms or antecedent lower grade pathology. In contrast, secondary GBM derives consistently from the progressive transformation of lower grade astrocytomas, with approximately 70% of grade II gliomas transforming into grade III/IV disease within 5–10 year of diagnosis [14]. Moreover, only 5% of all cases estimated as secondary glioblastomas have histopathological evidence of a precursor low-grade or anaplastic astrocytoma [16]. Remarkably, despite their distinct clinical histories, primary and secondary GBMs are morphologically and clinically indistinguishable as reflected by an equally poor prognosis when adjusted for patient age [14].

In majority of patients, GBM occurs as a local disease affecting different parts of the brain. However, in rare cases, distant metastases in other organs may develop. For example, the presence of metastatic lesions of GBM have been described in bones [17–20], lungs [21,18,22–24], heart [24], spleen [25], liver [26], cutaneous and subcutaneous tissue [27,28], parotid gland [29–31] and lymph nodes [32–34]. It is believed that metastatic glioblastoma cells, like other

types of cancer cells, use common dissemination pathways, such as blood and lymphatic vessels [35]. Also, next to previously described ways, extraneural spread of GBM has been reported [36].

1.4. HEREDITARY SYNDROMES AND GENETIC PROFILE

Most GBMs appear to be sporadic, without any genetic predisposition. Nevertheless, a number of hereditary syndromes are associated with an increased risk of glioma, including Cowden, Turcot, Li-Fraumeni and von Hippel-Lindau syndromes, as well as neurofibromatosis type 1 and type 2, tuberous sclerosis and familial schwannomatosis [9]. This suggests a putative genetic relationship but in a small minority, approximately 5% of GBM patients [37].

Loss of heterozygosity (LOH) on chromosome arm 10q is the most frequent gene alteration for both primary and secondary GBMs [16]. LOH occurs in up to 80% of GBM cases and it is reported to be more extensive in primary than in secondary GBMs [38]. Mutations in *p53,* a tumor suppressor gene, are more commonly seen in secondary GBM [39]. These mutations are present in more than two-thirds of secondary GBMs but rarely seen in primary GBM [16]. Epidermal growth factor receptor (EGFR) amplification has been identified as a genetic hallmark of primary glioblastomas that occurs in 40–60% of cases [40,41]. Amplification or overexpression of *MDM2* gene constitutes an alternative mechanism to escape from *p53*-regulated control of cell growth. Overexpression of *MDM2* is the second most common gene mutation in GBM [16]. Platelet-derived growth factor alpha (*PDGFA*) gene acts as a major mitogen for glial cells. Platelet-derived growth factor receptor alpha (PDGFRA) overexpression and isocitrate dehydrogenase 1 (IDH1) mutations are some of the major genetic alterations found in low grade gliomas, as well as secondary GBMs. When the low grade tumors progress toward the high grade secondary GBMs, additional changes such as CDK-dependent kinase inhibitor (CDKN2A/CDKN2B) deletion are acquired [42]. *PTEN* (phosphatase and tensin homolog also known as *MMAC* and *TEP1*) encodes a tyrosine phosphatase located at band 10q23.3. Loss of PTEN function is the most common alteration in primary but not in secondary GBMs [43].

1.5. RISK FACTORS

The specific cause of GBM is unknown and identifying various risk factors has proven difficult. However, the cause-effect relationship between ionizing radiation and the development of GBM was established with studies that demonstrated that children treated with radiotherapy for malignancies like leukemia have a markedly increased risk for developing GBM [44]. Additionally, the use of cell phones that are known to release a small amount of non-ionizing electromagnetic radiation, has been suggested as possible risk factor for glio-

mas, especially in the cases of long-term use [45]. Pesticide exposure (possibly also during pregnancy), is associated with an increased childhood brain tumors risk but no positive association of farm pesticide exposure and glioma risks in adults has been identified [46,47]. Head trauma has been suggested as a possible risk factor for developing GBM. Although epidemiological studies do not support a definitive connection between head injury and intracranial glioma, there are few case reports of post-traumatic gliomas published [48–53]. Family history of any cancer probably is not an important risk factor for adult glioma, however, a family history of brain tumors may play a role [54]. Previous studies have also found that gliomas are inversely associated with the presence of atopic diseases such as asthma, eczema, and hay fever [55]. It has also been suggested that viral infections (e.g. human herpesvirus 6, cytomegalovirus, simian virus 40) may be associated with the development of GBM by tumorigenesis through integration of viral genetic material into normal DNA [56–58].

Preventive measures, such as lifestyle and dietary changes, are ineffective in averting gliomas [59]. Moreover, early diagnosis and treatment unfortunately do not improve outcomes, precluding the utility of screening for this disease [60].

1.6. CLINICAL PRESENTATION

The clinical history of patients with GBM is usually short, being less than 3 months in more than 50% of patients, unless the neoplasm has developed from a lower-grade astrocytoma [61,62]. Figure 2 illustrates a clinical course of GBM patient treated at Tartu University Hospital. In this patient, symptoms (mainly headache) lasted for 3 months before final diagnosis of GBM was established after surgery.

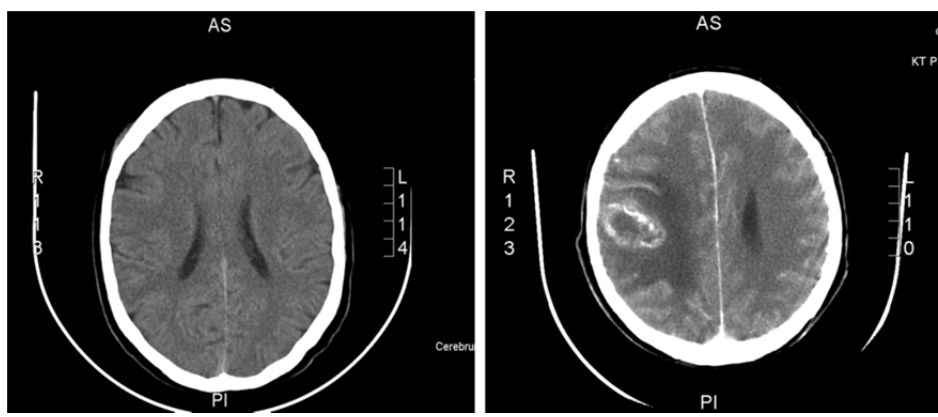

Figure 2. Clinical course of GBM patient treated at Tartu University Hospital
Computer tomography images were obtained at the time of first onset of headache (left, January 2012) and 3 months later (right, April 2012). On the first CT image, tumor was not visible but 3 months later contrast-enhancing tumor and very extensive peritumoral oedema were present.

The character of the symptoms depends highly on the location of the tumor. Common symptoms of GBM include headache, seizures, nausea, vomiting and hemiparesis, however, due to temporal and frontal lobe involvement (most frequent sites of GBM) symptoms such as progressive memory and neurological deficits, as well as personality changes may develop [63,64].

Headaches are relatively frequent, present in about 50% of patients at diagnosis, but usually with a nonspecific pain pattern [65]. Progressive severity, unilateral localization, and new-onset headache in a patient older than 50 years are some of the features that may distinguish a tumor-associated headache from a benign headache [9]. Seizures manifest in about 20% to 40% of patients, and usually a focal onset is reported [66]. As described earlier, cognitive difficulties and personality changes may develop and are often mistaken for psychiatric disorders or dementia, particularly in elderly individuals [9]. Focal signs such as hemiparesis, sensory loss, or visual field disturbances are common and reflect tumor location. Also, gait imbalance and incontinence may be present, usually in larger tumors with significant mass effect. Papilledema is associated with significantly increased intracranial pressure and is now rarely seen because imaging is usually obtained at earlier disease stages. Occasionally, the development of symptoms is rapid, mimicking a stroke. Speech disorder may be mistaken for confusion or delirium [9].

1.7. PROGNOSTIC FACTORS

Radiation Therapy Oncology Group (RTOG) has proposed a prognostic score of malignant gliomas based on patient and tumor features (age, Karnofsky performance Status (KPS), extent of surgery) [67]. The European Organisation for Research and Treatment of Cancer and NCIC Clinical Trials Group (EORTC/NCIC) confirmed the prognostic value of recursive partitioning analysis including only GBM patients [68]. Additionally, German Glioma Network findings underline the association of GBM long-term survival with prognostically favourable clinical factors, in particular young age and good initial performance score, as well as O6-methylguanine-DNA methyltransferase (MGMT) gene promoter hypermethylation [8]. Also, a 20% reduction in the hazards of mortality in patients with a specific histological form (giant cell GBM) compared to GBM has been reported [69].

1.8. TREATMENT

Unfortunately, none of GBM treatment choices is curative.

Upon initial diagnosis of GBM, standard treatment consists of maximal surgical resection, radiotherapy, and concomitant and adjuvant chemotherapy with temozolomide (TMZ) [14,7,9]. For older patients, less aggressive therapy is sometimes suggested, using radiation or chemotherapy alone [70,71].

1.8.1. Surgery

Surgery is the first therapeutic modality for GBM.

After neuroimaging, patients with suspected malignant glioma should be considered for surgical resection, aiming at relieving mass effect, achieving cytoreduction, and providing adequate tissue for histologic and molecular tumor characterization [9]. In inoperable tumors, stereotactic biopsy may be performed for histologic diagnosis, but the limited amount of tissue acquired may preclude full molecular characterization.

The goal of surgery is to achieve gross total resection of the contrast enhancing component of the tumor (seen in Magnetic Resonance Imaging, MRI), without compromising neurological function (maximal safe resection) [72]. In some circumstances, gross total resection may not be possible based on anatomic structures invaded by the tumor. Advances in surgical imaging techniques, such as intraoperative ultrasound and MRI, diffusion tensor imaging, awake craniotomy, cortical mapping, stereotactic guidance, and fluorescent-guided resection, have facilitated delineation of tumor borders and can help optimize maximal safe surgical resection [73,74,72]. Therefore, whenever possible, patients should be referred for surgery in tertiary care facilities, which provide optimized surgical tools (advanced intraoperative monitoring, awake mapping, and functional and intraoperative MRI) and allow for adequate handling, processing, and storage of the tissue, including comprehensive molecular characterization and tissue profiling that may guide subsequent treatments [9,75].

1.8.2. Radiotherapy

Although maximal surgical resection remains important, more than 90% of patients with glioma show recurrence at the original tumor location or within 2 to 3 cm from the border of the original lesion [76–78]. Therefore, surgical resection is combined with adjuvant therapy to prolong survival.

Since 1978, patients with GBM have been treated by debulking surgery (to the extent that is safely feasible) and postoperative radiotherapy [79]. Radiation therapy (RT) uses controlled high-energy rays to kill cancer cells by damaging directly or indirectly the DNA inside cells making them unable to divide or reproduce and delay a recurrence of the tumor. Abnormal cancer cells are more sensitive to radiation because they divide more quickly than normal cells. Over time, the abnormal cells die and the tumor shrinks. Normal cells can also be damaged by RT, but they can repair themselves more effectively. The area where the radiation is delivered (called the radiation field) is carefully calculated to include the smallest possible amount of normal brain as possible, so called the "involved field" (the original area of the tumor plus a small margin around).

