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Membrane Hsp70 expression in gliomas: a novel, hypoxia-related marker for primary glioblastoma

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List of abbreviations

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CA IX	carboanhydrase 9
CM	conditioned medium
CNS	central nervous system
CSC	cancer stem cells
d	days
DAB	3,3'-Diaminobenzidine
DMEM	Dulbecco’s MEM medium
EC	endothelial cells
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FGF	fibroblast growth factor
FITC	fluoresceinisothiocyanate
GBM	glioblastoma
Gb3	glycosphingolipid 3
Glut-1	glucose transporter-1
h	hours
hEGF	human epidermal growth factor

HOPE	hepes-glutamic acid buffer-mediated organic solvent protection effect
HIF-1	hypoxia inducible factor-1
HSPs	heat shock proteins
Hsp70	heat shock protein 70
IDH	isocitrate dehydrogenase
IgG	immunoglobulin G
LDH-5	lactat-dehydrogenase-5
MCT-1,4	monocarboxylate transporter 1,4
MGMT	O^6-methylguanine methyltransferase
MSC	mesenchymal stem cells
NF	neurofibromatosis
PDGFR	platelet-derived growth factor receptor
PK	primary culture
PTEN	phosphatase-and tensin-homolog-on-chromosome-ten
Rb	retinoblastoma
TBS	tris-buffered saline
TP53	Tumor protein 53
VEGF	vascuoar endothelial cell growth factor
VEGFR-2	vascuoar endothelial cell growth factor receptor 2
vWF	Von Willebrand factor
WHO	the world health organization

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a novel, hypoxia-related marker for primary glioblastoma

1 Introduction

1.1 Gliomas: definition, epidemiology, classification and molecular biomarkers

Primary brain tumors range from benign pilocytic astrocytomas to malignant glioblastoma multiforme. In contrast to brain metastatic tumors which are from tumors originated in other organs or tissues in body, primary brain tumor arise from different cells in brain, such as gliomas arise from glial cells (Pytel and Lukas, 2009). In United States, gliomas account for nearly 31% of all primary CNS (central nervous system) tumors and 61% of gliomas occur in temporal, frontal, parietal and occipital lobes (www.cbtrus.org. 2011) (Brem et al., 2005). The incidence rates for gliomas seem to be higher in males than in females and significant higher among whites than among blacks (www.cbtrus.org. 2011). The world health organization (WHO) classifies gliomas on the basis of histological features into four grades: grade I (pilocytic astrocytoma), grade II (diffuse astrocytoma), grade III (anaplastic astrocytoma), and grade IV (glioblastoma)(Louis et al., 2007). Glioblastoma (GBM, WHO grade IV) accounts for the majority (>50%) of gliomas (www.cbtrus.org. 2011) (Preusser et al., 2011). Anaplastic astrocytoma and GBM account for malignant gliomas which have worse clinical outcome. Despite improved combined therapeutic strategies, including surgical resection, radiation and chemotherapy, the median survival is 13.2 months to 16.8 months (median, 14.6 months) for the cases of GBMs and 2 to 5 years for the cases of anaplastic gliomas (Preusser et al., 2011; Stupp et al., 2005; Wen and Kesari, 2008).

On the basis of biologic and genetic differences, GBM can be subdivided into two subtypes: primary GBM (account for >90%) and secondary GBM (account for 10%) (Preusser et al., 2011). Primary GBMs occur more frequently in elder patients (median age, 64 years) (Preusser et al., 2011) and do not have previous history of low-grade precursors, they are characterized by epidermal growth factor receptor (EGFR) amplification and mutations, loss of heterozygosity of chromosome 10q, deletion of phosphatase-and tensin-homolog-on-chromosome-ten (PTEN) and p16 deletion (Kleihues and Ohgaki, 1999; Preusser et al., 2011). Secondary GBMs occur in young patients (median age, 45 years) (Preusser et al., 2011) and arise from low-grade astrocytomas, they are characterized by mutations in the p53 gene, overexpression of the platelet-derived growth factor receptor (PDGFR), abnormalities in the p16 and retinoblastoma (Rb) pathways, and loss of heterozygosity of chromosome 10q (Kleihues and Ohgaki, 1999; Preusser et al., 2011; Wen and Kesari, 2008). Recently, the methylation status of O^6-methylguanine methyltransferase (MGMT) gene promoter has emerged as a promosing prognostic and predictive biomarker for GBM patients treated with alkylating drugs like nitrosoureas or temozolomide (Hegi et al., 2005). After treatments with radiotherapy plus temozolomide, GBM patient without MGMT promoter methylation has a 12.7-month overall survival and 5.9-month progression-free survival, patient having a methylated GBM has better overall survival (median, 21.7 months) and progression-free survival (median, 10.3 months) (Hegi et al., 2005; Stupp et al., 2009). Meanwhile, in over 80% of all secondary GBM, mutations of the isocitrate dehydrogenase 1 (IDH1) and IDH2 (a lesser extent) genes, which are specific mutated on amino acid R132 for IDH1 and R172 for IDH2, have been identified (Parsons et al., 2008; Yan et al., 2009). At the same time, the precursors of secondary GBM, WHO grade II (88%) and grade III gliomas (78%), also show IDH1 or IDH2 mutation (Watanabe et al., 2009; Yan

et al., 2009). In contrast, only 5% of primary GBM, which do not have low-grade precursors, have been shown to have IDH1 or IDH2 mutation (Watanabe et al., 2009; Yan et al., 2009). This suggested that IDH1/2 mutation might be an early event in tumor development of secondary GBM (Watanabe et al., 2009; Yan et al., 2009). Furthermore, gene expression patterns of EGFR, NF1, PDGFR and IDH1 are used for subclassification of GBMs into proneural, neural, classical and mesenchymal subtypes. On the basis of this classification, GBM patients with tumor classified as classical and mesenchymal subtypes show better responses to aggressive treatments than GBM patients with tumor classified as proneural and neural subtypes (Verhaak et al., 2010).

1.2 Microenvironment of malignant brain tumors

Tumor microenvironment in general is described by Judithe Leibovici as the tumor stroma, which contains distinct cell types as well as extracellular matrix, a variety of soluble molecules, constituting a complex organ-like structure which is involved in tumorigenesis (Leibovici et al., 2011). Except proliferating malignant cancer cells, cancer stem cells, endothelial cells, pericytes, immune inflammatory cells, cancer associated fibroblasts and recruited stem/progenitor cells have already been described to contribute to the biology of non-glial tumors (Hanahan and Weinberg, 2011). The brain tumor microenvironment recently also has been reviewed(Charles et al., 2011). In brain tumor microenvironment, not only distinct cell types but also the intercellular cross-talk may contribute to the formation, invasion, progression and therapy-responses of brain tumors (Charles et al., 2011).

1.2.1 Glioma stem cells

In 1997, Bonnet and Dick published a conclusive conception of cancer stem cells for the first time in Nature Medicine (Bonnet and Dick, 1997). In acute myeloid leukemia, they defined CD34+CD38- sorted cells as cancer stem cells (Bonnet and Dick, 1997). In brain tumor, concept of cancer stem cells is used to describe a relatively small cell subpopulation with characteristics of self renewal and differentiation, which has similarities to normal neural stem cells but resides in tumors (Reya et al., 2001). Since 2004, glioma stem cells were isolated by CD133 positive-sorting and showed neurosphere-like morphology within growth factors (Singh et al., 2004). They have been thought as the most tumorigenic component in gliomas，only hundreds of glioma stem cells showed the ability of tumor formation (Singh et al., 2004; Stiles and Rowitch, 2008). However, other groups show that CD133 expression is not limited to glioma stem cells and can be regulated by hypoxia *via* HIF-1alpha (Soeda et al., 2009). Surprisingly, CD133 negative cells also have been shown to have the ability of tumor formation (Lottaz et al., 2010). Moreover, with the passaging and culture in *vivo*, CD133 negative glioma cells may increase CD133 expression and finally give rise to CD133 positive tumors (Wang et al., 2008). By now, glioma stem cells are defined as a neurosphere-like population with the ability of self-renewal and differentiation into neurons, astrocytes and oligodendrocytes in *vitro* and the ability of tumor formation (Jordan, 2009), but these criteria are also been challenged (Barrett et al., 2012). On the other hand, it is hard to say which cell type can be served as the origin for glioma stem cells among mature dedifferentiated glia, “restricted” neural progenitors and multipotent neural progenitors (Stiles and Rowitch, 2008). On the clinical side, glioma stem cells are suggested to contribute to radioresistance and chemoresistance, resulting in the maintenance and recurrence of gliomas (Bao et al., 2006; Liu et al., 2006).

1.2.2 Mesenchymal stem cells

Mesenchymal stem cells were firstly isolated from bone marrow and were cultured as adherent cells by Friedenstein et al (Friedenstein et al., 1966). In normal conditions, mesenchymal stem cells always reside in bone marrow and other tissues, such as adipose tissues, fetal skin, bone, umbilical cord blood (Bianco et al., 2008). But in pathological conditions such as inflammation and cancer, mesenchymal stem cells have been shown to be recruited to the lesion (Hung et al., 2005; Schichor et al., 2006). There are several literature describing mesenchymal stem cells can be recruited to tumor microenvironment as the result of tumor-secreted factors like SDF-1, VEGF, IL-8 in different tumor models like colon carcinoma, lung cancer, breast cancer, and so on (Birnbaum et al., 2007). When recruited into tumor microenvironment, these cells would take multi-faces roles in tumor formation and development (Birnbaum et al., 2007). For example, they may be involved in cancer stem cell niche formation and maintenance (Gilbertson and Rich, 2007), they can promote tumor vessel formation (Gilbertson and Rich, 2007) and suppress the immune system to help tumor cells to escape the killing of immunity (Bianchi et al., 2011; Klopp et al., 2011); they also can promote tumor invasion, migration and metastasis (Bianchi et al., 2011). In gliomas, there is no clear conclusion whether mesenchymal stem cells support or suppress the growth of tumor cells in different animal models. Adipose tissue derived mesenchymal stem cells can increase tumor size via inhibition of apoptosis in a U87 glioma model (Klopp et al., 2011). But on the other hand, umbilical cord blood derived mesenchymal stem cells can decrease tumor size via upregulation of PTEN in U251, SNB19 glioma models (Bianchi et al., 2011). Mesenchymal stem cells are not found in human brain until recently a population of mesenchymal stem-like cells are successfully isolated from brain tumor, and this population has been shown to promote the growth of glioma (Clavreul et al., 2012).

1.2.3 Endothelial cells

In general, the sprouting and proliferation of endothelial cells play an important role in tumor vasculature which supports the growing tumor mass with blood, nutrition and oxygen. In gliomas, the process of neovascularization is discussed as distinct phenomenon, angiogenesis and vasculogenesis (Jain et al., 2007). Angiogenesis, "the sprouting of new blood vessels *via* proliferation of pre-exsiting endothelial cells", is supposed to play a major role in gliomas (Jain et al., 2007). Vasculogenesis, "increasing recruitment of circulating endothelial progenitor cells or bone marrow derived hematopoietic cells", also contribute to new vessel formation to support the progression of gliomas (Greenfield et al., 2010). Angiogenesis and vasculogenesis are two parallel processes in glioma tumorigenesis (Jain et al., 2007). Except them, recent research show CD133+ GBM stem-like cells can differentiate into endothelial progenitor cells (Wang et al., 2010). Glioma-isolated endothelial cells show similar typical endothelial markers, such as vWF, CD105, CD31, VE-cadherin, as endothelial cells in normal tissues (Charalambous et al., 2006). However, in contrast to endothelial cells in normal tissues, glioma-derived endothelial cells have larger, flat morphology and express high intercellular adhesion molecules (Charalambous et al., 2006). Moreover, glioma-derived endothelial cells show higher ability of cytotoxic resistance, migration and proliferation, and can produce higher mounts of growth factors like VEGF, IL-8 (Charalambous et al., 2006). Like endothelial cells in non-glial tumor (Garcia-Barros et al., 2003), glioma-derived endothelial cell not only contribute to the vessel formation but also regulate the therapy responses (Jain et al., 2007).