The current standard of care for RT in GBM is focal, fractionated external beam radiation therapy (EBRT) to the surgical resection cavity and to a 2–3 cm margin of surrounding brain tissue. Usually, 60 Gy of RT is delivered in fractions of 2 Gy over 6 weeks [7]. Although radiotherapy is still the corner-

stone adjuvant treatment in GBM, at the present, the combination of RT plus chemotherapy with temozolomide (TMZ) is the most efficacious adjuvant therapy after primary resection. Treatment following surgery usually consists of 6 weeks of RT to the surgical cavity and concomitant TMZ, followed by 6 adjuvant cycles of TMZ [7].

1.8.3. Chemotherapy

Chemotherapy refers to the use of medicines to stop or slow the growth of cancer cells. Chemotherapy works by interfering with the ability of rapidly growing cells (like cancer cells) to divide. Because most of an adult's normal cells are not actively growing, they are not affected by chemotherapy, with the exception of bone marrow (where blood cells are produced), the hair, and the lining of the gastrointestinal tract. Effects of chemotherapy on these and other normal tissues cause side effects during treatment.

Up to 2005, chemotherapy had no demonstrable clinical benefit in GBM, and RT alone remained the standard of care after surgical resection [72]. However, in 2005, a clinical trial demonstrated that concurrent RT and TMZ followed by adjuvant TMZ significantly prolonged the median survival more than that of radiation alone (14.6 months versus 12.1 months; $p < 0.001$). These findings established the therapeutic benefit of TMZ in combination with RT, establishing the so-called "Stupp regimen", standard of care for GBM treatment [7].

TMZ is an oral alkylating chemotherapeutic agent that causes DNA damage and triggers a cascade of events leading to tumor cell apoptosis [72]. Previous studies have shown that patients with an unmethylated DNA repair enzyme O6-methylguanine-DNA methyltransferase (MGMT) gene are much less responsive to TMZ, whereas MGMT methylation confers sensitivity to TMZ in patients with GBM [9,72].

Implantation of carmustine wafers into the resection cavity is another approved treatment of GBM [80]. Similar to TMZ, carmustine is a DNA alkylating agent. Carmustine is released into the surrounding brain tissue immediately after tumor resection and its effect last for several weeks [73]. In clinical trials, carmustine wafers used in combination with radiation and TMZ have been shown to modestly prolong survival in subsets of patients. However, because there are complications associated with the use of wafers, including infection, swelling, need for removal, and impairment of wound healing, they are not used routinely at most centers [81,82,80].

After first-line treatment, virtually all glioblastoma patients experience disease progression after a median progression free survival of 7 to 10 months [83]. Salvage treatments include surgical re-resection, re-irradiation and chemotherapy (bevacizumab, TMZ rechallenge, carmustine, lomustine, carboplatin, irinotecan) [9,84–86]. Unfortunately, none of the available salvage treatments has clearly shown improved survival and likely only benefit in selected patients. Treatment choices should be therefore individualized, and clinical trials strongly considered.

2. INTRODUCTION TO THE STUDY

Since 1978, local radiotherapy, administered after debulking surgery, has been a mainstay of standard treatment of GBM patients [79]. Although radiotherapy results in excellent local control and cure rates in most solid tumors [87], the efficacy of this treatment modality in GBM is extremely limited, resulting only in disease stabilization for a few months [79,80,7]. Almost all GBM patients (99%) develop fast disease progression and tumor recurrence within or immediately adjacent to the high-dose radiation (60 Gy) volumes [88,89], whereas local recurrences are also described after very high doses such as 90 Gy [90]. Therefore, GBM, by nature one of the most radioresistant tumors, represents a major challenge in neuro-oncology.

Detailed information about molecular mechanisms of radioresistance of GBM is not known. The basis of radioresistance may involve many tumor cell and surrounding microenvironment processes, including changes in growth factors and their receptors, different signaling and apoptotic pathways and DNA repair mechanisms [91–95]. Since there is an urgent need for new treatment strategies to improve the chance of survival for the patients of this fast-killing disease, precise knowledge about these resistance mechanisms is of great importance.

2.1. PARP-1 and DNA-PK

Radiotherapy causes a variety of DNA lesions, including single-strand brakes (SSB) and double-strand brakes (DSB) [96]. The lethal lesion is an unrepaired or misrepaired DSB produced as part of a complex lesion [97].

PARP-1 (Poly(ADP-ribose) polymerase 1) is an enzyme of PARP superfamily that is responsible for most of PARP activity [98]. The most well-known role of PARP-1 is the detection of SSB [99]. After binding to radiation-induced SSB (damage detection), activated PARP recruits repair enzymes (X-ray repair cross-complementing group 1, DNA polymerase-β, DNA ligase III) that are involved in base excision repair (BER) pathway. Recruited enzymes process broken DNA ends, synthesize missing DNA and seal the gap in DNA [100, 101].

DNA-PK (DNA-dependent protein kinase) plays an important role in DNA DSB repair by nonhomologous end joining (NHEJ) pathway [102]. DNA-PK is a kinase that binds to DNA DSB, phosphorylates, and activates DNA-binding proteins (X-ray repair cross-complementing protein 4, DNA ligase IV) [103]. Due to interaction of these enzymes, double strand break ends are directly ligated [103,104]. Since DNA repair enzyme inhibitors enhance the cytotoxic effects of DNA-damaging agents (radiation, chemotherapy), their role in cancer therapy is increasingly explored [105].

2.2. CD133

According to the brain tumor cancer stem cell model, a subpopulation of cancer cells possesses the capacity of self-renewal, tumor formation and the capability to form progeny with a more restricted fate [106]. In GBM, several stem cell candidate markers have been explored, however, out of these, CD133 is the most studied [93,107].

CD133 is a transmembrane glycoprotein which is expressed in different type of progenitor cells, including hematopoietic stem cells. CD133+ GBM cells are considered stem cells because of their ability to self-renew, differentiate and to initiate tumor formation *in vivo* [108]. An injection of as few as 100 CD133+ cells has been shown to produce a tumor that could be serially transplanted and resembled phenotypically the patient's original tumor [108].

Previous *in vitro* and *in vivo* studies have proposed that CD133+ tumor cells represent the cellular population that confers GBM radioresistance and could therefore be the source of tumor recurrence after radiation [109].

2.3. CD63

Radioresistance of GBM involves tumor-cell related changes, as well as changes that occur in tumor surrounding microenvironment. It has been reported that both constituents of tumor microenvironment, inflammatory and immune response markers are expressed in GBM [110,111]. Nevertheless, their exact role and impact on radiotherapy efficacy is not known.

CD63 is a lysosomal glycoprotein that is expressed on activated platelets, monocytes, macrophages, as well as on granulocytes, T-cells and B-cells [112]. Therefore, CD63 represents one of the markers of inflammation and immune response that might influence tumor micromilieu and thereby cancer cells.

2.4. ANGIOGENESIS, VEGFR-2

GBM is one of the most angiogenic tumors. Therefore, in recent years, the inhibition of tumor angiogenesis has been an extremely attractive and dominating experimental therapeutic strategy in neuro-oncology [113,114].

In GBM, at least five mechanisms by which tumors achieve neovascularization have been described: vascular co-option, angiogenesis, vasculogenesis, vascular mimicry, and glioblastoma-endothelial cell transdifferentiation [115]. Out of these, angiogenesis and vasculogenesis have been most extensively studied and described. During angiogenesis, blood vessels arise from sprouting and proliferation of endothelial cells from pre-existing vascular network, whereas in vasculogenesis, *de novo* blood vessels are formed through colonization of circulating bone marrow-derived endothelial progenitor cells that are recruited to the tumor [115]. Both previously mentioned mechanisms of neo-

vascularization are largely regulated via vascular endothelial growth factor (VEGF) and its receptor 2 (VEGFR-2) [116].

Downstream effects of VEGFR-2 activation in the vascular endothelium include cell proliferation, migration, permeability and survival, resulting in neovascularization processes, such as angiogenesis and vasculogenesis [116]. Consequently, this receptor has been very attractive target in the development of antiangiogenic drugs (e.g. bevacizumab, sunitinib, sorafenib, vatalanib, vandetanib, recentin, cediranib) [116]. Unfortunately, a number of these antiangiogenic drugs (vandetanib, cediranib, sorafenib, sunitinib) have failed to show clinical efficacy in different phases of clinical trials both in newly diagnosed and recurrent glioblastoma [117–121]. Moreover, the most advanced antiangiogenic drug in glioblastoma – bevacizumab – did not get approval from The European Medicines Agency Committee for Medicinal Products for Human Use (CHMP) due to the lack of clinically relevant efficacy [122,123]. All these negative trials have caused a lot of frustration since the results do not coincidence with the initial expectations. The reasons of the lack of significant clinical efficacy of antiangiogenig drugs, however, are not fully elucidated.

It has been shown that tumor microenvironment influences GBM treatment outcome [95]. Whether inflammatory tumor microenvironment, which is one of the characteristic histological features of GBM, affects the expression of VEGFR-2 is not clear.

3. AIMS OF THE STUDY

The general aim of the study was to identify possibilities to improve the efficacy of radiotherapy and chemotherapy in patients with GBM.

Accordingly, the specific aims were:

1. To explore, whether higher tumor levels of DNA repair enzymes (PARP-1, DNA-PK) contribute to worse treatment results of GBM patients after postoperative radiotherapy.
2. To test, whether higher proportion of CD133+ GBM cells (stem cell population) contributes to worse treatment results after postoperative radiotherapy.
3. To evaluate the impact of tumor infiltrating CD63+ inflammatory and immune cells on radiotherapy treatment response and survival of GBM patients.
4. To evaluate the impact of tumor microenvironment, particularly inflammatory reaction, on the expression of VEGFR-2 – one of the main targets of antiangiogenic drugs.

4. MATERIAL AND METHODS

4.1. PATIENTS

All the studies were approved by the Research Ethics Committee of the University of Tartu, Estonia.

Between January 2006 and December 2008, maximum of 42 patients with GBM were treated with postoperative three-dimensional (3D) radiotherapy at Tartu University Hospital or North Estonia Medical Centre. Characteristics of patients are listed in table 1.

Table 1: Characteristics of 42 patients with glioblastoma multiforme

Variable	No of patients (n=42)	Percentage (%)
Gender		
▪ Male	23	55%
▪ Female	19	45%
Age, years (mean)*	19–77 (57 years)	
Radiotherapy dose (mean)	30–60 Gy (54 Gy)	
Chemotherapy**		
▪ No	16	38%
▪ Yes	26	62%

* Age at the time of operation
** Used for recurrent disease

4.2. TREATMENT PLANNING AND TREATMENT PARAMETERS

Treatment planning was performed using CT/MRI scans and TPS XiO CMS treatment planning system. The gross tumor volume (GTV) encompassed the resection cavity and any residual tumor. A 2–3 cm margin was added to create clinical target volume (CTV). Critical tissues were spared (brainstem, chiasma). For planned target volume (PTV), 0.5 cm margin was included. Treatments were performed using linear accelerators (30–60 Gy in 2.0 Gy fractions; mean dose 54 Gy). The prescribed dose was normalized to 100% at the isocenter and PTV was covered by 95% isodose surface (ICRU Report 50). None of the patients received concomitant and adjuvant chemotherapy with TMZ (available since 2010). However, for recurrent disease, 26 patients received chemotherapy with lomustine (CCNU).

4.3. HISTOLOGY

Surgically excised GBM specimens were immediately fixed in the buffered 10% formaline (pH 7.4) for 24 hours and subsequently embedded into paraffin wax as routinely performed. From the resulting tissue blocks, serial paraffin sections of 4 μm were cut and placed on glass slides for standard hematoxylin-eosin (H&E) stain and immunohistochemistry. The diagnosis of GBM was confirmed in the H&E stained slides by 2 independent pathologists.