1.2.4 Hypoxia in gliomas

As conflict between demand of a fast growing tumor mass and support of relatively slow neovascularization, regional hypoxia play an important role in malignancy and becomes “a negative prognostic and predictive factor of tumor progression” (Wilson and Hay, 2011). In gliomas, the characteristic necrosis areas are considered to be hypoxia regions, which are frequent in malignant GBM (Jensen, 2009). In glioma cells, hypoxia may regulate gene alterations, invasion, migration, responses of radiotherapy and chemotherapy (Jensen, 2009). Clinical data show that hypoxia is correlated with therapy-resistance and predicts worse clinical outcome in patients with GBM (Flynn et al., 2008; Jensen, 2009). Hypoxia can regulate the expression of several molecules such as hypoxia inducible factor-1alpha (HIF-1α), Glucose transporter-1 (Glut-1), carbonic anhydrase 9 (CA IX) in gliomas (Flynn et al., 2008). These hypoxia-regulated proteins have grade-dependent expression in gliomas and increased expression of these molecules correlated with worse survival (Flynn et al., 2008). Moreover, hypoxia-regulated molecules are involved in several processes during tumor development, for example, HIF-1α can regulate the expression of genes that mediate adaptive responses (Jensen, 2009; Semenza, 2011). Expression of Glut-1 is not only linked to microvascular density and cell proliferation, but also limited to peri-necrotic regions in gliomas (Airley et al., 2010; Flynn et al., 2008).

1.2.5 Cross-talk in tumor microenvironment of gliomas

In general, a signaling interaction between various cell types in tumor microenvironment is critical importance of tumor formation, development and progression (Hanahan and Weinberg, 2011). The cross-talk is mediated through soluble factors and cell-cell contacts (Charles et al., 2011; Hanahan

and Weinberg, 2011). In gliomas, tumor cells can activate and educate the surrounding resident stromal cells to facilitate the tumor progression (Charles et al., 2011; Clavreul et al., 2012), they also can recruit stem/progenitor cells from host tissues by tumor-derived factors into tumor microenvironment, such as bone marrow derived mesenchymal stem cells (Hung et al., 2005; Schichor et al., 2006) . Stromal cells can secrete several factors including cytokines, growth factors, chemokines, then promote the initiation, invasion, proliferation of tumors (Parsons et al., 2008). Stromal cells also recruit immune cells to constitute an immunosuppressive environment to promote the tumor progression (Bianchi et al., 2011). Glioma stem cells have been shown to recruit endothelial cells into glioma stem cell niche, and these endothelial cells and other glioma cells are supposed to contribute to maintenance of the self-renewal ability of glioma stem cells (Gilbertson and Rich, 2007).

1.3 Heat shock protein 70

Heat shock proteins (Hsps) are members of intracellular proteins, which mainly function as molecular chaperones to assist protein folding and translocation under stress conditions (Asea, 2006; Wallin et al., 2002). Heat shock proteins can be divided into different families according to their molecular weights, including Hsp10, Hsp27, Hsp40, Hsp60, Hsp70, Hsp90 and Hsp110. Heat shock protein 70(Hsp70) is the major stress-inducible heat shock protein with molecular weights of 70 kDa (Calderwood et al., 2005). Hsp70 can be expressed in intracellular location and on the plasma membrane of several tumor cells, Hsp70 can also be secreted into extracellular space in the form of exosomes by tumor cells (Gehrmann et al., 2008b), as shown in Fig. 1-1.

Intracellular Hsp70 possesses a C-terminal domain, unfolding proteins and peptides, and a N-terminal ATPase domain which controls the peptide binding (Calderwood et al., 2005). Following stress, intracellular Hsp70, which is constitutively expressed in many tumor cell lines and tumor biopsies, fulfills protective functions and thus prevents lethal damage (Yaglom et al., 2007). In tumor cells, intracellular Hsp70 also participates in multi-steps in tumorigenesis, including the formation of a malignant phenotype, inhibition of programmed cell death, angiogenesis, invasion and metastasis (Calderwood et al., 2006). For example, high levels of intracellular Hsp70 might suppress senescence in colon carcinoma, breast cancer, prostate carcinoma cells, whereas depletion of intracellular Hsp70 has been shown to induce senescence via activation of TP53 pathway in colon and prostate carcinoma cell lines(Yaglom et al., 2007). In different types of cancer, strong intracellular Hsp70 expression is assumed to contribute to worsened clinical prognosis (Ciocca and Calderwood, 2005; Gehrmann et al., 2008b; Yaglom et al., 2007). For example, prostate carcinoma cells with high expression of intracellular Hsp70 are more resistant to chemotherapy (Roigas et al., 1998). Intracellular Hsp70, which expressed in bladder urothelial carcinoma cell lines and clinical specimens, is associated with tumor progression such as migration and invasion (Garg et al., 2010)

Extracellular Hsp70 which is secreted by necrotic tumor cells binds to adjacent cells or enters into bloodstream to distant sites (Calderwood et al., 2007). In viable tumor cells, the mechanisms for the secretion of extracellular Hsp70 are complex and incompletely understood (Mambula et al., 2007), but extracellular Hsp70 secretion can be enhanced following exogenous stress, such as pro-inflammatory cytokines (Barreto et al., 2003). For example, interferon-gamma has been shown to induce secretion of extracellular Hsp70 in breast adenocarcinoma cells and erythroleukemic cells

(Barreto et al., 2003; Bausero et al., 2005). Extracellular Hsp70 functions as an inducer of signal transduction cascades and transport of cargo molecules. On the other side, it acts as a danger signal and elicits immune responses (Calderwood et al., 2005; Multhoff, 2007). For example, extracellular Hsp70 from human liver carcinoma cell line HepG2 activate macrophage, dendritic cells and natural killer cells by a receptor-mediated pathway (Vega et al., 2008).

In non-glial tumors, membrane Hsp70 expression has been described uniquely on the surface of tumor cells, but not in the surrounding non-neoplastic tissues or normal cells. The externalization of intracellular Hsp70 to the membrane-associated form under stress conditions serving as a "danger-signal" is investigated (Hightower and Guidon, 1989; Vega et al., 2008). Multhoff et al show the minimal membrane Hsp70 sequence is just a 14-mer Hsp70 peptide (TKDNNLLGRFELSG) termed TKD, thus a special antibody cmHsp70.1 targeting membrane Hsp70 is generated by using TKD as immunogen (Multhoff and Hightower, 2011). Both on viable human and mouse tumor cells and in tumor-bearing mice, the cmHsp70.1 antibody is used to selectively detect the C-terminal substrate binding domain (Multhoff and Hightower, 2011; Radons and Multhoff, 2005; Stangl et al., 2011b). Membrane Hsp70 expression was shown to be regulated by anti-inflammatory drugs and antineoplastic agents (Calderwood et al., 2005; Gehrmann et al., 2004; Multhoff, 2007; Pfister et al., 2007). For instance, in a leukemic cell line and colon carcinoma cell line, compared to DNA-interacting antitumor agents (cytarabine, ifosfamid), vincristine and paclitaxel can increase not only the cytoplasmic but also the membrane Hsp70 expression (Gehrmann et al., 2002). Membrane Hsp70 is correlated with different clinical outcomes in different cancers. For example, the membrane Hsp70-positive phenotype has been associated with a higher

metastatic potential and an unfavourable prognosis in malignant melanoma, acute myeloid leukaemia as well as lower rectal and squamous cell carcinoma of the lung (Farkas et al., 2003; Pfister et al., 2007). However, in colon and gastric cancer, membrane Hsp70 expression correlates with a significantly improved survival (Pfister et al., 2007).

Membrane Hsp70 exosomes, which can associate with lipid raft sphingolipid globoyltriaosylceramide Gb3 and phosphatidylceramide components in membrane of tumor cells, can be released from membrane Hsp70 positive tumor cells (Multhoff and Hightower, 2011). The secretion of membrane Hsp70 was described to be in an ATP binding cassette-dependent lysosomal/endosomal pathway (Mambula et al., 2007; Multhoff and Hightower, 2011). The secretion is induced by neither high/low concentration of salt nor PH change (Vega et al., 2008). Membrane Hsp70 exosomes can induce the migratory and cytolytic activity of natural killer cells to initiate apoptosis in tumor cells (Elsner et al., 2007; Multhoff, 2007; Radons and Multhoff, 2005). Membrane Hsp70 exosomes have been shown to induce the accumulation and activation of suppressive immune cells to help tumor cells escape from immunity killing in human and mouse (Chalmin et al., 2010). So clear functions of membrane Hsp70 is still unknown.

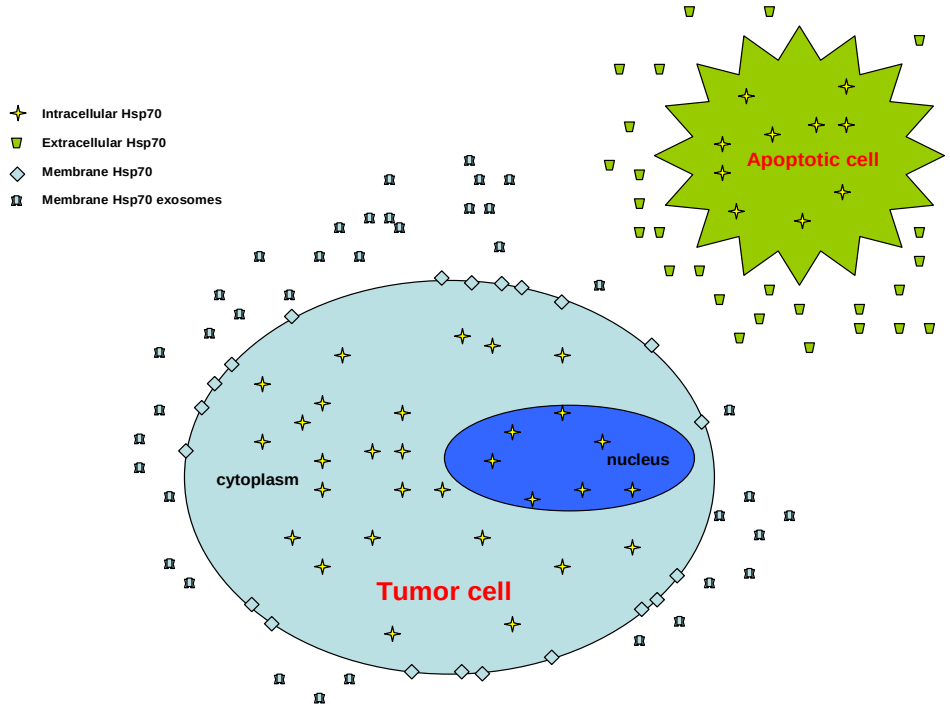

Fig 1-1: The distribution of different forms of Hsp70 in cells: intracellular Hsp70, extracellular Hsp70, membrane Hsp70 and membrane Hsp70 exosomes. Intracellular Hsp70 can be found in cytoplasm and nucleus of tumor cells and apoptotic cells, extracellular Hsp70 can be secreted from apoptotic cells, membrane Hsp70 can be expressed on the surface of tumor cells and be secreted by tumor cells in form of exosomes.

1.4 Aims of this study

Several studies investigated the expression of intracellular Hsp70 in GBM cell lines and in tumor specimens from GBM patients (Hermisson et al., 2000), but not the membrane Hsp70 (Elsner et al., 2007; Hermisson et al., 2000). But there was no direct correlation between intracellular Hsp70 expression and radioresistance of glioma cell lines (Fedrigo et al., 2011) or clinical outcome of patients with GBMs (Hermisson et al., 2000). Thus, the expression of membrane Hsp70 in gliomas was investigated in the current study.

The aims of this study:

1. To investigate the expression of the specific membrane Hsp70 in gliomas regarding grade-dependency and to compare its expression in primary GBM and secondary GBM.
2. To detect the localization of membrane Hsp70 within GBM tissues and to investigate a potential co-expression with hypoxic markers.
3. To define the membrane Hsp70-positive subpopulations isolated from primary GBM.
4. To determine whether hypoxia can induce membrane Hsp70 expression in GBM-derived subpopulations.
5. To investigate secretion of membrane Hsp70 into conditioned media of different isolated populations and to compare the secretion of membrane Hsp70 in serum of patients with primary and secondary GBM.

2 Materials and methods

2.1 Tissue samples

Tumor probes for cell isolation and tissue specimens were acquired directly from patients with gliomas (n = 65), non-neoplastic brain were aquired from temporal lobe resections of epilepsy patients in Department of Neurosurgery, University of Munich. The study was approved by the local ethical committee, written informed consent was obtained from all patients. Diagnosis was made by the Dept. of Neuropathology, University of Munich, according to WHO criteria. N= 55 tissue samples (non-neoplastic brain n = 3, astrocytoma WHO II n = 5, astrocytoma WHO III n = 11, primary GBM n = 23, secondary GBM n = 13) were investigated for immunohistochemistry and n=10 samples were investigated for isolation of cell subpopulations.

2.2 Immunohistochemistry

Tumor tissues were obtained from tumor resections of glioma patients and non-neoplastic brain samples were obtained from temporal lobe resections of epilepsy patients. Fresh tissues were fixed according the manufacturer's protocol of HOPE (Hepes-glutamic acid buffer-mediated organic solvent protection effect) formula (Wiedorn et al., 2002) and embedded in paraffine. All immunohistochemical stainings of non-neoplastic and gliomas tissues were performed in the tumorbiology laboratory, Department of Neurosurgery by using DAB staining kit. In brief, HOPE-treated, paraffin-embedded tissue sections were deparaffined and unmasked the epitope by

treating the sections in 100% isopropanol at 60 ℃ for 30 min, then fix in 70% ice-cold acetone at -20 ℃ for 10 min, washed in water for 10min, the slides were then incubated with peroxidase blocking buffer at room temperature for 10 min for peroxidase blocking. After a washing step in phosphate-buffered saline for two times, the slides were incubated with protein blocking buffer at room temperature for 30 min for nonspecific binding blocking. Then the slides were incubated overnight at 4 ℃ with the primary antibodies shown in Table 2-1. Following three 10-min washing step in TBS, the slides were incubated with the anti-mouse biotinylated antibody at room temperature for 15 min. After 5-min washing in TBS, the slides were incubated with horse radish peroxidase at room temperature for 20 min. After three 10-min washings in TBS, the expression was detected by using DAB Chromogen and nuclei were counterstained with haematoxylin. After dehydration with methanol for 30 min, the slides were coverslipped with entellan and microscopically photographed for further analysis.

Table 2-1: Antibodies list for immunohistochemistry.

Antibody	**Company**	**Cat.No**	**Quantity**	**Dilution**
IDH1	Dianova,Germany	DIAH09	0.2mg/ml	1:50
cmHsp70.1	Multimmune,Germany		2.4mg/ml	1:50
cytoHsp70	Enzo life Sciences, Switzerland	ADI-SPA-810	1.0mg/ml	1:50
Glut-1	Dako,Germany	A3536	2.2mg/ml	1:100
HIF-1alpha	Santa Cruz,USA	sc-53546	0.2mg/ml	1:50

2.3 Isolation and cultivation of Cell samples

2.3.1 Isolation of primary GBM cell culture

All tumor tissues used for cell isolation were obtained from patients with primary GBM. Tumor tissues were kept on ice immediately after resection and processed to cell isolation in 2 hours. In brief, tumor tissue was minced into small pieces and then homogenized in Dulbecco's MEM medium supplemented with 20% fetal calf serum, antibiotics mix consisting of penicillin, streptomycin and glutamine as well as non-essential amino acids. After 60-min enzyme digestion at 37 ℃ and filtration with 70-μm filter, the single-cell suspension was obtained. To isolate glioblastoma primary culture, this suspension was directly cultured in DMEM supplemented with 20% FCS medium.

2.3.2 Isolation of CD133-positive cells

To isolate CD133-positive cells, tumor tissue-derived single-cell suspension was separated using MACS-column according to manufacturer's protocol with anti-CD133 antibody, the positive isolated cells were cultured with DMEM/ F12 supplemented with N2, B27, hEGF and FGF in Gelatine-coated plastic flasks. The expression of CD133 was determined by flow cytometry with two different phenotype CD133 antibodies (Miltenyi, Germany).

2.3.3 Isolation of endothelial cells

To isolate CD-31-positive microvascular endothelial cells from GBM, tumor tissue-derived single-cell suspension was separated by using Dynabeads magnetic beads with anti-CD31 antibody. CD31 positive endothelial cells were plated in 2% gelatine coated plastic flasks and cultured with Microvascular Endothelial Cell Growth Medium (Provitro Company, Germany).

2.3.4 Isolation of mesenchymal stem-like cells

To isolate GBM-derived mesenchymal stem cells, tumor tissue-derived single-cell suspension was used. After Ficoll-gradient sorting, the mononuclear cells were obtained and cultured in DMEM supplemented with 20%FCS. The glioblastoma-derived mesenchymal stem-like cells analyzed for mesenchymal stem-like cell markers by flowcytometry and the ability of osteogenic and adipogenic differentiation was determined in *vitro*.

2.3.5 Normoxia and hypoxia cultivation

GBM cell lines U87 and U373 were purchased from ATCC (Manassas, VA, USA) and cultured in Dulbecco's MEM medium supplemented with 10% fetal calf serum, antibiotics mix and MEM non-essential amino acids.

All cells were cultured in plastic flasks and kept in humidified atmosphere, 5% CO_2 at 37 °C. Trypsin/EDTA solution was used for passaging . Medium was changed every 48 hours. All cells were analyzed at passage 1-3. To treat cells with hypoxia incubation (0.01% O_2, humidified atmosphere and 5% CO_2 at 37 ℃ in the hypoxia incubator) for 72h, cells were cultured in normal condition at first and change to fresh medium before hypoxia incubation.

2.4 Characterization of cell samples

2.4.1 Osteogenic and adipogenic differentiation of mesenchymal stem cells

To detect the differentiation potential of isolated mesenchymal-like stem cells, osteogenic and adipogenic differentiation were performed as described previously(Hong et al., 2005; Kim et al., 2003; Oswald et al., 2004). In brief, for osteogenic differentiation, $1x10^4$ cells were seeded in

12-well plates with DMEM medium for 3 days, then switched to differentiation medium which supplemented with 0.1μM Dexamethasone, 10mM beta-glycerophosphate, 0.05mM L-ascorbic acid, with medium changed every 3 days. After 14 days, plates were stained with Kossa staining and photographed. For adipogenic differentiation, $1x10^5$ cells were seeded in 12-well plate with normal DMEM medium for 3 days, then were switched to differentiation medium which supplemented with 1μM dexamethasone, 0.5μm isobutylmethylxanthine, 100μm indomethacin, 10μg/ml insulin, with medium changed every 3 days. After 14 days, plates were stained with Oil Red O and photographed.

2.4.2 Tube formation of endothelial cells

To detect the ability for three dimensional cellular organization potential of isolated endothelial cells, tube formation assay on Ibidi angiogenesis slide (ibidi GmbH, Germany) was performed as described previously (Kumar et al., 2003). In brief, matrigel was plated on Ibidi angiogenesis slide after thrawing on ice, then was incubated at 37℃ for 30 min to allow matrigel to polymerize. $1x10^4$ endothelial cells with 50μl medium were seed into coated wells. After incubation for 24 hours at 37℃ in 5% CO_2 humidified atmosphere, the plates were photographed.

2.5 Fluorescence activated cell sorting (FACS)

Cultured cells were washed with PBS and detached from flask using Trypsin/ EDTA at 37 ℃ for 3min. For Hsp70-FACS analysis, after washing with FACS buffer (PBS supplemented with 10% FCS) membrane-Hsp70-specific mouse monoclonal IgG1 antibody cmHsp70.1-FITC and FITC conjugated IgG1 isotype-matched negative control immunoglobulin for 30min on ice in the dark. After another washing step, cells were resuspended in 500μl FACS buffer. 5μl propidium iodide for

viability staining was added. Only propidium iodide negative cells were analyzed on a FACS Calibur flow cytometer (BD Biosciences, USA). For CD133-FACS-analysis, PE-coupled-antibody from Miltenyi Biotechnology with the corresponding IgG control was used. The following markers shown in table 2-2 for endothelial cells and mesenchymal stem-like cells were tested: CD31, CD34, CD44, CD45, CD90 and CD105. Data analysis was performed with flowjo® 6.7.1 (Treestar, California, USA).

2.6 Enzyme-linked immunosorbent assay (ELISA)

Serum from patients with epilepsy and primary and secondary GBM and conditioned medium from isolated cell populations, including primary tumor cell culture, endothelial cells, and mesenchymal stem-like cells, were used to detect the concentration of membrane Hsp70 in exosome form. To obtain serum samples, blood samples were centrifuged for 10 min at 15,000 g to remove all debris. The patients list is shown in Table 3-3. To obtain conditioned medium from isolated cell subpopulations, $1x10^5$ cells were seeded into 24-well plates with 500ul medium supplemented without FCS or growth factors. After 48-hour incubation in humidified atmosphere at 37℃, conditioned medium was collected and centrifuged at 15,000 g to remove all debris, the list of conditioned medium is also shown in Table 3-2. All serum and conditioned medium samples were stored at -80℃ for tests.

A sensitive sandwich enzyme-linked immunosorbent assay was performed by using Hsp70 Duo Set IC kit according to the protocol of the manufacturer. Briefly, samples and standards were added to a 96-well plate coated with a mouse anti-human Hsp70 capture antibody and incubated for 2h at

room temperature. After being washed, the plate was incubated with anti-Hsp70 detection antibody conjugated to horseradish peroxidase at room temperature. Color reaction was performed by using a substrate solution and stopped with an acid solution. Finally, absorbance was measured with a microplate reader at a wavelength of 450nm. A standard curve should be generated for samples assayed and calculated.

2.7 Statistical analyses

The ELISA data were summarized as the mean ± SEM (standard error of the mean). Statistical analysis was performed using student's test (as indicated in the figure legend or the text). Statistical significance was accepted at the $P<0.05$ level.

Table.2-2: Antibodies list for FACS.