4.4. IMMUNOHISTOCHEMISTRY

Additional sections were cut from archived paraffin blocks and stained according to standard immunohistochemistry (IHC) protocol.

For immunostaining, solutions and buffers provided by DAKO (Hamburg, Germany) were used. The sections were deparaffinized and incubated in the target retrieval solution (pH 9.0) in the 96°C thermostated water bath for 40 min and afterwards in peroxidase blocking solution for 5 min at room temperature. Subsequently, the tissue sections were incubated with the specific antibodies at room temperature for 1 hour in humid conditions. After several washings, the antigen-antibody complex was visualized by using DAKO REALTM EnVision Detection System, Peroxidase/DAB+, Rabbit/Mouse. Diaminobenzidine was used as chromogen. Slides were counterstained with hematoxyline, dehydrated and coverslipped for light microscopy.

Characteristics of primary antibodies used for immunohistochemical staining are listed in table 2.

Table 2: Characteristics of primary antibodies used for immunohistochemical staining

Primary antibody	Supplier	Catalogue number	Dilution
anti-PARP-1 antibody	Bethyl Laboratories	#IHC-00279	1:250
anti-DNA-PKcs antibody	Bethyl Laboratories	#IHC-00044	1:250
anti-CD133 antibody	Biorbyt Ltd.	#orb18124	1:50
anti-CD63 antibody	Santa Cruz Biotechnology, Inc.	#sc-5275	1:150
anti-ICAM-1 antibody	Santa Cruz Biotechnology, Inc.	#sc-8439	1:100
anti-VEGFR-2 antibody	Santa Cruz Biotechnology, Inc.	#sc-6251	1:100

4.5. EVALUATION AND SCORING

Evaluation and scoring of slides are shortly described in table 3.

Table 3: Evaluation and scoring of slides

Parameter	Staining	Scoring	Description
Inflammatory reaction	H&E	Arbitrary score 1–3	1= weak inflammation 2= moderate inflammation 3= strong inflammation
Necrosis	H&E	Percentage (%)	Overall extent of necrosis
PARP-1 staining intensity (tumor cells)	IHC	Arbitrary score 0–3	0= no staining 1= weak staining intensity 2= moderate staining intensity 3= strong staining intensity
DNA-PK staining intensity (tumor cells)	IHC	Arbitrary score 0–3	0= no staining 1= weak staining intensity 2= moderate staining intensity 3= strong staining intensity
CD133+ cells (stem like cells)	IHC	Percentage (%)	Proportion of CD133+ cells per microscopic field
CD63+ cells (inflammatory and immune cells)	IHC	Number	Number of CD63+ cells per microscopic field
ICAM-1 (tissue expression)	IHC	Optical density (0–255)	Pixel values measured by digital image analysis
VEGFR-2 staining intensity (endothelial)	IHC	Arbitrary score 0–3	0= no staining 1= weak staining intensity 2= moderate staining intensity 3= strong staining intensity

4.5.1. Histology

H&E stained sections were used to assess two parameters, both determined by an experienced pathologist.

First, the overall extent of necrosis was assessed. For this, the whole section of tumor tissue was evaluated and overall proportion (%) of necrosis estimated.

Afterwards, overall extent of inflammatory reaction was determined. This was based on typical visual appearance of inflammation, including presence of edema and inflammatory cell infiltration. For the evaluation, an arbitrary score ranging from 1 to 3 was applied (1= weak, 2= moderate, 3= strong inflammatory reaction).

4.5.2. Immunohistochemistry

The evaluation and scoring of immunohistochemically stained slides were carried out in a blinded fashion.

4.5.2.1. PARP-1 and DNA-PK

The evaluation and scoring of slides were carried out in 5 randomly taken microscopic fields at a magnification of × 40. Scoring was performed twice by one researcher.

Immunohistochemical staining intensities of PARP-1 and DNA-PK were quantified using an arbitrary score ranging from 0 to 3 (0= no staining; 1= weak, 2= moderate, 3= strong staining intensity). GBM tissue displayed mainly cytoplasmic expression of PARP-1 and nuclear expression of DNA-PK. Therefore, for PARP-1 cytoplasmic and for DNA-PK nuclear staining intensities were determined. Staining intensities of PARP-1 and DNA-PK positive cells were scored for each of five microscopic fields (excluding negative cells and necrotic areas). For individual values, the mean of 10 scores (2 x 5 fields) was calculated.

Individual means were used to determine group mean and median values. According to median values of PARP-1 and DNA-PK, patients were divided into subgroups ≥ median (equal and more than median) and < median (less than median). These subgroups were used in survival analysis.

4.5.2.2. CD133

The proportion of CD133-positive (CD133+) cells was determined in 6 randomly taken microscopic fields at a magnification of × 40. The evaluation and scoring of slides were carried out by 2 independent researchers.

For individual values, the mean proportion of CD133+ cells (%) was determined in 6 microscopic fields. These values were used to evaluate the correlation between the assessments of 2 independent researchers. Afterwards, the mean proportion of CD133+ cells in 12 microscopic fields (2 × 6 fields) was calculated. The proportion of CD133+ GBM cells was determined in areas with vital tumor tissue (excluding necrotic areas).

Individual means of CD133+ cell proportions were used to determine group mean and median values. According to the median value of CD133+ proportions, patients were divided into subgroups ≥ median (equal and more than median) and < median (less than median). These subgroups were used in survival analysis.

4.5.2.3. CD63

The evaluation and scoring of slides were carried out in 5 randomly taken microscopic fields at a magnification of × 40. The evaluation was carried out by 2 independent researchers.

For individual values, the mean number of tumor infiltrating CD63-positive (CD63+) inflammatory and immune cells per microscopic field was determined in 5 microscopic fields. These values were used to evaluate the correlation between the assessments of 2 independent researchers. Afterwards, the mean number of tumor infiltrating CD63+ cells in 10 microscopic fields (2 × 5 fields) was calculated. The number of CD63+ cells was determined in areas with vital tumor tissue (excluding necrotic areas).

Individual means of CD63+ inflammatory and immune cell numbers were used to determine group mean and median values. According to the median value of CD63+ numbers, patients were divided into subgroups ≥ median (equal and more than median) and < median (less than median). These groups were used in survival analysis.

4.5.2.4. ICAM-1

For intercellular adhesion molecule 1 (ICAM-1) expression, digital IHC image analysis was performed. Tissue expression of ICAM-1 was determined at a magnification of × 10.

IHC digital image analysis was carried out in 6 selected images from each slide by using the freeware program ImageJ. The brown-colored area, occupied by the immunohistochemical reaction was selected by the color threshold filtering tool to substract the hematoxylin-stained areas at the background. Then the images were converted to the greyscale and the optical density by the area method was measured in pixel values ranging from 0–255. Value 0 represents the lightest shade of the color while 255 the darkest shade of the color in the image.

4.5.2.5. VEGFR-2

The evaluation and scoring of slides were carried out in 5 randomly taken microscopic fields at a magnification of × 40. The evaluation was performed by 2 independent researchers.

For VEGFR-2 expression, two parameters were assessed. First, the number of VEGFR-2 positive (VEGFR-2+) blood vessels per microscopic field was determined. Additionally, endothelial VEGFR-2 staining intensity was evaluated using an arbitrary score ranging from 0 to 3 (0= no staining; 1= weak, 2= moderate, 3= strong staining intensity).

For individual values, both parameters were determined in 5 microscopic fields. These values were used to evaluate the correlation between the assessments of 2 independent researchers. Afterwards, the mean number of VEGFR-2+ blood vessels and VEGFR-2 staining intensity in 10 microscopic fields (2 ×

5 fields) were calculated. All VEGFR-2 parameters were determined in areas with vital tumor tissue (excluding necrotic areas).

4.6. STATISTICAL ANALYSIS

The SPSS statistical software was used to calculate individual means, group means, and standard deviations of the mean as well as median values. In addition, the author used Pearson correlation analysis.

The magnitude of pre-irradiation PARP-1 and DNA-PK expression, the proportion CD133+ GBM cells and the number of tumor infiltrating CD63+ inflammatory and immune cells were correlated with the overall survival that was defined as the period from the date of operation to the date of death resulting from GBM or to the date of last analysis. Survival curves were created using the Kaplan-Meier method and differences between the groups were compared using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards model. Due to small number of patients, maximum of 4 variables were included into multivariate analysis. A p-value <0.05 was regarded statistically significant.

5. RESULTS

5.1. PARP-1 and DNA-PK (Paper I)

5.1.1. Expression of PARP-1 and DNA-PK

In a normal brain, a weak constitutive expression of PARP-1 and DNA-PK was seen. GBM tissue displayed various levels of PARP-1 (mainly cytoplasmic) and DNA-PK (nuclear) expression. Figure 3 illustrates low (score 1) and high (score 3) expression of PARP-1 and DNA-PK in the tumor tissue.

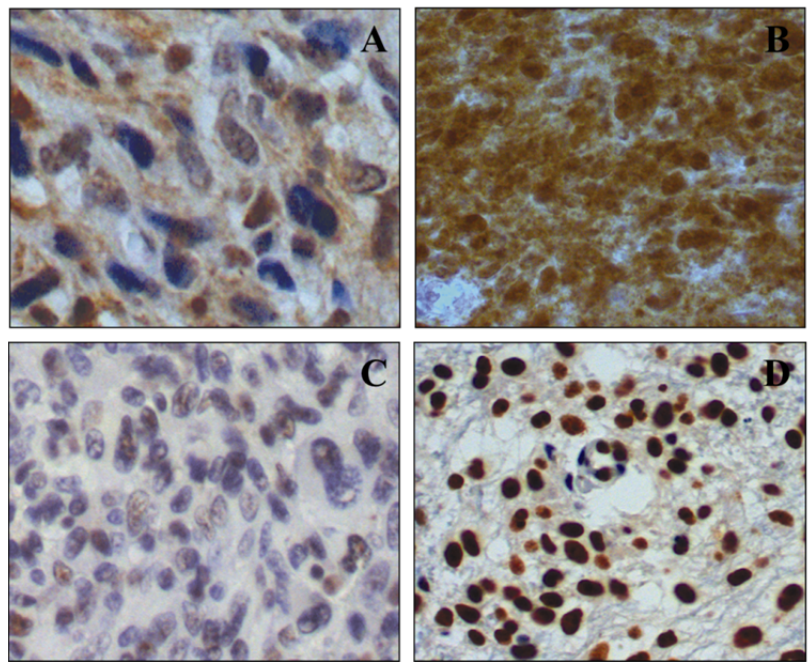

Figure 3. Expression of PARP-1 and DNA-PK in GBM
Photos illustrate PARP-1 and DNA-PK immunohistochemical staining intensities in GBM tissue. A: weak cytoplasmic (score 1) staining intensity of PARP-1, B: strong cytoplasmic (score 3) staining intensity of PARP-1, C: weak nuclear (score 1) staining intensity of DNA-PK, D: strong nuclear (score 3) staining intensity of DNA-PK.