Antibody	**Company**
PE labelled-CD45	Serotec
PE labelled-CD44	Serotec
PE labelled-CD90	Serotec
PE labelled-CD105	Serotec
PE labelled-CD34	BD
PE labelled-CD29	Serotec
PE labelled-CD31	Serotec
PE labelled-CD133/1	miltenyi
PE labelled-CD133/2	miltenyi
FITC labelled-cmHsp70	multimmune
PE labelled- IgG1	Serotec
PE labelled-IgG2a	Serotec
PE labelled-IgG1	MACS
PE labelled-IgG1	BD
FITC labelled-IgG1	BD

3 Results

3.1 Membrane Hsp70 is over-expressed in (primary) GBM tissues, but not in normal brain and WHO grade II and III gliomas

To investigate the expression of membrane Hsp70 in gliomas, immunohistochemistry staining of membrane Hsp70 in primary GBM (n=23), non-neoplastic brain (n=3), diffuse astrocytoma (n=5) and anaplastic astrocytoma (n=11) was studied, as shown in Table. 3-1. In non-neoplastic brain, no positive expression of membrane Hsp70 could be detected. In WHO grade II and III gliomas, there was also no positive signal for membrane Hsp70 (Fig. 3-1). Compared to normal brain and WHO grade II and III gliomas, all primary GBM tissues (n=23) showed strong positive expression of membrane Hsp70. In primary GBMs, the membrane Hsp70 positive expression always localized within tumor parenchyma, and strongest expression was found in peri-necrotic regions. There was no positive signal for membrane bound Hsp70 in tumor vessels neither in normal brain nor in gliomas of different grades.

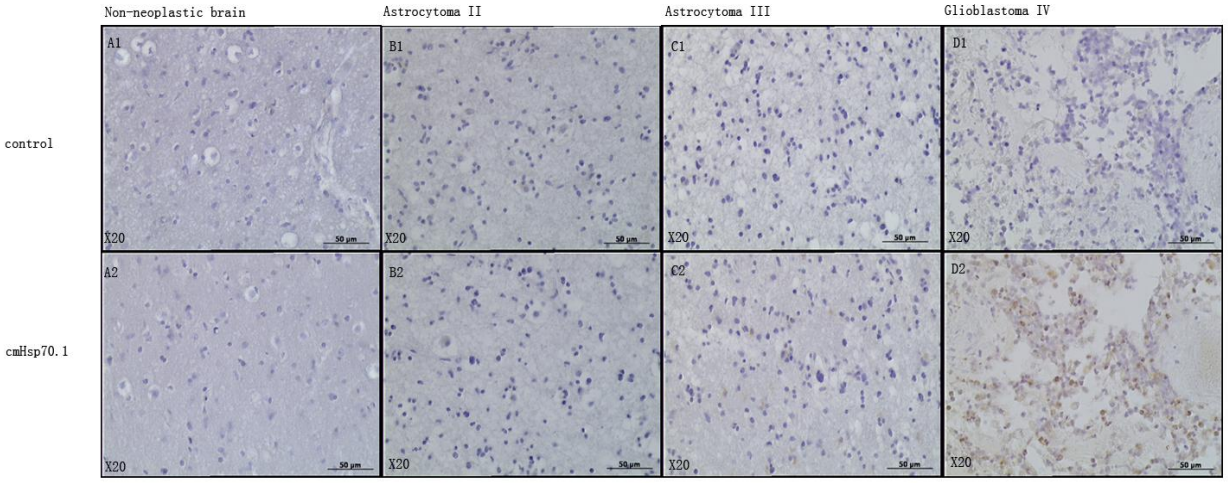

Fig.3-1: Expression of membrane Hsp70 and corresponding negative control in non-neoplastic brain (No.TB409), diffuse astrocytoma (WHO grade II, No.TB400), anaplastic astrocytoma (WHO grade III, No.TB665) and primary GBM (WHO grade IV, No.TB771). In normal brain (A2) and WHO grade II (B2) and III (C2) astrocytomas, there was no detectable expression of membrane Hsp70. Only in primary GBM (D2), strong brown signals could be detected within the tumor and were supposed to be specific positive membrane Hsp70 expression. Magnification x20.

3.2 Expression of intracellular Hsp70 and membrane Hsp70 in normal brain and WHO grade II or III astrocytomas

Heat shock protein 70 was previously described in the intracellular localization of tumor cells, but was not only found on the membrane of tumor cells (Calderwood et al., 2005). To compare the expression of intracellular Hsp70 and membrane Hsp70 in normal brain and gliomas (including diffuse astrocytoma and anaplastic astrocytoma), different antibodies which separately aim to the membrane or intracellular localized antigens of Hsp70 were used for immunohistochemistry. In our immunohistochemical staining, intracellular Hsp70 could be found in a few cells within normal brain and grade II and III glioma tissues and also vessel-associated cells (Fig.3-2; Table.3-1). The expression of intracellular Hsp70 did not show any correlation to the tumor grade, we found positive signals in most of diffuse astrocytomas and anaplastic astrocytomas (Fig.3-2; Table.3-1), which is in line with previous publications (Hermisson et al., 2000). In comparison with the results of intracellular Hsp70, there was no positive signal for membrane Hsp70 in non-neoplastic brain or WHO grade II or III glioma tissues. In line with the histopathological diagnosis obtained by the neuropathological department, we found positive signal for IDH-1 mutation which was uniquely expressed in the parenchyma of WHO grade II (5 positive samples of 5 samples) and III gliomas (8 positive samples of 11 samples). WHO grade II and III gliomas expressed mutated IDH-1 in a variety of cells within the tumor, whereas no positive signal was detectable in normal brain (Fig.3-2; Table.3-1).

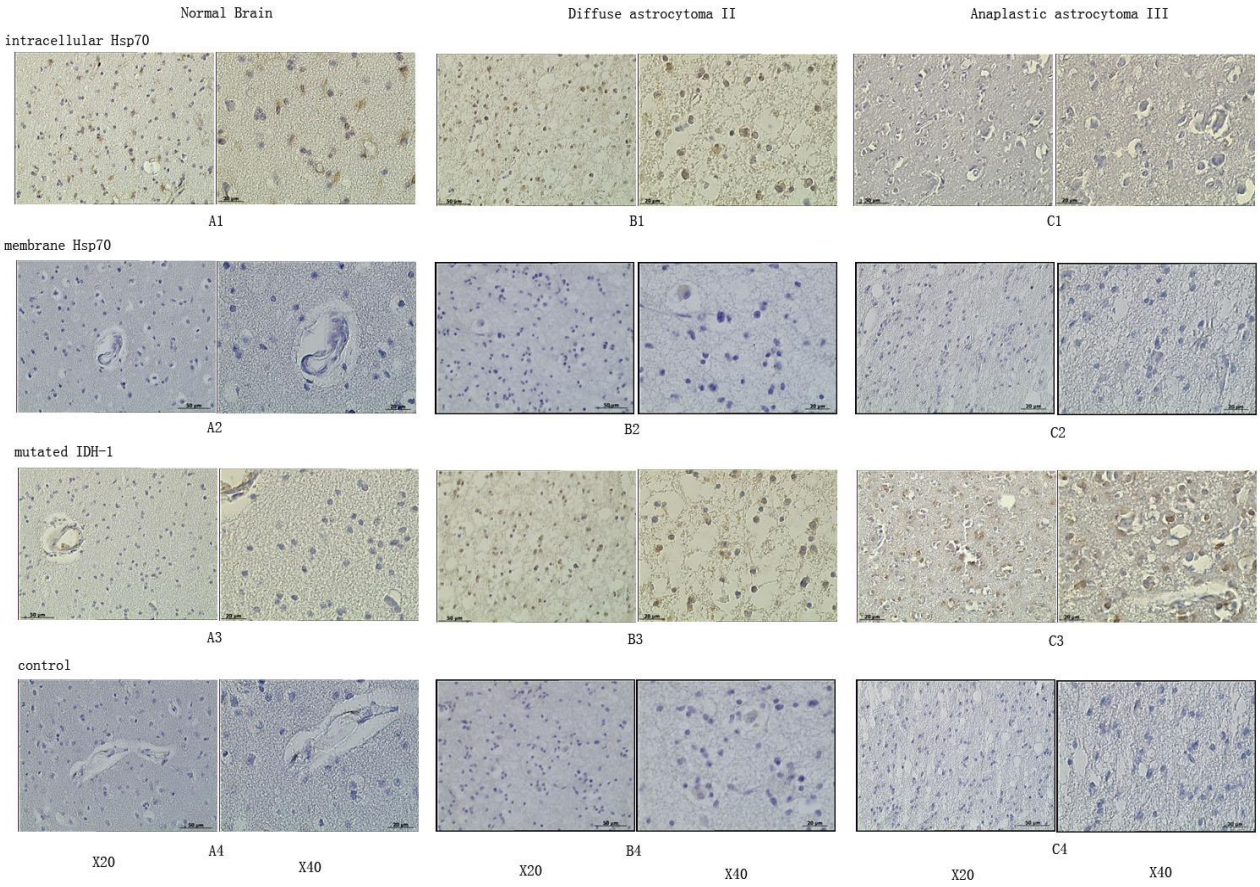

Fig.3-2: Immunohistochemical staining of intracellular Hsp70 (A1, B1, C1), membrane Hsp70 (A2, B2, C2), mutated IDH-1 (A3, B3, C3) and corresponding negative control (A4, B4, C4) in normal brain (No.TB409), diffuse astrocytoma (WHO grade II, No.TB400), anaplastic astrocytoma (WHO grade III, No.TB579). Expression of intracellular Hsp70 was not grade-dependent. It could be positive or negative in normal brain (A1) and WHO grade II (A2) and III (A3) astrocytomas. Compared with intracellular Hsp70, membrane Hsp70 showed definite negative expression in normal brain (A2) and WHO grade II (B2) and III (C2) astrocytomas. Expression of Mutated IDH-1 could be found both in WHO grade II (B3) and III (C3) astrocytomas, but not in normal brain (A3). (Magnification: X20, X40).

3.3 Expression of membrane Hsp70 is detectable in primary GBM but not secondary GBM

To compare the membrane Hsp70 expression in primary GBMs and secondary GBMs, 23 primary GBMs and 13 secondary GBMs were immunohistochemical stained. In all primary GBMs (N=23; table.3-1), we detected clear upregulation of membrane Hsp70 expression compared to the low grade tumors (Fig. 3-1). Interestingly, secondary GBMs tissue did not show any positive signal for membrane Hsp70 (n = 13; Fig. 3-3; Table 3-1). In line with previous studies (Watanabe et al., 2009; Yan et al., 2009) and the histopathological diagnosis obtained by the neuropathological department, mutated IDH-1 staining was performed in every sample as a marker for secondary GBM, ten of thirteen samples of 13 were positive. Selectively tumor cells expressed mutated IDH-1 in secondary GBM tissues (10 positive samples of 13 samples). As expected, none of the primary GBM tissues expressed mutated IDH-1 (n = 23).

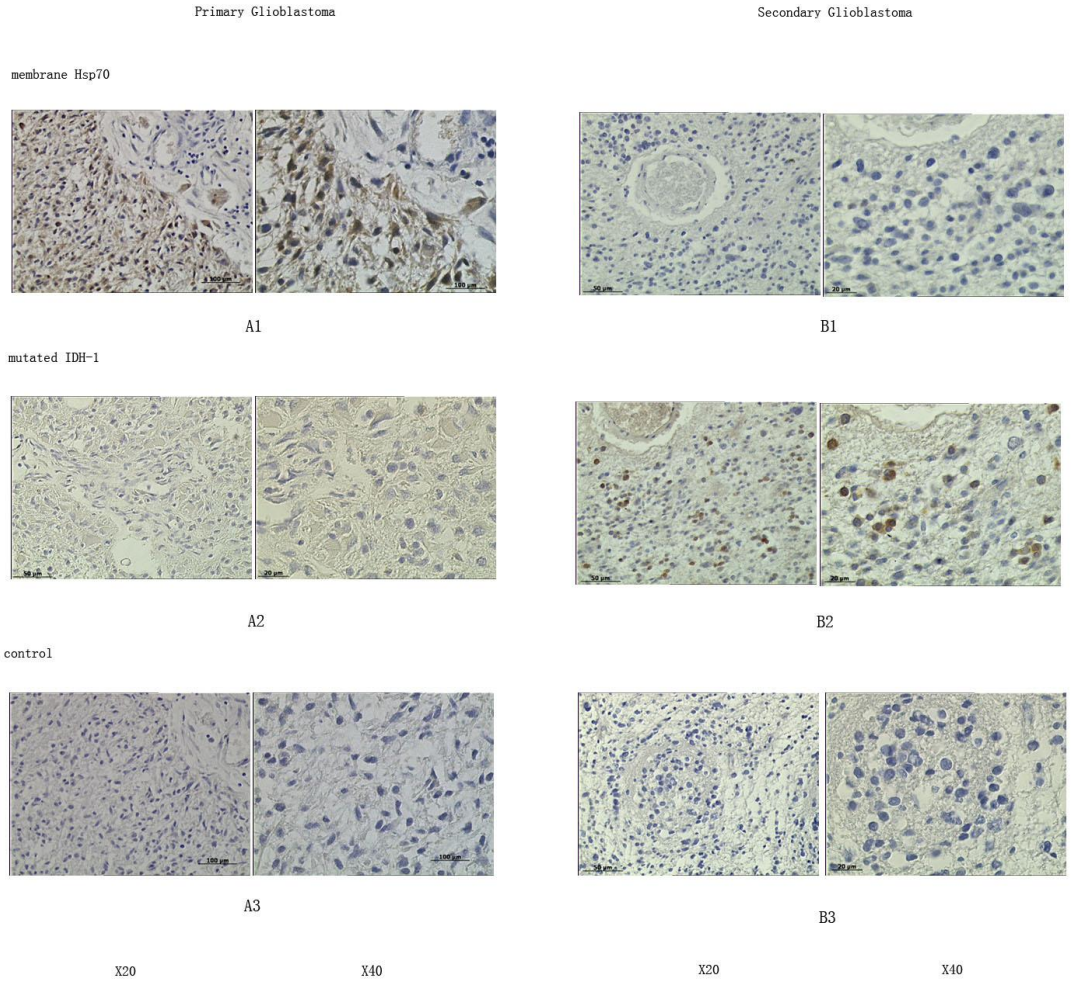

Fig.3-3: Immunohistochemical stainings of membrane Hsp70 (A1, B1), mutaed IDH-1 (A2, B2) and corresponding negative control (A3, B3) in primary GBM (No.TB410) and secondary GBM (No.TB490).

Membrane Hsp70 was clearly upregulated in primary GBM (A1) within tumor tissue but not in gliomeruloid vessel proliferation area. But there was no expression in secondary GBM (B1). Mutated IDH-1 was expressed uniquely in secondary GBM (B2) but not in primary GBM (A2), as already described in literatures. (Magnification: X20, X40)

3.4 Membrane Hsp70 is expressed in GBM cells lines, primary tumor cell cultures, and CD133-positive cells, but not in mesenchymal stem-like cells, endothelial cells and bone-marrow derived stem cells

To further investigate the expression of membrane Hsp70 in the tumor microenvironment of primary GBM, different subpopulations (including primary tumor cell cultures, CD133 positive cells, mesenchymal stem-like cells, endothelial cells) were isolated from resected tumor specimens in primary GBM patients and checked for membrane Hsp70 expression by FACS. GBM cell lines U87 and U373 and Bone marrow derived mesenchymal stem cells, as negative control for GBM derived mesenchymal stem-like cells, were also determined.

3.4.1 Membrane Hsp70 expression in GBM cell lines and primary tumor cell cultures

GBM cell lines U87 and U373 were analysed for membrane Hsp70 expression. The GBM cell line U87 and U373 showed strong positive signal for FITC-conjugated membrane Hsp70 with mean fluorescent intensities of 35 – 65 (data not shown). Primary tumor cell cultures, isolated from primary GBM also showed positive signals for FITC-conjugated-membrane Hsp70 in general, but in different extents regarding the mean fluorescent intensity (Fig3-7.A).

3.4.2 Membrane Hsp70 expression in CD133 positive cells

CD133 positive cells, which were isolated from GBM patients and culture with growth factors, grown in plastic-adherent morphology on coated flask and stably expressed CD133 markers (Fig.3-4). The expression of membrane Hsp70 on CD133-positive cells was positive. Compared with GBM-isolated primary tumor cell cultures, CD133 positive cells showed weaker positive expression of membrane Hsp70 (Fig.3-7.B).

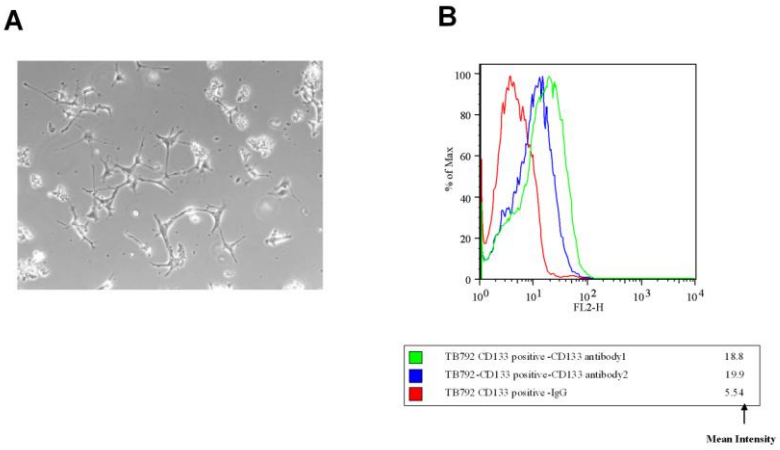

Fig.3-4: Characterization of CD133 positive cells. (A) Cells were isolated by using MACS-column and cultivated in Gletine-coated flask with DMEM/F12 medium supplemented with growth factors, after 15 days, cells growth as adherent morphology. (B) Cells were still CD133 positive after cultivation for several passages in *vitro*, the expression were determined by FACS.

3.4.3 Membrane Hsp70 expression in GBM derived endothelial cells

In general, endothelial cells always show CD31 and VEGFR-2 positive expression, which were shown by immunohistochemisty staining of tumor tissues and FACS (Fig.3-5). Moreover, the tube formation assay of endothelial cells which isolated from GBM was performed on matrigel to confirm its vessel formation capability, as shown in Fig 3-5. No expression of membrane Hsp70 on GBM derived endothelial cells was shown (Fig .3-7.C).

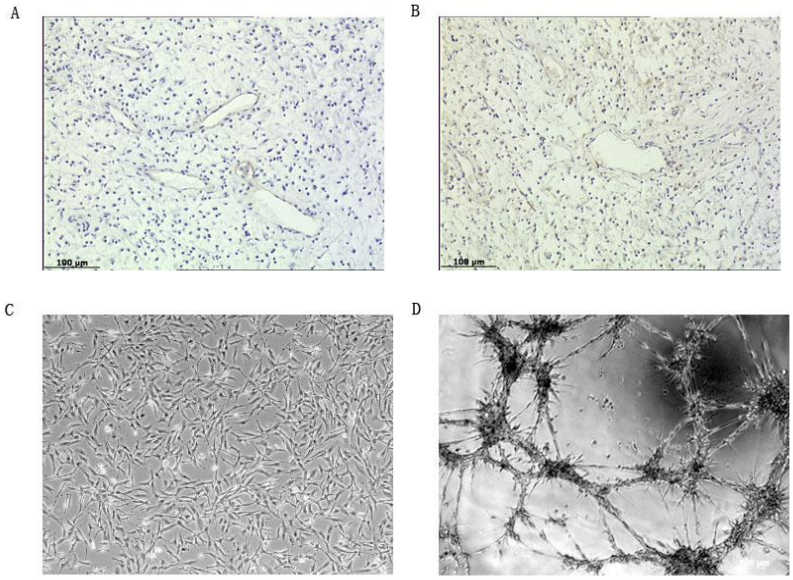

Fig.3-5: Characterization of endothelial cells. Endothelial cells in GBM tissues expressed CD31 (A) and VEGFR-2 (B). After several passages cultivation in *vitro*, endothelial cells derived from GBM showed similar morphology (C) and tube formation ability in matrigel assay (D).

3.4.4 Membrane Hsp70 expression in GBM derived and bone marrow derived mesenchymal stem-like cells

Mesenchymal stem cells were defined as plastic-adherent cells under standard culture condition; in standard culture conditions, cells have positive expression of CD105, CD90, CD44, and negative expression of CD14, CD34, CD45; In *vitro*, mesenchymal stem cells can differentiate into osteocytes, adipocytes, and chondrocytes under specific stimuli (Horwitz et al., 2005). As shown in Fig.3-6, this subpopulation isolated from primary GBM was identified to be mesenchymal stem-like cells. The expression of membrane Hsp70 in mesenchymal stem-like cells isolated from GBM tissue was negative, as shown in Fig.3-7.D, and was similar with the expression in mesenchymal stem cells isolated from bone marrow (Fig.3-7.E).

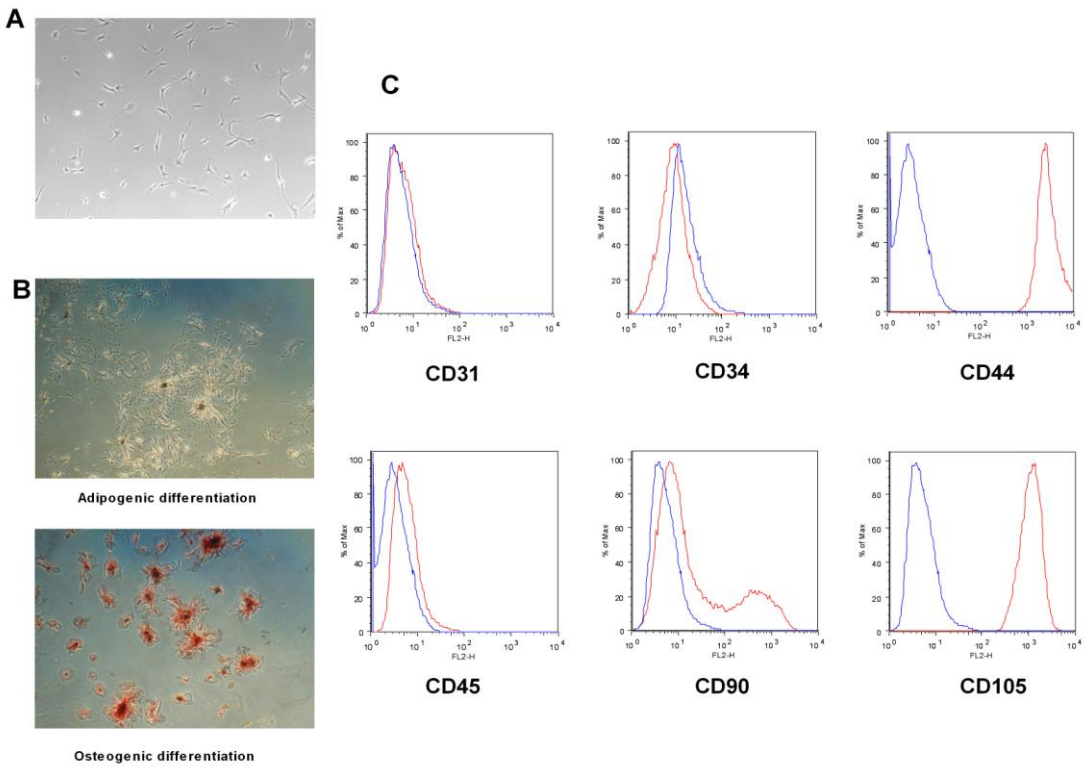

Fig.3-6: Characterization of GBM derived mesenchymal stem-like cells. (A) Isolated mesenchymal stem-like cells were plastic-adherent in DMEM medium supplemented with 20% FCS. (B) In adipogenic differentiation assay, isolated mesenchymal stem-like cells were cultured in differentiation medium for 14 days, and stained following kossa staining (upper) and Oil red S (lower). (C) Isolated mesenchymal stem-like cells were checked for typical markers by FACS analysis. Comparing with FITC labelled IgG (blue line), FITC labelled CD31, CD34, CD45 (red line) were negative and CD44, CD90, CD105 (red line) were positive.