Among individual GBM patients, the magnitude of PARP-1 immunoreactivity in the tumor tissue ranged from 1.2 to 2.8 (individual means). The mean and median values of PARP-1 expression of the entire study group were 1.96±0.50 (mean±SD) and 2.0 respectively. There were slightly more patients with high (≥median) expression levels of PARP-1 (56%) in the study group. Individual levels of DNA-PK expression ranged from 0.8 to 2.8 (individual means). The

mean and median values of DNA-PK expression of the entire study group were 2.02±0.49 (mean±SD) and 2.0, respectively. Similarly to PARP-1, there were more patients with high (≥median) expression levels of DNA-PK (65%) in the whole study group.

5.1.2. Correlation of tumor PARP-1 and DNA-PK expression with overall survival

Thirty two patients had died by the time of analysis. The median overall survival of the whole study group was 10.0 months (95% CI 8.1–11.9). The overall survival among the patients with low (<median) and high (≥median) expression levels of PARP-1 is represented in figure 4. The median survival of the patients with low and high tumor PARP-1 levels did not differ significantly, being 12.0 months (95% CI 8.3–15.7) and 10.0 months (95% CI 7.9–12.1) respectively (p=0.93). In contrast, figure 5 illustrates significant overall survival difference between the patients with low (<median) and high (≥median) expression levels of DNA-PK. The median survival of the patients with low and high tumor DNA-PK levels were 13.0 months (95% CI 10.7–15.3) and 9.0 months (95% CI 7.2–10.8) respectively (p=0.02).

DNA-PK expression (HR 3.9, 95% CI 1.5–10.7, p=0.01) and Karnofsky Performance Score (KPS; HR 3.3, 95% CI 1.4–8.4, p=0.01) emerged as the significant independent prognostic factors for overall survival in the multivariate analysis (Table 4).

Table 4: Multivariate analysis for overall survival

		Overall survival	
Variable		p	HR (95% CI)
DNA-PK*	IHC range 0.8–2.8	0.01	3.9 [1.5–10.7]
PARP-1*	IHC range 1.2–2.8	0.12	0.5 [0.2–1.2]
Chemotherapy	yes vs no	0.89	1.1 [0.4–2.6]
Karnofsky performance score	<70% vs ≥70%	0.01	3.3 [1.4–8.4]

*Continuous variable; IHC – Immunohistochemical staining intensity; HR – Hazard Ratio;
CI – Confidence Interval

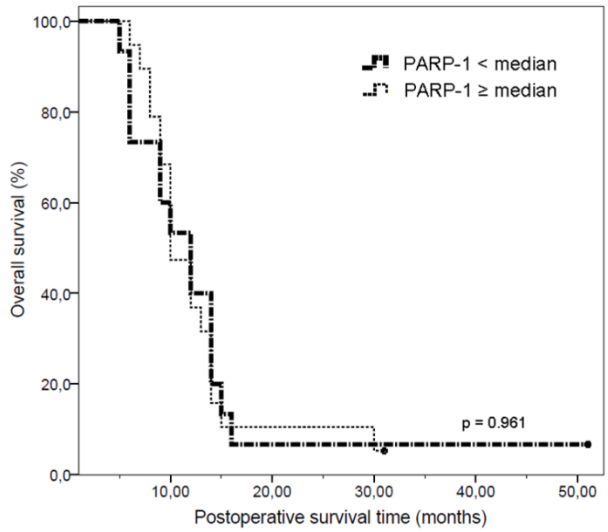

Figure 4. Kaplan-Meier analysis of overall survival according to PARP-1 expression
The median survival of patients with low and high tumor PARP-1 levels did not differ (p=0.961).

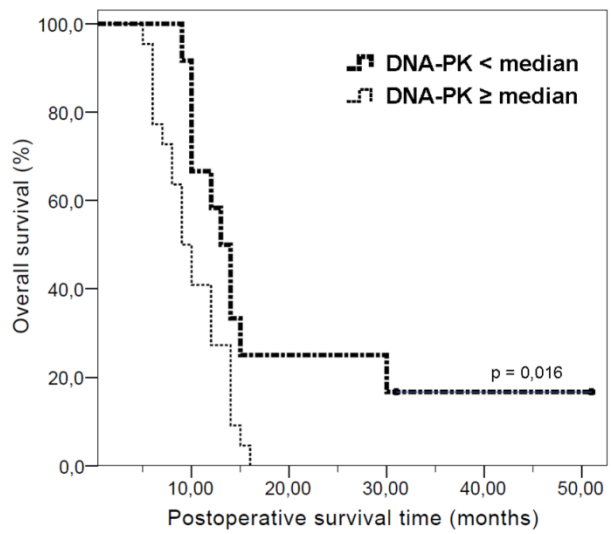

Figure 5. Kaplan-Meier analysis of overall survival according to DNA-PK expression
The median survival of patients with low and high tumor DNA-PK levels differed significantly (p=0.016). The median survival of the patients with low and high tumor DNA-PK levels were 13.0 months (95% CI 10.7–15.3) and 9.0 months (95% CI 7.2–10.8) respectively.

5.2. CD 133 (Paper II)

5.2.1. Proportion of CD133+ GBM cells

In GBM tumor samples, the proportion of CD133+ cells varied greatly between patients. Figure 6 illustrates low (<median) and high (≥median) CD133+ cell proportions in the tumor tissue.

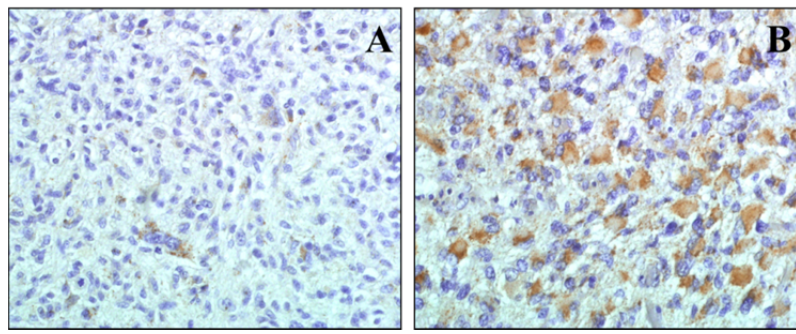

Figure 6. CD133+ stem cells in GBM
Photos illustrate different proportions of CD133+ stem cells (A: low, B: high) in GBM tissue.

The proportion of CD133+ GBM stem cells was determined by 2 independent researchers whose results were in good accordance ($R=0.8$, $p<0.0001$).

Among individual GBM patients, the proportion of CD133+ stem cells in tumor tissue ranged from 0.5% to 82% (individual means). Mean and median proportions of CD133+ cells of the entire study group were 33%±24% (mean± SD) and 28% respectively. According to individual values, patients were divided into two groups: patients with low (<median) and high (≥median) proportion of CD133+ GBM cells. Groups were sufficiently balanced, since there were 48% of patients with low (<median) and 52% of patients with high (≥median) proportion of CD133+ cells.

Additionally to IHC the overall proportion of necrosis (%) was determined in haematoxylin-eosin stained tissue sections. The mean proportion of necrosis of the entire study group was 38%±31% (mean±SD). Correlation analysis, based on the individual values, revealed a significant association between the proportion of stem-cells and the percentage of necrosis ($R=0.5$, $p<0.01$).

5.2.2. Correlation of CD133+GBM cell proportion with overall survival

Forty patients had died by the time of analysis. The median overall survival of the whole study group was 10.0 months (95% CI 9.0–11.0). Figure 7 illustrates the overall survival among patients with low and high proportion of CD133+ GBM cells. Their survival times clearly depended on the proportion of CD133+ cells (log rank test, p=0.02). Median survival times for patients with low (<median) and high (≥median) proportion of CD133+ cells were 9.0 months (95% CI 7.6–10.5) and 12.0 months (95% CI 9.3–14.7) respectively.

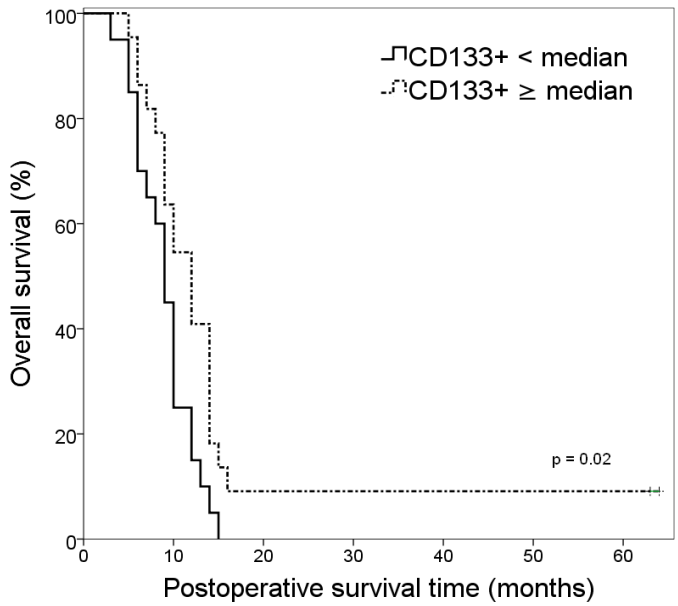

Figure 7. Kaplan-Meier analysis of overall survival according to CD133+ GBM stem cell proportions
The median survival of patients with low and high proportions of CD133+ stem cells differed significantly (p=0.02). Median survival times for patients with low (<median) and high (≥median) proportion of CD133+ cells were 9.0 months (95% CI 7.6–10.5) and 12.0 months (95% CI 9.3–14.7) respectively.

In multivariate analysis (table 5), the proportion of CD133+ cells (HR 2.0, 95% CI 1.0–3.8, p=0.04) and Karnofsky Performance Score (HR 2.2, 95% CI 1.0–4.8, p=0.04) emerged as significant independent prognostic factors for overall survival.

Table 5: Multivariate analysis for overall survival

Variable		Overall survival	
		p	HR (95% CI)
CD133+	<median vs ≥median	0.04	2.0 [1.0–3.8]
Radiotherapy dose*	range 30–60 Gy	0.24	1.0 [0.9–1.0]
Chemotherapy	yes vs no	0.75	1.1 [0.5–2.4]
Karnofsky performance score	<70% vs ≥70%	0.04	2.2 [1.0–4.8]

* Continuous variable; HR- Hazard Ratio; CI- Confidence Interval

5.3. CD63 (Paper III)

5.3.1. Numbers of tumor infiltrating CD63+ inflammatory and immune cells

In GBM tumor samples, the numbers of CD63+ cells varied greatly between patients. Figure 8 illustrates low (<median) and high (≥median) CD63+ inflammatory and immune cell numbers in tumor tissue.

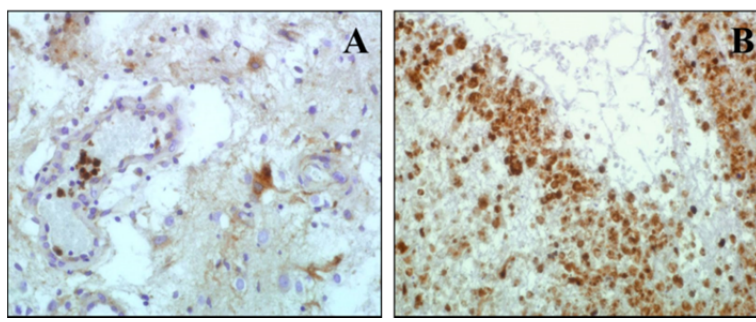

Figure 8. CD63+ inflammatory and immune cells in GBM
Photos illustrate different numbers of CD63+ inflammatory and immune cells (A: low, B: high) in GBM tissue.

The numbers of CD63+ cells was determined by 2 independent researchers whose results were in good accordance ($R=0.8$, $p<0.0001$).