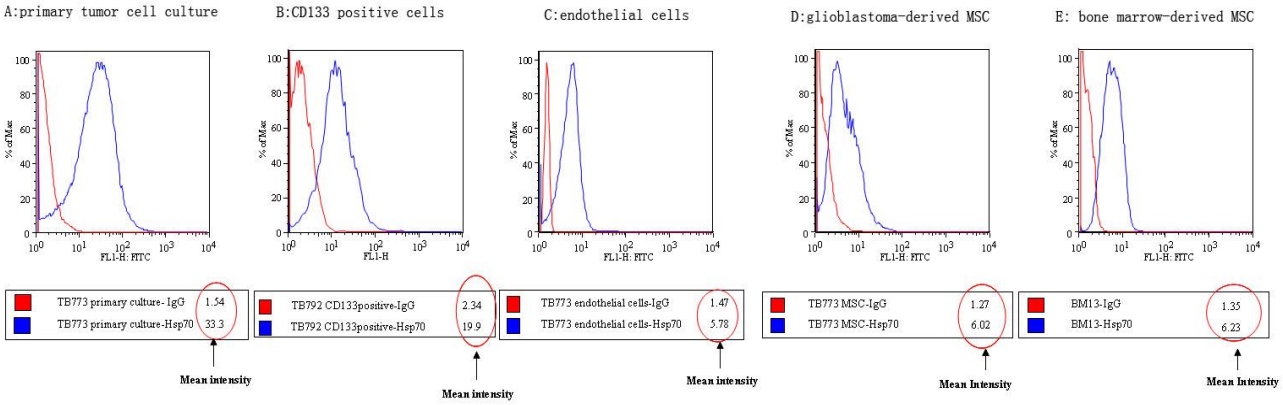

Fig.3-7: Expression of membrane Hsp70 in isolated subpopulations. Among these subpopulations, the expression in primary tumor cell culture (A) was strong positive, the expression in CD133 positive cells (B) was positive but was not as strong as primary tumor cell culture. On the other hand, the expression in endothelial cells (C), mesenchymal stem-like cells derived from GBM tissue (D) and bone marrow (E) was negative. The expression was analyzed by FACS.

3.5 Hypoxia increases the expression of membrane Hsp70 in GBM cell lines and primary tumor cell cultures.

Hypoxia, which contributes to invasion, necorsis, therapy-resistance, recurrence, is a major feature in GBM (Oliver et al., 2009). Herein, the effect of hypoxia on the expression of membrane Hsp70 in primary GBM was investigated. After cultivation of GBM cell lines U87 under normoxia and hypoxia (0.01% O_2 saturation) for 72h, cells were analyzed for FITC-conjugated-membrane Hsp70 expression. Compared to the normoxic group, mean fluorescent intensity was doubled in the hypoxic group (Fig 3-8A).

For primary tumo cell culture from GBM, the induction of FITC-conjugated- membrane Hsp70 expression phenomenon could also be achieved (Fig.3-8B). In the other subpopulations including GBM derived mesenchymal stem-like cells ((Fig.3-9A), endothelial cells (Fig.3-9B) and bone marrow-derived stem cells (Fig.3-9C), which did not show any membrane Hsp70 expression under normoxia, membrane Hsp70 expression could not be induced under hypoxia cultivation. Thus, hypoxia can only increase the expression of membrane Hsp70 in positive cell subpopulations, but not in membrane Hsp70 negative cell subpopulations.

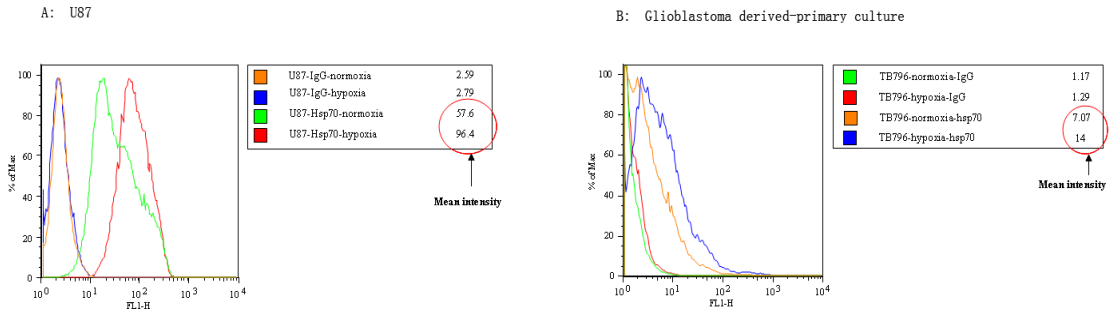

Fig.3-8: Hypoxia incubation effect on membrane Hsp70 expression in GBM tumor cell line U87 (A) and GBM derived primary tumor cell cultures (B). U87 and primary tumor cell culture was positive for membrane Hsp70. After 72-hour cultivation in hypoxia, the expression increased. The expression was analyzed by FACS.

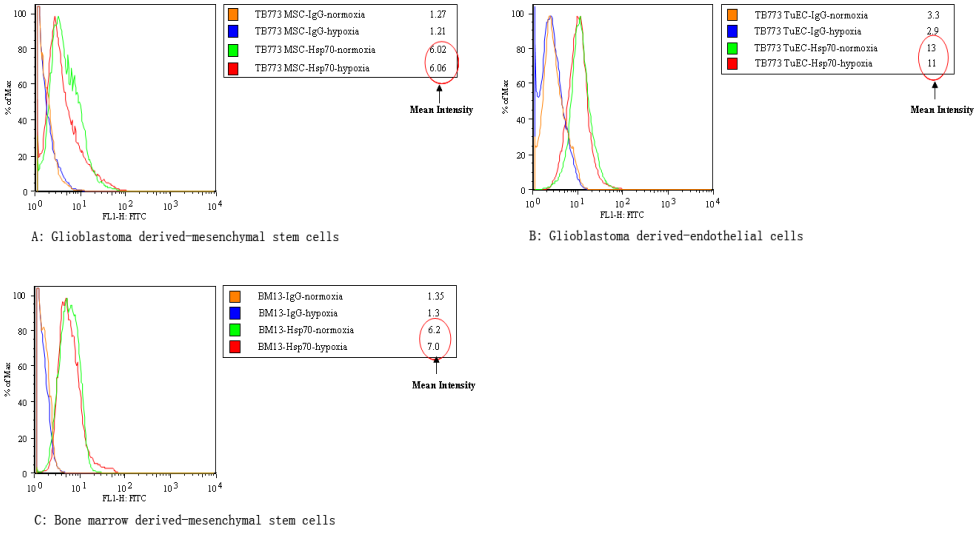

Fig.3-9: Hypoxia incubation has no effect on membrane Hsp70 expression on membrane Hsp70 negative expression isolated subpopulations. GBM derived endothelial cells (B) and mesenchymal stem-like cells (A) showed negative membrane Hsp70 expression and the expression did not increase after hypoxia cultivation. Bone marrow derived mesenchymal stem-like cells (C) showed similar result under hypoxia cultivation. The expression was analyzed by FACS.

3.6 Expression of membrane Hsp70 in GBM tissue regarding localization to hypoxic areas

To detect hypoxia regions in gliomas of different grades, hypoxia marker Glut-1 was investigated. In normal brain and WHO grade II and III gliomas, Glut-1 was shown to be expressed in the erythrocytes and endothelial cells but not in the tumor parenchyma, shown in Fig.3-10. These results were in line with already pulished studies (Rademakers et al., 2011; Vaupel and Mayer, 2007). To further investigate the relationship between hypoxia and the expression of membrane Hsp70 in primary GBM tissues, expression of hypoxia markers (Glut-1 and HIF-1alpha) and membrane Hsp70 on primary GBM tissue were analyzed by immunohistochemistry. In Glut-1 staining of consecutive slides in primary GBM tissue (n = 23), a coexpression of membrane Hsp70 (Fig. 3-11, D1) and Glut-1 (Fig. 3-11, B1) was detected in perinecrotic areas and weaker in non-hypoxic tumour parenchyma. In vascularized areas, no membrane Hsp70 or Glut-1 expression could be found on endothelial cells and the surrounding tumour tissue (Fig. 3-11, D2 and B2). HIF-1α, another marker for hypoxia, showed similar localization for positive signal compared to Glut-1 (Fig. 3-11, C1). But HIF-1α was expressed much weaker than Glut-1 and positive signal for HIF-1α could also be found in well vascularized areas within the tumor tissue (Fig. 3-11, C2).

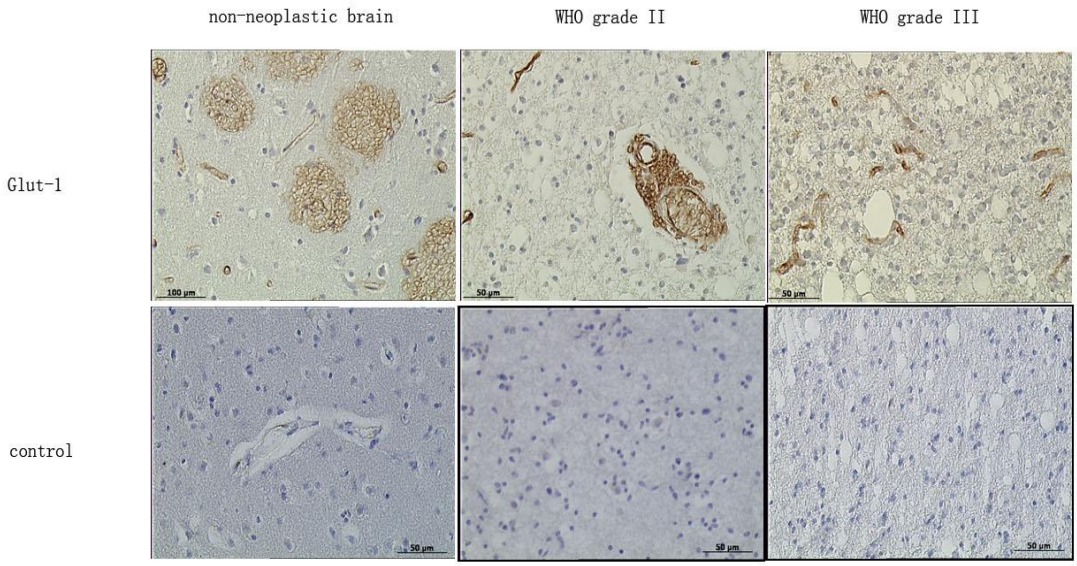

Fig.3-10: Immunohistochemical stainings of hypoxia marker Glut-1 (upper line) as well as corresponding negative control (lower line) was performed in non-neoplastic brain, WHO grade II and III gliomas. In normal brain and WHO grade II and III gliomas, Glut-1 was shown to be expressed in the erythrocytes and endothelial cells but not in the tumor parenchyma.

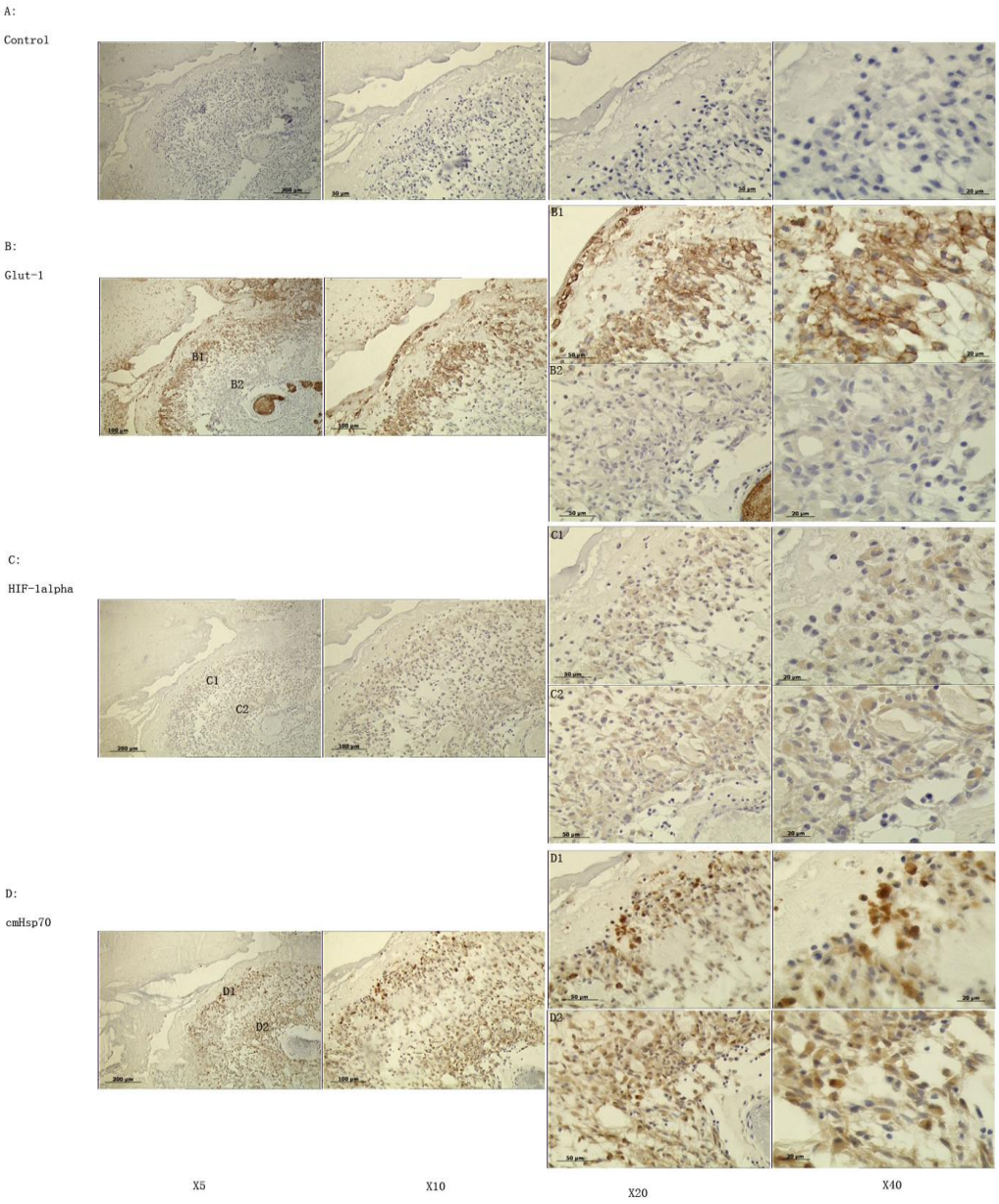

Fig. 3-11: Immunohistochemical stainings of hypoxia markers Glut-1 and HIF-1α as well as membrane Hsp70 were performed in consecutive slides of primary GBM. Positive statining of Glut-1 and HIF-1α was localized in peri-necrotic areas of the tissue (B1, C1, D1). Also membrane Hsp70 expression could be found in peri-necrotic zone. In good vascularized tumour areas around a big vessel (B2) no Glut-1 expression was detected whereas HIF-1α expression was present (C2). Membrane Hsp70 showed colocalization with Glut-1 signal and could not be detected in vital tumour areas in vascularized areas (D2).

3.7 Membrane Hsp70 is highly secreted by primary tumor cell cultures, but not by endothelial cells and mesenchymal stem-like cells

As membrane Hsp70 on tumor cells could be secreted into the blood stream as a form of exosomes (Calderwood et al., 2005; Multhoff and Hightower, 2011). To investigate the secretion of membrane Hsp70 in different subpopulations, conditioned media were investigated *via* ELISA (Fig.3-12, Table.3-2). Only conditioned media from primary tumor cell cultures (n=4) showed high concentration of membrane Hsp70 protein, but much lower levels in conditioned media from endothelial cells (n=3) or mesenchymal stem-like cells (n=3). Three of four primary tumor cell cultures secreted more than 2000 pg/ml membrane Hsp70 into conditioned media (1803 pg/ml to 9834pg/ml, mean 5754pg/ml), endothelial cells (699pg/ml to 1885pg/ml, mean 1203pg/ml) and mesenchymal stem-like cells (883pg/ml to 2028pg/ml, mean 1544pg/ml) secreted significantly lower levels of membrane Hsp70 into conditioned media.

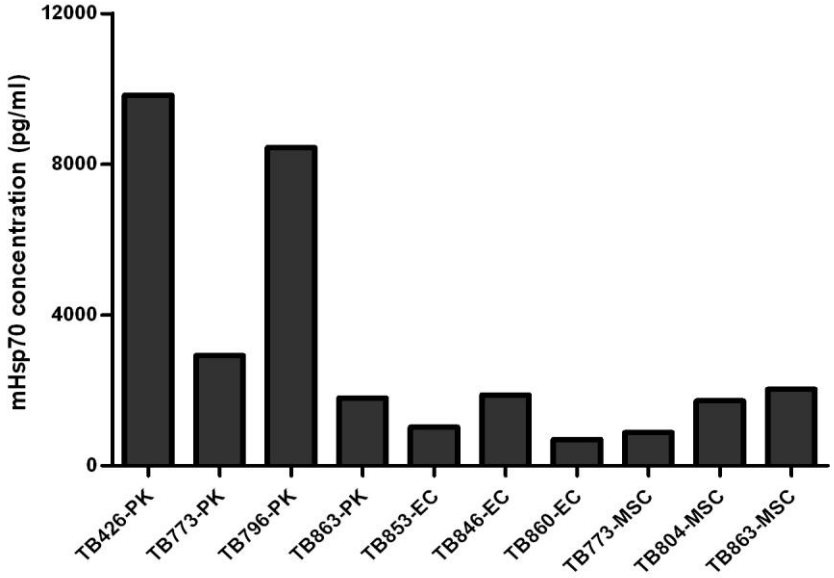

Fig.3-12: Determination of membrane Hsp70 in the conditioned mediums of primary GBM-derived subpopulations, including primary tumor cell cultures, endothelial cells, mesenchymal stem cells. Detail in Table.3-2. Membrane Hsp70 expression was analyzed by ELISA. Membrane Hsp70 concentration in three of four conditioned media of primary tumor cell cultures was >2000 pg/ml. But the values in the conditioned media of endothelial cells, mesenchymal stem-like cells were in a lower range, which were considered as basic levels.

3.8 Membrane Hsp70 is increased in serum of patients with primary GBM but not secondary GBM

In order to further validate the membrane Hsp70 secretion in *vivo* in primary GBM and secondary GBM, the concentration of membrane Hsp70 in serum samples was determined by using ELISA. As shown in Fig.3-13, compared with secondary GBM (8254±2277pg/ml) and normal control (9782±2646pg/ml), primary GBM (19009±1614pg/ml) showed significant higher membrane Hsp70 expression ($p<0.01$) in serum. So in the line with immunohistochemical staining results, primary GBM patients had higher levels of secreted membrane bound Hsp70 in the serum than secondary GBM patients.

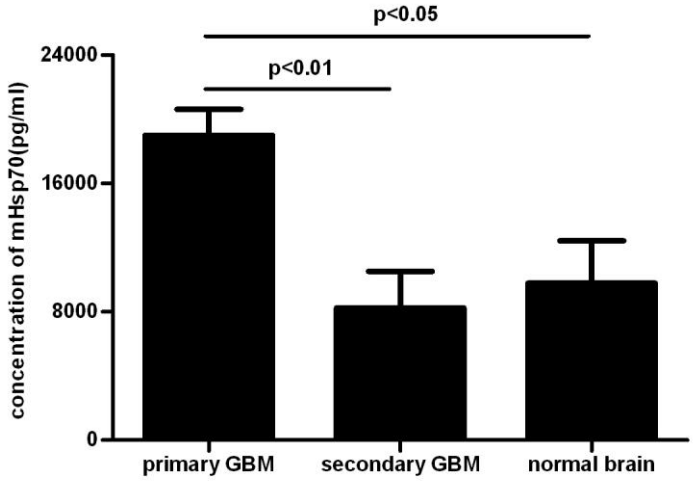

Fig.3-13: Membrane Hsp70 expression in serum of normal brain, primary GBM and secondary GBM. Serum were obtained from blood of 5 normal brain, 16 primary GBM and 9 secondary GBM patients, membrane Hsp70 concentration was determined by ELISA kits. The result showed concentration of secreted membrane Hsp70 was much higher in serum of patients with primary GBM than patients with secondary GBM ($P<0.01$) and control ($P<0.05$).

Table. 3-1: Overview of immunohistochemistry tissue

	antibody				
Number	**IDH1**	**Glut-1**	**mHsp70**	**iHsp70**	**pathology**
TB409	negative	positive	negative	positive	normal brain
TB547	negative	positive	negative		normal brain
TB601	negative	positive	negative		normal brain
TB400	positive	positive	negative	positive	astrocytoma II
TB411	positive	positive	negative		astrocytoma II
TB425	positive	positive	negative		astrocytoma II
TB509	positive	positive	negative		astrocytoma II
TB729	positive	positive	negative		astrocytoma II
TB538	positive	positive	negative		astrocytoma III
TB560	positive	positive	negative	negative	astrocytoma III
TB578	negative (previous grade II)	positive	negative		astrocytoma III
TB579	positive	positive	negative	negative	astrocytoma III
TB625	negative (previous grade II)	positive	negative		astrocytoma III
TB637	positive	positive	negative		astrocytoma III
TB665	positive	positive	negative	positive	astrocytoma III
TB672	positive	positive	negative		astrocytoma III
TB707	negative	positive	negative		astrocytoma III
TB750	positive (PCR)	positive	negative		astrocytoma III
TB781	positive	positive	negative	positive	astrocytoma III
TB402	negative	positive	positive		primary GBM
TB410	negative	positive	positive	positive	primary GBM
TB426	negative	positive	positive		primary GBM
TB500	negative	positive	positive		primary GBM
TB503	negative	positive	positive		primary GBM
TB541	negative	positive	positive weakly	negative	primary GBM
TB650	negative	positive	positive	negative	primary GBM
TB697	negative	positive	positive		primary GBM
TB704	negative	positive	positive		primary GBM
TB771	negative	positive	positive	positive	primary GBM
TB773	negative	positive	positive		primary GBM
TB792	negative	positive	positive	positive	primary GBM
TB804	negative	positive	positive	positive	primary GBM
TB817	negative	positive	positive		primary GBM
TB824	negative	positive	positive		primary GBM