Among individual GBM patients, the numbers of CD63+ cells per microscopic field ranged from 10.3 to 134.5 (individual means). The mean and median numbers of CD63+ inflammatory and immune cells of the entire study group were 45.3±27.2 (mean±SD) and 39.6, respectively. According to individual values, patients were divided into two groups: patients with low (<median) and high (≥median) numbers of CD63+ tumor infiltrating cells. Groups were balanced, since there were 50% of patients with low (<median) and 50% of patients with high (≥median) numbers of CD63+ cells.

In addition to immunohistochemistry, the overall proportion of necrosis (%) was determined in haematoxylin-eosin stained tissue sections by an experienced pathologist. The mean proportion of necrosis of the entire study group was 38%±31% (mean±SD). Correlation analysis, based on individual values, revealed a significant association between the numbers of CD63+ tumor infiltrating inflammatory and immune cells and the percentage of necrosis (R=0.5, p=0.004).

5.3.2. Correlation of tumor infiltrating CD63+ inflammatory and immune cell numbers with overall survival

Thirty eight patients had died by the time of analysis. The median overall survival of the whole study group was 10.0 months (95% CI 9.0–11.0). Figure 9 illustrates the overall survival among patients with low and high numbers of CD63+ tumor infiltrating inflammatory and immune cells. The survival times clearly depended on the number of CD63+ cells (log rank test, p=0.003). Median survival times for patients with low (<median) and high (≥median) numbers of CD63+ cells were 9.0 months (95% CI 8.1–9.9) and 12.0 months (95% CI 8.5–15.5), respectively.

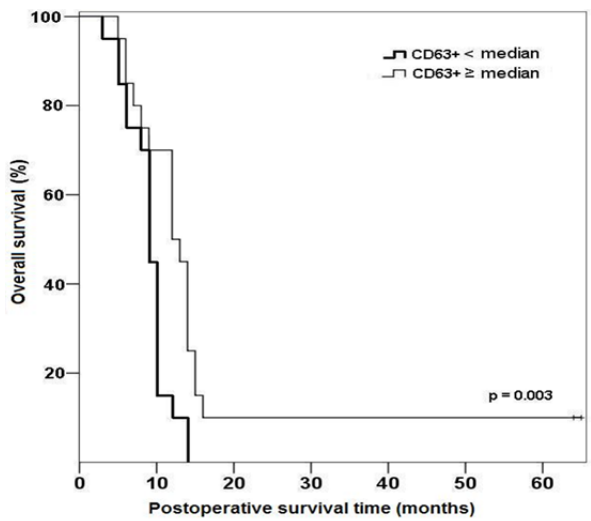

Figure 9. Kaplan-Meier analysis of overall survival according to CD63+ inflammatory and immune cell numbers
The median survival of patients with low and high numbers of CD63+ inflammatory and immune cells differed significantly (p=0.003). Median survival times for patients with low (<median) and high (≥median) numbers of CD63+ cells were 9.0 months (95% CI 8.1–9.9) and 12.0 months (95% CI 8.5–15.5) respectively.

In multivariate analysis (Table 6), the number of CD63+ tumor infiltrating inflamematory and immune cells (HR 2.4, 95% CI 1.2–5.1, p=0.02) emerged as significant independent prognostic factor for OS.

Table 6: Multivariate analysis for overall survival

Variable		Overall survival	
		p	HR (95% CI)
CD63+	<median vs ≥median	0.02	2.4 [1.2–5.1]
Radiotherapy dose*	range 40–60 Gy	0.59	1.0 [0.9–1.1]
Chemotherapy	yes vs no	0.66	1.2 [0.6–2.5]
Karnofsky performance score	<70% vs ≥70%	0.13	1.9 [0.8–4.5]

* Continuous variable; HR- Hazard Ratio; CI- Confidence Interval

5.4. Inflammation, ICAM-1 and VEGFR-2 (Paper IV)

5.4.1. Inflammation and ICAM-1

In individual GBM samples, the extent of inflammation varied, being in the whole group 1.9±0.7 (mean±SD). Figure 10 represents GBM tissues with weak (A), moderate (B), and strong (C) visual inflammatory reaction.

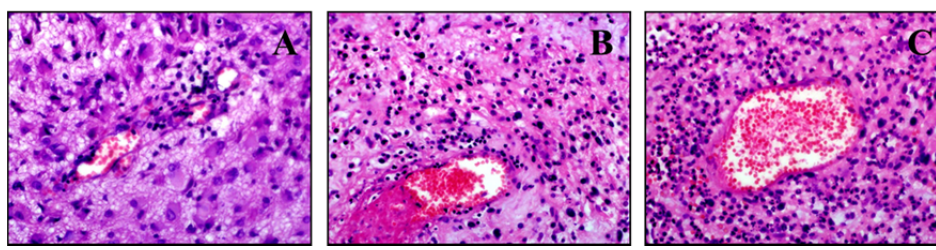

Figure 10. Inflammatory reaction in GBM
Photos illustrate GBM tissues with inflammatory reaction. A: weak (score 1) inflammation, B: moderate (score 2) inflammation, C: strong (score 3) inflammation. Note different numbers of tumor infiltrating inflammatory cells.

Similarly, individual optical densities of ICAM-1 in GBM tissue varied, ranging from to 17.6 to 154.9 pixel values. Group mean optical density of ICAM-1 was 57,0±27.1 (mean±SD). Figure 11 illustrates GBM tissues with weak (A), moderate (B), and strong (C) optical density of ICAM-1.

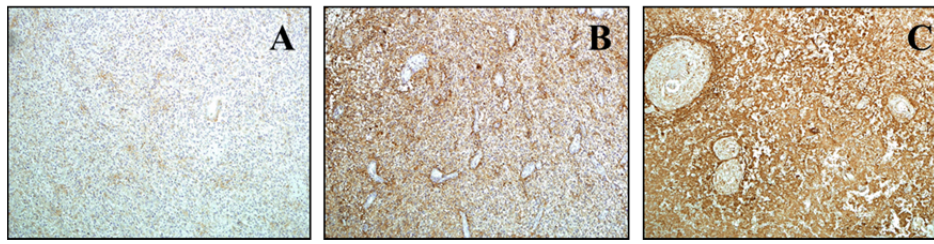

Figure 11. ICAM-1 expression in GBM
Photos illustrate GBM tissues with different extent of ICAM-1 expression. A: weak optical density, B: moderate optical density, C: strong optical density.

5.4.2. VEGFR-2

VEGFR-2 parameters were determined by 2 independent researchers whose results were in good accordance ($R=0.8$, $p<0.0001$).

In GBM tissue sections, the number of VEGFR-2+ blood vessels per microscopic field and endothelial VEGFR-2 staining intensity were 6.2±2.4 (mean±SD) and 1.2±0.8 (mean±SD) respectively. Figure 12 illustrates GBM tissues with weak (A), moderate (B), and strong (C) expression level of VEGFR-2 in tumor blood vessels.

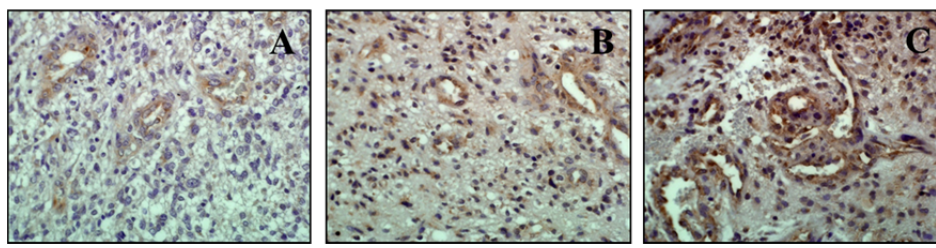

Figure 12. VEGFR-2 expression in GBM blood vessels
Photos illustrate GBM tissues with different endothelial staining intensities of VEGFR-2 in blood vessels. A: weak (score 1) staining intensity, B: moderate (score 2) staining intensity, C: strong (score 3) staining intensity. Note also different numbers of VEGFR-2+ blood vessels.

5.4.3. Correlation of VEGFR-2 expression with inflammation and ICAM-1

The results of correlation analysis are described in table 7. A positive association was found between the extent of visual inflammation and VEGFR-2 staining intensity ($R=0.4$, $p=0.005$). Moreover, VEGFR-2 staining intensity correlated with the expression level (optical density) of tissue ICAM-1 ($R=0.4$, $p=0.026$). Additionally, there was a trend toward significant association

between the number of VEGFR-2 positive blood vessels and VEGFR-2 staining intensity in GBM tissue (R=0.3, p=0.065).

Table 7: Correlation of VEGFR-2 expression with inflammation and ICAM-1*

Correlations	p value
Visual inflammatory reaction and VEGFR-2 staining intensity	p=0.005
Tissue ICAM-1 expression and VEGFR-2 staining intensity	p=0.026
The number of VEGFR-2+ blood vessels and VEGFR-2 staining intensity	p=0.065

* Bivariate Pearson correlation test

6. DISCUSSION

6.1. PARP-1 and DNA-PK

In the present study, we assessed 2 DNA-repair enzymes (PARP-1, DNA-PK) in GBM tissue before standard radiotherapy. Our study revealed a weak constitutive expression of PARP-1 and DNA-PK in normal brain tissue. Similarly, constitutive expression of PARP-1 and DNA-PK within normal brain has been documented in previous human studies [124,125]. A primary function of PARP-1 and DNA-PK under basal condition is the detection of DNA damage and the facilitation of DNA repair to maintain genomic integrity [126,127]. Similarly to our findings, the high expression of DNA repair enzymes in GBM tissue has been described in other studies [125,128].

In the present study, the median survival of the entire study group was 10.0 months. This is in a good accordance with previous studies where postoperative radiotherapy has resulted in median survival of 9.0–11.6 months [79,129,80]. Since GBM patients had different tumor levels of PARP-1 and DNA-PK, we looked whether differences in protein expression influence the treatment outcome. Our study showed that survival did not depend on PARP-1 expression in tumor tissue, since median survival of GBM patients with high or low levels of PARP-1 did not differ. In contrast, survival was significantly influenced by the expression level of DNA-PK, showing much shorter median survival of patients with high tumor levels (9.0 months) compared to patients with low levels (13.0 months). Moreover, multivariate analysis showed that next to the well-established prognostic factor KPS [130], DNA-PK expression emerged as a significant independent predictor for overall survival. This suggests that GBM patients with high tumor levels of DNA-PK are more resistant and respond less to standard radiotherapy.

No clinical studies have been published which compare treatment outcome of GBM patients after radiotherapy depending on tumor PARP-1 and DNA-PK expression levels. Therefore, the data of the present study cannot be compared with similar studies. In spite of that, there are some preclinical studies where inhibition of these DNA repair enzymes (resulting in low levels of these enzymes) has been tested in GBM cell cultures. The effect of PARP-1 inhibitor Olaparib in combination with radiotherapy has been evaluated in four GBM cell lines (T98G, U373-MG, UVW, U87-MG) [131]. In three GBM cell lines, decrease in surviving fraction of tumor cells, radiosensitization and delayed repair of radiation-induced DNA-brakes were seen. However, in U87-MG cell line, Olaparib had no effect on radiation sensitivity in 2 independent studies [131,132]. This shows that the inhibition of PARP-1 might be insufficient to increase the radiosensitivity of GBM, which is also suggested in our study.

Previous *in vitro* studies have demonstrated that DNA-PK-deficient cell line (MO59J) is approximately 30-fold more sensitive to radiation than DNA-PK-proficient cell line (MO59K) [133]. Moreover, specific DNA-PK inhibitors SU11752 and wortmannin have demonstrated to sensitize GBM cells (MO59K)

to radiotherapy [134]. Interestingly, SU11752 alone did not affect GBM cell survival. However, in combination, SU11752 sensitized cells fivefold to ionizing radiation at 2 Gy with even more pronounced effect at higher radiation doses. Most recent *in vitro* study confirmed that novel DNA-PK inhibitor NU7741 increased the cytotoxicity of irradiation by twofold in MO59-Fus-1 cells (DNA-PK-proficient) whereas it had no effect on MO59J cells [135]. NU7741 decreased the repair of radiation-induced DSB and inhibited also homologous recombination activity (assessed by Rad51 foci) in a DNA-PK dependent manner. This shows that there is a potential cross talk of DNA-PK with another important DNA repair pathway – homologous recombination. Additionally, specific inhibition of DNA-PK with short hairpin RNA (shRNA) has been shown to radiosensitize glioma-initiating cells [136].

Since only high expression of DNA-PK correlated with poor survival of GBM patients, the repair of DSB rather than SSB might have a prognostic value. Similarly, previous experimental studies have shown that the incidence of cell killing, and higher response to radiotherapy do not correlate with the induced number of SSB, but relates better to the incidence of DSB [15]. Radiotherapy has been shown to produce equivalent number of DNA lesions in MO59J (DNA-PK-deficient) and MO59K (DNA-PK-proficient) GBM cell lines [137]. Nevertheless, significant DNA damage repair was evident for MO59K cells with a 5.8 fold increase in relative survival, whereas MO59J GBM cells showed little repair capacity. More DSB were repaired by 30 min in MO59K cells than in MO59J cells, suggesting that deficient DSB repair may be a major determinant of radiosensitivity of GBM cells.

Present study measured only baseline (preirradiation) levels of PARP-1 and DNA-PK expression. Due to study design, changes in these proteins that might occur during radiotherapy cannot be described. However, previous *in vitro* study has shown that there is a radiation-induced increase in the activity of PARP in glioblastoma cell line A172 [138]. Also, in MO59K cell line, both DNA-PK relative protein level and DNA-PK activity have been shown to increase in response to irradiation [139]. In contrast, there was no increase in DNA-PK protein level and no detectable kinase activity in DNA-PK deficient MO59J cells, either with or without irradiation.

In the present study, only postoperative radiotherapy was used. However, radiotherapy alone is no longer standard adjuvant treatment of GBM. In countries with access to temozolomide, combined treatment consisting of radiotherapy and concomitant as well as adjuvant chemotherapy (radiochemotherapy) is preferably used [7]. Whether tumor PARP-1 and DNA-PK expression have the prognostic value for patients treated with combined treatment schedule, is not clear. Nevertheless, the effect of DNA repair enzymes on combined treatment results is rather plausible since TMZ has been shown to produce DNA lesions that are substrates for base excision repair and homologous recombination DNA repair pathways [140,141]. *In vivo*, mice treated with combined treatment of PARP inhibitor E7016 plus radiotherapy and temozolomide, showed additional growth delay of six days compared with

the combination of radiotherapy and temozolomide [142]. Additionally, previous *in vitro* studies have reported that in glioblastoma cells (MO59K, MO59J), the sensitivity of temozolomide depends on DSB repair efficiency since DSB are critically involved in drug-induced apoptosis [143].

In conclusion, this hypothesis generating study showed that the survival of GBM patients receiving postoperative radiotherapy depends on the tumor expression of DNA-PK. Further studies are needed to clarify whether DNA-PK inhibitors might have a potential to radiosensitize GBM and improve the treatment outcome of this devastating disease.

6.2. CD 133

In the current study, the presence of CD133+ cells in GBM tissue was detected by immunohistochemical staining method. The proportion of CD133+ GBM stem cells was determined in surgically excised tumor tissue, i.e. prior radiotherapy. The study revealed wide variability in the proportion of these cells. Among evaluated GBM samples, there were tumors that contained only 0.5% CD133+ GBM cells but also tissues in which the proportion of CD133+ cells was as high as 82%. The variability in CD133+ GBM stem cell proportions has also been reported in studies of 37 and 44 consecutive GBM patients, where CD133 expression ranged between 0.5% and 10.0% [144,145]. However, in our study, somewhat higher CD133+ GBM cell proportions were detected (median 28%) that might be related to the use of different primary CD133 antibody clone [146].

Present study revealed the correlation between the proportion of CD133+ stem cells and the overall proportion of tissue necrosis. It is widely accepted that necrosis typically develops in hypoxic (low-oxygen) environments. In GBM, the expression of hypoxia markers (CAIX and HIF-1α) has been shown to be especially high in tumor regions containing 10% to 45% necrosis of total area [147]. Additionally, it has been reported that tumor-initiating CD133+ GBM stem cells are preferentially expanded in hypoxic conditions [147,148]. Therefore, hypoxia might have also influenced the proportion of CD133+ GBM cells in the present study.

Additionally to the determination of CD133+ cell proportions, tumor CD133 expression levels were correlated with GBM patients overall survival. The median survival of the entire study group was 10.0 months. However, the survival time clearly depended on the proportion of CD133+ GBM stem cells. Median survival times for patients with low (<median) and high (≥median) proportion of CD133+ cells were 9.0 months and 12.0 months respectively. In contrast to what was expected, significantly longer survival times after postoperative radiotherapy were achieved in patients with higher stem cell proportion. To the knowledge of authors, there are no other clinical studies that would have evaluated the prognostic significance of CD133 expression after GBM radiotherapy. Nevertheless, clinical series that have used radiochemo-

therapy (radiotherapy and concomitant plus adjuvant TMZ), which currently represents standard-of-care treatment for GBM, have shown opposite results. In clinical study of 44 GBM patients, the CD133+ tumor cell proportion of ≥2% negatively correlated with overall survival [144]. Additionally, mRNA expression analysis in 48 GBM patients showed that high sample CD133 mRNA expression was a significant prognostic factor for adverse overall survival independent of the extent of resection and O(6)-methylguanine-DNA methyltransferase (MGMT) gene methylation status [149]. These opposite results may be related to other treatment protocol (radiochemotherapy), different primary antibody used for CD133 immunohistochemical detection, as well as to the fact that mRNA expression study samples contained up to 50% of non-tumor tissue, which may also contain CD133 [150].

Similarly to our findings, different clinical outcomes were documented in a study that divided GBM patients into 2 groups (CD133-low, CD133-high) according to CD133+ cell ratio either <3% or ≥3%, as detected by FACS analysis (fluorescence activated cell scanning) of primary tumor cultures. Namely, tumors from CD133-low GBM patients were shown to have tendency to be localized within the deeper structures of the brain, to show more invasive growth patterns and ventricle involvement, as well as relatively higher rate of disease progression after radiotherapy and chemotherapy [151]. Also, although not in primary GBM, significantly longer survival times were detected in recurrent GBM patients with higher proportion of CD133+ cells [145]. In addition, the multivariate analysis of the present study revealed that next to the well-established prognostic factor KPS, CD133+ GBM stem cell proportion emerged as a significant independent predictor for overall survival. This clearly suggests that GBM patients with high proportion of CD133+ tumor cells respond better to radiotherapy and achieve better treatment response that consequently result in longer survival times.

It has been widely accepted that CD133+ GBM stem cells are especially radioresistant [109]. The findings of our study point toward the possibility that these cells might be, in contrast to what has been believed, radiosensitive. The radioresistant nature of CD133+ GBM stem cells has been mainly documented in studies that compare isolated CD133+ and CD133- GBM cell lines [109,152]. However, when compared to the traditional glioblastoma established cell lines that contain heterogeneous cell subpopulations, higher radiosensitivity of CD133+ GBM stem cells has been seen. It has been previously reported that CD133+ GBM stem cells have a reduced capacity to repair radiation-induced double strand brakes (DSBs), which is likely to be a major contributor to the relatively greater degree of radiosensitivity [153]. Therefore, the radiosensitivity of CD133+ GBM stem cells might be greatly underestimated.

As mentioned earlier, a correlation between the proportion of CD133+ GBM stem cells and the overall proportion of tissue necrosis was found. This shows indirectly that also a surrounding microenvironment may contribute to the radiation response of GBM stem cells. Indeed, recent publications have confirmed this relationship. It has been demonstrated that GBM stem cells

irradiated *in vivo* within orthotopic xenografts are less susceptible to DSB induction and have greater capacity to repair damage as compared to same tumor cells irradiated under *in vitro* growth conditions [154]. Moreover, close correlation between CD133+ GBM cells and hypoxia [147], vascular structures [91], extracellular matrix (ECM) components [93], as well as inflammation and immunoregulatory markers [155] have been reported. This all shows that radiation response of CD133+ GBM stem cells are determined by a numerous known and unknown processes that can be collectively named as "micro-environment-stem cell unit" [93].

In conclusion, our study showed that there is no association between higher proportion of stem cells and the aggressiveness of GBM. In contrast, in patients with higher stem cell proportion, significantly longer survival times after post-operative radiotherapy were achieved.

6.3. CD63

It has been shown that both tumor-cell related changes, as well as changes in tumor surrounding microenvironment contribute to the inefficacy of standard radiotherapy.

In the current study, we evaluated the role of tumor infiltrating CD63+ inflammatory and immune cells, representing one constituent of tumor micromilieu, on radiotherapy treatment response and survival of GBM patients. The presence of CD63+ cells was detected by immunohistochemical staining procedure, which revealed wide variability in these cell numbers: evaluated tumor samples contained from 10.3 CD63+ cells to as much as 134.5 CD63 cells per microscopic field. In previous studies, CD63 expression has been evaluated in a different manner. For example, CD63 expression has been reported in GBM tissue microarray cores as labeling indexes, representing the percentage of positively immunostained tissue area [156]. Also, gene expression analyses in GBM tumor tissue that always contain also some proportions of non-tumor components have revealed higher levels of CD63 [157,158]. However, in these previously described studies, it is not exactly clear, whether CD63 expression relates more to tumor cells and/or normal tissue compartments, including inflammatory and immune cells, which makes the comparison of the studies difficult. Our study additionally revealed the association between the numbers of CD63+ tumor infiltrating inflammatory and immune cells and the percentage of necrosis in GBM tissue. This shows that next to cancer cells, inflammatory and immune response might be mediated through other components of the tumor microenvironment.

Median survival time of the entire study group was 10.0 months. Present study revealed, however, that the survival time clearly depended on the number of tumor infiltrating CD63+ inflammatory and immune cells. Median survival times for patients with low (<median) and high (≥median) numbers of CD63+ inflammatory and immune cells were 9.0 months and 12.0 months respectively.

Therefore, patients whose tumors were infiltrated with higher number of inflammatory and immune cells, had better treatment response and lived significantly longer compared to those whose tumors had fewer tumor infiltrating CD63+ cells. Given the dismal prognosis of GBM, the gain in overall survival of 3 months is remarkable. To the knowledge of authors, there are no other clinical studies that would have evaluated the prognostic signifycance of the amount of tumor infiltrating CD63+ inflammatory and immune cells after GBM radiotherapy. However, it has been shown earlier that co-expression of tissue inhibitor of metalloproteinase-4 (TIMP-4) and CD63 is associated with reduced survival of GBM patients [156]. In the latter study, however, CD63 expression was evaluated in only 3 small GBM tissue microarray cores and reported as labeling indexes, representing the percentage of positively immunostained tissue area comprising both tumor and inevitably also non-tumor tissue. Moreover, in univariate analysis, TIMP-4 and CD63 labeling indexes alone did not reach statistical significance in relation to median cancer specific survival.

Our study raises the possibility that both inflammation and immune reaction might influence radiation sensitivity of GBM cells and thereby treatment outcome. According to a dominating belief, targeting the inflammatory signaling pathways might offer a good opportunity to improve clinical cancer outcome, since irradiation itself leads to additional synthesis of several pro-inflammatory factors and inflammatory response is one of the hallmark of radiation-induced normal tissue side effects [159,160]. However, there are several clinical studies, although in other types of cancer, showing that blocking of inflammatory pathways does not enhance treatment response of radiotherapy and concomitant chemotherapy. For example, blocking of inflammatory enzyme cyclooxygenase-2 (COX-2), which is also expressed in GBM tissues, with a selective inhibitor of COX-2 celexocib does not improve treatment efficacy and survival in patients with stage IIIA/B non-small cell lung cancer [111,161]. Similar disappointing results with COX-2 inhibitor celexocib have been reported in pancreatic cancer, as well as in rectal cancer [162,163]. In contrast to latter studies but in line with our data, a higher grade of inflammatory infiltration in tumor tissue that was defined by 2 pathologists in a blinded fashion in 10–20 microscopic fields in hematoxylin stained sections, related to favorable survival of rectal cancer patients receiving preoperative radiotherapy [164].

In the tumor microenvironment, an intensive interaction between tumor cells and infiltrating immune cells, most frequently macrophages and T-cells occur [165, 160]. The presence of tumor infiltrating T-cells has also been reported in GBM tissues [110]. Similarly to our study, increased immune cell infiltration is a significant independent variable contributing to longer survival in high grade astrocytomas and glioblastoma [166,167]. Additionally, the expression of immune genes in GBM has been associated with prolonged progression-free survival and immunohistochemically detected expression of CD3 and CD68 cells (markers of T-cells and macrophages) is significantly more frequent in responders to radiotherapy than in non-responders, confirming the role of tumor infiltrating immune cells in modulating radiation effects in GBM [168]. It is

believed that tumor infiltrating resident leukocytes detect danger signals form cytotoxic therapy that results in subsequent activation of both innate and adaptive immune cells [169,170]. Indeed, it has been previously reported that irradiation influences tumor immune response through release of proinflammatory molecules and cytokines, antigen presentation, increased homing of inflammatory cells, enhanced T-cell activation and cytotoxic immune response, as well as through inhibition of immunosuppressive cells [170].

Previous studies have shown that pro-inflammatory and immune-based mechanisms might have a potential to influence the response to conventional cytotoxic therapy and thereby diminish tumor progression and prolong patients survival. In fact, signs from early clinical trials confirm that the combination of immune-based therapies and chemotherapy, which is one form of cytotoxic treatment, result indeed in synergistic effects. In GBM patients (mostly with recurrent tumors), autologous dentritic cell vaccination prior chemotherapy significantly prolonged time to tumor recurrence and survival [171]. Moreover, vaccine responders have been shown to exhibit significantly longer times to post chemotherapy tumor progression and survival than nonresponders [172]. Therefore, concomitant immune-therapy with cytotoxic anti-cancer treatment holds great promise and the exact sequencing and combination of different treatment modalities (including radiotherapy) should be defined in the near future.

In conclusion, enhanced inflammatory and immune response in GBM tissue corresponds to better survival after postoperative radiotherapy. Although CD63 immunohistochemical expression does not distinguish exact cell types that have the greatest impact on GBM treatment response, our study created a good platform for further clarifying studies.

6.4. Inflammation, ICAM-1 and VEGFR-2

In the present study, we evaluated the impact of tumor microenvironment on the expression level of VEGFR-2 – one of the main targets of antiangiogenic drugs. Foremost, the possible role of inflammatory reaction was assessed. Inflammatory reaction was evaluated by two means. First, visual inflammation (based on the presence of tissue edema and inflammatory cell infiltration) was estimated in hematoxylin-eosin stained sections by experienced pathologist. Afterwards, to reduce subjectivity, a digital IHC image analysis was performed in ICAM-1 stained sections. ICAM-1 was chosen as a marker of inflammation since this transmembrane glycoprotein can be induced in response to a number of stimuli, including inflammatory mediators, hormones and cellular stresses [173,174]. Moreover, endothelial ICAM-1 is considered to represent the most important adhesion molecule for leukocyte recruitment to inflamed sites [175–177].

All glioblastoma samples showed various levels of visually confirmed inflammatory reaction. This is not surprising since inflammation is considered one of the characteristic histopathological features of glioblastoma [178]. Also,

the expression of ICAM-1 was present in all digitally analyzed individual tumor samples, which is in good accordance with previous studies, where compared to peritumoral ICAM-1 expression significantly higher expression of ICAM-1 has been detected in GBM tumor areas both in gene and protein levels [179–181]. In GBM cells, ICAM-1 expression has been shown to increase following stimulation with pro-inflammatory cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNFα) and interferon-gamma (IFN-γ) [179,182], indicating that ICAM-1 is one of the inflammatory mediators also in this type of cancer.

In GBM tissues, different numbers of VEGFR-2+ blood vessels and endothelial levels of VEGFR-2 were detected. Previous studies have shown that in normal brain, low or undetectable endothelial expression of VEGFR-2 can be found, however, in gliomas, the proportion of VEGFR-2+ vessels and endothelial VEGFR-2 expression increases with tumor grade, being the highest in GBM [183,184]. Our study revealed that also in most aggressive glioma – GBM – the extent of VEGFR-2 expression may vary. Additionally, present study showed that the expression of VEGFR-2 depends on inflammatory reaction in tumor tissue: the higher endothelial VEGFR-2 expression the higher extent of inflammation. Moreover, this association was seen for both assessments of inflammatory reaction (visual and computer software based).

Angiogenesis is a tightly controlled process that in a number of pathological conditions, including cancer and inflammation, may become aberrant [185]. Different factors produced by tissues are capable of promoting or inhibiting blood vessel proliferation, whereas in normal status, the balance between angiogenic and angiostatic factors exists. In inflammation, this balance is clearly inclined toward angiogenic factors and angiogenesis [186].

Although the link between inflammation and angiogenesis has received much attention only recently, there is a substantial body of evidence showing close association between these two processes. Previous studies have described that angiogenic factors exhibit both pro-angiogenic and pro-inflammatory effects, inflammatory cells are able to produce large quantities of pro-angiogenic factors and both processes (inflammation and angiogenesis) are capable of potentiating each other [186]. For example, VEGF that exerts majority of its angiogenic effects by binding to VEGFR-2, has also been shown to induce adhesion molecules on endothelial cells during inflammation [187]. In endothelial cells, treatment with VEGF results in an increase of both ICAM-1 mRNA, as well as protein expression [188]. Moreover, VEGF increases leukocyte adhesiveness to endothelial cells, which is the first step of leukocyte trafficking into inflamed tissue [188]. Next to these effects, VEGF enhances vascular permeability and causes vasodilatation, potentiating thereby inflammation through formation of tissue edema [116,189]. At the same time, hyperpermeability is also involved in pathological angiogenesis [189]. Additionally, inflammatory and angiogenic processes involve similar cell types. Inflammatory cells, namely monocytes, macrophages, T lymphocytes and neutrophils, participate in the angiogenesis by secreting cytokines that affect endothelial cell

functions, proliferation, migration and activation [190]. Macrophages, present in the inflammatory infiltrate, produce a broad array of angiogenic growth factors and cytokines, generate channels for blood flow through proteolytic mechanisms, and promote the remodeling of arterioles into arteries [185]. Inflammatory dendritic cells stimulate similarly angiogenesis by secreting angiogenic factors and cytokines, as well as by promoting pro-angiogenic activity of T lymphocytes [185]. Previous studies have also shown that pro-inflammatory cytokines, which are always present in inflamed tissue, mediate also endothelial expression of VEGFR-2 [191,192]. Latter is in line with our findings since positive correlation was found between the extent of VEGFR-2 expression and inflammatory response in GBM tissue.

There are several clinical situations, where inflammatory reaction in GBM may be suppressed. These particularly include the use of anti-inflammatory drugs, such as steroids and nonsteroidal anti-inflammatory drugs (NSAIDs) to manage tumor surrounding inflammation and edema [193]. Whether these very commonly used medicines influence also treatment efficacy of antiangiogenic drugs through diminishing inflammatory response and thereby the expression of VEGFR-2, remains unclear. Nevertheless, our data point toward the possibility that this association might exist. This is also supported by studies where dexamethasone, most frequently used steroid in GBM patients, has been shown to inhibit the effects of pro-inflammatory cytokines, VEGF mRNA expression, VEGFR-2 expression, as well as macrophage infiltration [192,194,195].

In conclusion, our study showed that the expression of VEGFR-2 – one of the main targets of antiangiogenic drugs - depends on GBM microenvironment. Importantly, higher endothelial VEGFR-2 levels were seen in the presence of more pronounced inflammation, whereas in less inflamed tissues only weak expression of VEGFR-2 was found. Latter has to be taken into consideration when treatment approaches that block VEGFR-2 signaling are designed.

The present PhD study has several limitations. These include retrospective data collection and small number of patients, which considered small Estonian population and rare tumor type is inevitable. Also, some important variables, such as tumor O6-methylguanine-DNA methyltransferase (MGMT) methylation status, isocitrate dehydrogenase 1 (IDH1) gene mutation status, recursive partitioning analysis (RPA) and patient's quality of life scores were not recorded. Nevertheless, this study showed that several aspects in GBM therapy might be improved and modification of not only GBM cells but also stem cells and tumor microenvironment might be necessary to find more efficacious treatment strategies to fight this devastating disease. Latter, however, has to be clarified in further preclinical and clinical studies that may be based on ideas that rose in the present study.

7. CONCLUSIONS

1. The survival of GBM patients receiving postoperative radiotherapy depends on tumor expression of DNA-PK. GBM patients with high tumor levels of DNA-PK are more resistant and respond less to standard radiotherapy that consequently result in significantly shorter survival times. Further studies are needed to clarify whether DNA-PK inhibitors might have a potential to radiosensitize GBM and improve the treatment outcome of this disease.
2. In patients with GBM, there is no association between higher proportion of tumor stem cells and the aggressiveness of disease. In contrast, in patients with higher stem cell proportion, significantly longer survival times after postoperative radiotherapy were achieved. Underlying reasons and possible higher sensitivity of GBM stem cells to fractionated radiotherapy should be clarified in further studies.
3. Enhanced inflammatory and immune response in GBM tissue corresponds to better survival after postoperative radiotherapy. Therefore, pro-inflammatory and immune-based therapies might have a potential to influence the response to conventional cytotoxic therapy and thereby diminish tumor progression and prolong patients survival.
4. The expression of VEGFR-2 – one of the main targets of antiangiogenic drugs – depends on GBM microenvironment. Higher endothelial VEGFR-2 levels were seen in the presence of more pronounced inflammation, whereas in less inflamed tissues only weak expression of VEGFR-2 was found. Latter may be one of the reasons of inefficacy of antiangiogenic drugs and should be taken into consideration when GBM treatment approaches that block VEGFR-2 signaling are designed.

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9. SUMMARY IN ESTONIAN

Multiformne glioblastoom: võimalused parandamaks kasvajavastase ravi efektiivsust

Multiformne glioblastoom (MGB) on kõige agressiivsem aju primaarne pahaloomuline kasvaja täiskasvanutel, mille ravitulemused on siiani ebarahuldavad (keskmine elulemus ca 1 aasta). MGB diagnoosiga haigete elulemust ei ole siiani suudetud märkimisväärselt pikendada vaatamata aastakümneid kestnud uurimustöödele. Alates 1978. aastast on MGB standardraviks olnud kasvaja kirurgiline eemaldamine koos sellele järgneva postoperatiivse kiiritusraviga ning medikamentoosse raviga (keemiaravi, sihtmärkravi). MGB on oma iseloomult resistentne haigus, sest nii postoperatiivse kiiritusravi kui ka medikamentoosse raviga on võimalik saavutada ainult haiguse lühiajalist stabilisatsiooni. Paraku surevad ravi järgselt enamus haigetest (99%) lokaalse retsidiivi tõttu ajukoes, mistõttu peetakse MGB üheks kõige raskemini ravitavaks pahaloomuliseks kasvajaks. Ravitulemuste parandamise üheks alustalaks on raviresistentsust põhjustavate mehhanismide väljaselgitamine.

Käesoleva töö põhieesmärgiks oli leida võimalusi MGB-vastase ravi efektiivsuse tõstmiseks ning selleks:

1. hinnati, kas MGB kõrge reparatsiooniensüümide (PARP-1 ja DNA-PK) tase on seotud halvema ravivastusega ning seeläbi haigete lühema elulemusega,
2. hinnati, kas CD133+ kasvaja tüvirakud, mida eelnevate eelkliiniliste uuringute põhjal on peetud kõige radioresistentsemateks kasvajarakkudeks, mõjutavad MGB diagnoosiga haigete ravitulemusi ja elulemust,
3. hinnati, kas CD63+ kasvajat infiltreerivad põletiku- ja immuunrakud mõjutavad MGB diagnoosiga haigete ravitulemusi ja elulemust,
4. hinnati kasvaja mikrokeskkonna, eelkõige põletiku mõju ühe kõige olulisema angiogeneesi inhibiitorite sihtmärgi, VEGFR-2 ekspressioonile.

Uuringusse kaasati MGB diagnoosiga haiged (max n=42), kes said postoperatiivset kiiritusravi Tartu Ülikooli Kliinikumis või Põhja-Eesti Regionaalhaiglas aastatel 2006–2008. Operatsiooni käigus eemaldatud kasvajakoes hinnati hematoksüliin-eosiin värvingu järgselt nekroosi ulatust (%) ning põletikulist reaktsiooni (1=nõrk, 2=mõõdukas, 3=raske põletikuline reaktsioon). Immuunhistokeemilise värvingu järgselt hinnati kasvajarakkudes DNA reparatsiooniensüümide (PARP-1, DNA-PK) värvumise taset (skoor 0–3), CD133+ MGB tüvirakkude osakaalu mikroskoobi vaatevälja kohta (%), CD63+ põletiku- ja immuunrakkude arvu mikroskoobi vaatevälja kohta, ICAM-1 ekspressiooni optilist tihedust (kvantitatiivse pikseli analüüsi tarkvara abil) ning VEGFR-2 endoteliaalse värvumise intensiivsust (skoor 0–3). Lisaks eelnevatele hinnati DNA reparatsiooniensüümide taseme, CD133+ tüvirakkude proportsiooni ning kasvajat infiltreerivate CD63+ põletiku- ja immuunrakkude seost patsientide üldise elulemusega (Kaplan-Meieri elulemuse analüüs, mitmemõõtmeline analüüs). Korrelatsioonide analüüsimisel kasutati Pearsoni korrelatsiooni testi.

Kasvajarakkude PARP-1 ja DNA-PK värvumise tasemed varieerusid vastavalt vahemikus 1,2–2,8 ja 0,8–2,8. PARP-1 keskmine ja mediaanväärtus olid vastavalt 1,96±0,50 (keskmine±standardhälve) ning 2,0. DNA-PK keskmine ja mediaanväärtus olid vastavalt 2,02±0,49 (keskmine±standardhälve) ja 2,0. Kogu MGB diagnoosiga haigete grupi üldise elulemuse mediaan oli 10,0 kuud (95% UV 8,1–11,9). Elulemus ei sõltunud PARP-1 tasemest (p=0,93), kuid sõltus oluliselt kasvajarakkude DNA-PK tasemest (p=0,02). Patsientide elulemus madala (<mediaan) ja kõrge (≥mediaan) DNA-PK tasemega oli vastavalt 13,0 kuud (95% UV 10,7–15,3) ja 9,0 kuud (95% UV 7,2–10,8). Mitmemõõtmeline analüüs kinnitas, et DNA-PK tase on üldise elulemuse oluline prognostiline tegur (riskisuhe 3,9, 95% UV 1,5–10,7, p=0,01).

CD133+ tüvirakkude osakaal oli MGB koes väga varieeruv, jäädes erinevatel patsientidel vahemikku 0,5–82%. Tüvirakkude osakaalu keskmine ja mediaanväärtus olid vastavalt 33%±24% (keskmine±standardhälve) ning 28%. Kogu grupi üldise elulemuse mediaan oli 10,0 kuud (95% UV 9,0–11,0). Elulemus sõltus märkimisväärselt CD133+ rakkude osakaalust (p=0,02). Patsientide elulemus madala (<mediaan) ja kõrge (≥mediaan) CD133+ tüvirakkude osakaaluga oli vastavalt 9,0 kuud (95% UV 7,6–10,5) ja 12,0 kuud (95% UV 9,3–14,7). Mitmemõõtmeline analüüs kinnitas, et CD133+ tüvirakkude osakaal on oluline prognostiline tegur (riskisuhe 2,0 95% UV 1,0–3,8, p=0,04).

Kasvajat infiltreerivate CD63+ põletiku- ja immuunrakkude arv mikroskoobi vaatevälja kohta varieerus MGB diagnoosiga haigetel vahemikus 10,3–134,5. CD63+ rakkude keskmine arv ja mediaanväärtus olid vastavalt 45,3±27,2 (keskmine±standardhälve) ning 39,6. Kogu grupi üldise elulemuse mediaan oli 10,0 kuud (95% UV 9,0–11,0). Elulemus sõltus märkimisväärselt kasvajat infiltreerivate CD63+ põletiku- ja immuunrakkude arvust (p=0,003). Patsientide elulemus madala (<mediaan) ja kõrge (≥mediaan) CD63+ põletikuliste rakkude arvuga olid vastavalt 9,0 kuud (95% UV 8,1–9,9) ja 12,0 kuud (95% UV 8,5–15,5). Mitmemõõtmeline analüüs kinnitas, et CD63+ põletikurakkude arv on oluline prognostiline tegur (riskisuhe 2,4 95% UV 1,2–5,1, p=0,02).

MGB koes visuaalselt hinnatud põletikulise reaktsiooni keskmine skoor oli uuringugrupis 1,9±0,7 (keskmine±standardhälve). Digitaalse pikseli analüüsiga hinnatud ICAM-1 ekspressiooni optiline tihedus varieerus vahemikus 17,6–154,9 ühikut. Grupi keskmine ICAM-1 optiline tihedus oli 57,0±27,1 (keskmine± standardhälve). Grupi keskmine endoteliaalse VEGFR-2 värvumise intensiivsus oli 1,2±0,8 (keskmine±standardhälve). Märkimisväärne korrelatsioon leiti endoteliaalse VEGFR-2 ekspressiooni ja põletiku ulatuse vahel (p<0,01). Samuti esines märkimisväärne seos endoteliaalse VEGFR-2 ekspressiooni ja kasvajakoe ICAM-1 optilise tiheduse vahel (p=0.03).

Käesolev doktoritöö näitas, et postoperatiivset kiiritusravi saavate MGB diagnoosiga haigete elulemus sõltub kasvaja DNA-PK tasemest. Patsiendid, kelle kasvajakoes oli kõrge DNA-PK tase, allusid ravile halvemini ning elasid lühemalt. Seetõttu tuleks edasistes uuringutes hinnata, kas DNA-PK inhibiitorid võiksid tõsta kasvajarakkude tundlikkust kiiritusravile ning parandada seeläbi

ravitulemust. Käesoleva töö raames täheldati, et kliinilised andmed ei kinnita seost MGB CD133+ tüvirakkude suurema osakaalu ning kasvaja agressiivsema kulu vahel. Suurema CD133+ tüvirakkude osakaaluga patsiendid saavutasid postoperatiivse kiiritusravi järgselt pikemalt kestva haiguse remissiooni ja pikema elulemuse. Selle põhjused vajavad selgitamist edasistes uuringutes. Lisaks eelnevale leiti, et kasvajat infiltreerivad CD63+ põletiku- ja immuunrakud omavad soodsat toimet MGB haigete elulemusele. Haigetel, kellel kasvajakude sisaldas postoperatiivse kiiritusravi eelselt rohkem põletiku- ja immuunrakke, oli oluliselt pikem elulemus. Eelnevast tulenevalt võiks seega edasistes uuringutes vaadelda, kas põletikku- ja immuunreaktsiooni võimendavad ravimeetodid koos standardse tsütotoksilise kasvajavastase raviga parandaksid MGB ravitulemust. Samuti selgus käesolevast tööst, et MGB ravis laialdaselt testitud angiogeneesi inhibiitorite ühe olulise sihtmärgi – VEGFR-2 – ekspressioon sõltub kasvajakoe põletiku ulatusest. VEGFR-2 ekspressiooni sõltuvus tuumori põletikulisest mikrokeskkonnast võib olla üheks angiogeneesi inhibiitorite ebaefektiivsuse põhjuseks.

Kokkuvõtvalt võib öelda, et MGB ravitulemuste parandamiseks on mitmeid võimalusi ning efektiivsema kasvajavastase ravi läbiviimiseks ei tuleks mõjutada ainult kasvajarakke, vaid ka kasvaja tüvirakke ning kasvaja mikrokeskkonda. Doktoritöö raames tekkinud ideid on kavas testida edasistes eelkliinilistes ning kliinilistes uuringutes.

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11. PUBLICATIONS

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1994–1998	University of Tartu, Faculty of Medicine, Course of Residency in Oncology, Degree in Oncology
2000–2001	London University, MS Course of Radiobiology, MSc of Radiobiology
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Professional employment:

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Scientific work and professional organisations:

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Publications:

1. Kase, M.; Adamson, A.; Saretok, M.; Minajeva, A.; Vardja, M.; Jõgi, T.; Asser, T.; Jaal, J. (2014). Impact of tumor infiltrating CD63 positive cells on survival in patients with glioblastoma multiforme. J Neurosurg Sci. 2014 Sep 12. [Epub ahead of print]
2. Kase, M.; Minajeva, A.; Niinepuu, K.; Kase, S.; Vardja, M.; Asser, T.; Jaal, J. (2013). Impact of CD133+ stem cell proportion on survival in patients with glioblastoma multiforme. Radiology and Oncology, 47(4), 405–410

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Liikmelisus:	*European Society for Radiotherapy and Oncology* – liige Eesti Kliiniliste Onkoloogide Selts – juhatuse liige Eesti Onkoloogide Selts – liige

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