TB833	negative	positive	positive		primary GBM
TB834	negative	positive	positive		primary GBM
TB837	negative	positive	positive		primary GBM
TB853	negative	positive	positive		primary GBM
TB869	negative	positive	positive		primary GBM
TB875	negative	positive	positive		primary GBM
TB877	negative	positive	positive		primary GBM
TB878	negative	positive	positive		primary GBM
TB348	positive	positive	negative	positive	secondary GBM
TB490	positive	positive	negative	positive	secondary GBM
TB551	positive	positive	negative	positive	secondary GBM
TB558	negative (previous grade III)	positive	negative	negative	secondary GBM
TB607	positive	positive	negative	negative	secondary GBM
TB715	negative (previous grade III)	positive	negative	negative	secondary GBM
TB716	positive	positive	negative		secondary GBM
TB742	positive	positive	negative		secondary GBM
TB747	positive	positive	negative		secondary GBM
TB785	negative (previous grade III)	positive	negative		secondary GBM
TB819	positive	positive	negative		secondary GBM
TB857	positive	positive	negative		secondary GBM
TB858	positive	positive	negative		secondary GBM

Table 3-1: This table is an overview of all stained tissue regarding the following markers: IDH-1, Glut-1, intracellular Hsp70 (iHsp70) and membrane Hsp70 (mHsp70). In WHO grade II and III gliomas, mutated IDH-1 expression was positive, Glut-1 and intracellular Hsp70 expression was variable positive, but membrane Hsp70 expression was negative. In primary GBM, mutated IDH-1 expression was negative whereas membrane Hsp70 expression was always positive. Intracellular Hsp70 expression was variable and Glut-1 expression could be deteced in vessels and necrotic areas. In secondary GBM, mutated IDH-1 expression (in immunohistochemistry of PCR) was positive whereas membrane Hsp70 expression was totally negative. Glut-1 expression could be found mainly in vessels and intracellular Hsp70 expression was variable.

Table.3-2: ELISA results of secreted membrane Hsp70 in conditioned media.

samples	mean of values (pg/ml)
TB853-EC	1026.4085
TB846-EC	1885.082
TB860-EC	698.9765
TB426-PK	9833.766
TB773-PK	2929.7645
TB796-PK	8451.011
TB863-PK	1803.284
TB773-MSC	883.2235
TB804-MSC	1721.488
TB863-MSC	2028.277

Table3-2: This table showed ELISA results of secreted membrane Hsp70 in conditioned media of subpopulations isolated from primary GBM: primary tumor cell cultures (PK, n=4), endothelial cells (EC, n=3) and mesenchymal stem-like cells (MSC, n=3). Values were extremely low except for three of four primary tumor cell cultures.

4 Discussion

Heat shock proteins, which are normally intracellularly localized conserved proteins, involved in folding and unfolding processes of proteins and peptides. Under physiological conditions, heat shock proteins might be involved in several processes including cell growth, differentiation and development (Lindquist and Craig, 1988). But under pathological conditions like cancer and inflammation, heat shock proteins could be highly expressed (Multhoff, 2007). They protect cells from lethal damage induced by stress stimuli like heat, hypoxia, radiotherapy and chemotherapy (De Maio, 1999). Hsp70 is a major inducible member of heat shock proteins in tumor cells, with the ability of protecting tumor cells from senescence (Yaglom et al., 2007). Recently, intracellular Hsp70 was found to be translocated to the plasma membrane, where it interacts with glycosphingolipid Gb3 and locates in cholesterol-rich domains (Gehrmann et al., 2008a; Vega et al., 2008). Further more, it was described to be secreted from intact tumor cells (Gehrmann et al., 2008a; Vega et al., 2008).

Several studies analyzed the expression of intracellular Hsp70 in GBM cell lines and in tumor specimens from GBM patients (Hermisson et al., 2000). Intracellular Hsp70, which is detected by using specially targeting intracellular Hsp70 antibody, is expressed in a variety of GBM cell lines like U373MG, LN-18, LN-229, U138MG and heat shock treatment could enhance its expression (Hermisson et al., 2000). But there was no direct correlation between intracellular Hsp70 expression and radioresistance of glioma cell lines (Fedrigo et al., 2011) or clinical outcome of patients with GBMs (Hermisson et al., 2000). In the current study, immunohistochemistry showed no grade-dependent expression of intracellular Hsp70 in gliomas. These results are in line with already

published studies (Elsner et al., 2007; Hermisson et al., 2000), but membrane Hsp70 expression in gliomas was investigated for the first time in the current study.

Membrane Hsp70 was detected on cell surface of a broad variety of human and mouse tumor cells in *vitro* and *vivo*, but not in non-neoplastic cells or normal tissues, thus served as a tumor-specific target structure (Multhoff, 2007; Multhoff and Hightower, 2011). The expression of membrane Hsp70 on tumor cells always served as “a danger signal” which might elicit antitumor immunity responses (Multhoff, 2007; Vega et al., 2008). In the plasma membrane of viable tumor cells, membrane Hsp70 localized in cholesterol-rich domains (Gehrmann et al., 2008a; Stangl et al., 2011b). The team of Prof. Multhoff generated the cmHsp70.1 antibody by using human 14-er TKD sequence as specific immunogen (Multhoff and Hightower, 2011; Stangl et al., 2011a). This antibody could directly recognize the epitope of membrane bound form of heat shock protein 70 (Multhoff and Hightower, 2011).

In previous studies, membrane Hsp70 was found to uniquely be expressed on tumor cells and was detected by flow cytometry analysis on single-cell suspensions from different tumor entities but not from corresponding non-neoplastic entities in patients with colon, gastric, lower rectal and squamous cell lung cancer (Pfister et al., 2007). The reason why membrane Hsp70 was not expressed in non-neoplastic tissue could be due to the fact that ceramide-based glycosphingolipids including Gb3 were not found in non-neoplastic cells (Gehrmann et al., 2008a). In such non-glial tumor entities, the membrane Hsp70-positive phenotype was associated with a higher metastatic potential and an unfavourable prognosis in malignant melanoma, acute myeloid leukaemia as well as lower rectal

carcinoma and lung squamous cell carcinoma (Farkas et al., 2003; Pfister et al., 2007). However, in colon and gastric cancer, membrane Hsp70 expression correlated with significant improved survival. The reason for these differing roles of membrane Hsp70 was supposed that the route of metastasis played a role, as colon and gastric tumor cells metastasize into the liver, where hepatic natural killer cells could provide a depletion of tumor cells (Pfister et al., 2007). In all mentioned tumors, membrane Hsp70-expression could be detected in the vast majority but not all tumor specimens (Pfister et al., 2007). For example, in head and neck cancer, the expression of membrane Hsp70 differed in a broad range in the various tumor localizations (Kleinjung et al., 2003). The reason why some tumor specimens did not express membrane Hsp70 is still unknown (Kleinjung et al., 2003).

In the current study, the immunohistochemical analysis showed that membrane Hsp70 expression was restricted to GBM tissues and could not be detected in non-neoplastic brains and WHO grade II or III astrocytomas. Surprisingly, membrane Hsp70 not only correlated with glioma grading but also differentiated secondary GBM from primary GBM. We found membrane Hsp70 positive signals in all of 23 primary GBM tissues, while in none of 13 secondary GBM tissues.

As we know, there are several genes, serving as molecular markers, altered in gliomas and tend to occur in a defined order during the progression to a high-grade tumor, such as TP53, PTEN and EGFR (Ohgaki and Kleihues, 2007; Yan et al., 2009). In low-grade astrocytomas, the mutation of TP53 appeared to be a relatively early event, whereas loss or mutation of PTEN and amplification of EGFR were not observed. “Primary GBM and secondary GBM subtypes achieved a common phenotypic endpoint, but recent genomic profiles revealed strikingly different transcriptional patterns and recurrent DNA copy number aberrations” (Furnari et al., 2007). In primary GBMs, EGFR gene amplification occurred in 40% of primary GBMs (Libermann et al., 1985; Ohgaki and

Kleihues, 2007). One special form of EGFR, the EGFR mutant allele with deletion of exons 2-7 (EGFRvIII), occurred in 20-30% of all human GBMs, making it to be the most common EGFR mutant (Nishikawa et al., 1994; Sugawa et al., 1990) and EGFR had been suggested as a prime target for therapeutic intervention in GBMs by using small molecule kinase inhibitors (Furnari et al., 2007; Huang et al., 2009). But as also mentioned, this marker was not a reliable marker for the characterization of a primary GBM due to its infrequent overexpression (Nishikawa et al., 1994; Ohgaki and Kleihues, 2007). In secondary GBMs, as present in 60% of precursor low-grade astrocytomas, TP53 mutation was the most frequently and detectable genetic alteration (Ohgaki and Kleihues, 2007), EGFR gene amplification was observed rarely in secondary GBMs (Libermann et al., 1985; Ohgaki and Kleihues, 2007). Recent studies showed that 70% of secondary GBMs had somatic mutations in the metabolic enzyme genes IDH1 (residue R132) and IDH2 (residue R172), but much more rarely primary GBM (5%) had such mutations (Watanabe et al., 2009; Yan et al., 2009). The finding that IDH1 or IDH2 was mutated in the secondary GBMs provided a biologic explanation for the clinical categorization and compromised a specific subgroup of GBMs (Yan et al., 2009). This genetic evidence suggested that IDH mutations were regarded as early genetic events in the tumor development of secondary GBM (Yan et al., 2009). IDH1 combined with EGFR, NF1, PDGFR genes were also used for subclassification of GBMs into proneural, neural, classical and mesenchymal subtypes (Verhaak et al., 2010). Also, many promoter methylation status of genes, like p16, p14, Rb1, TIMP-3, MGMT, were assessed in primary and secondary GBM (Ohgaki and Kleihues, 2007). For instance, O^6-methylguanine methyltransferase (MGMT) promoter methylation was detected in 75% of secondary GBMs and 36% of primary GBMs (Nakamura et al., 2001), and the methylation status had reached clinical significance as it predicted the response of patients with

GBMs to alkylating chemotherapeutic agents (Stupp et al., 2005). However, there was no marker which characterizes the more frequently existent primary GBM. As a result of the current study, immunohistochemistry of gliomas tissues showed a solitary expression of membrane Hsp70 in primary GBM but not in secondary GBM. This marker, membrane Hsp70, might serve as a reliable marker for primary GBM.

In non-glial tumors, membrane Hsp70 was found to be a specific structure on tumor cells but not on other cell types (Multhoff, 2007). In GBM, the current study further distinguished the cellular subtypes within the heterogenous GBM cell conglomerate which expressed the membrane Hsp70, so distinct subpopulations including primary tumor cell cultures, CD133-positive cells, endothelial cells and mesenchymal stem-like cells were isolated from primary GBM tissues for checking membrane Hsp70 expression. In FACS-analysis results, selectively CD133-positive cells and primary tumor cell cultures expressed membrane Hsp70, which also could be expressed on GBM cell lines U87 and U373. CD133-positive cells were identified as a subpopulation of so-called cancer stem cells within glioblastoma tissue with an unlimited capacity for self-renewal and tumor-initiation (Chen et al., 2010). Mesenchymal stem cells, which have the ability of homing to tumor tissue, differentiation in *vitro* and immunomodulatory potency (Motaln et al., 2010), and microvascular endothelial cells, which are involved in angiogenesis and vasculogenesis, did not express membrane Hsp70. As a consequence, it was supposed that the subpopulations with malignant potential including CD133-positive cells and primary tumor cell cultures express membrane Hsp70. So in GBM, the expression of membrane Hsp70 was also restricted to malignantly transformed cells such as CD133-positive cells, primary tumor cell cultures and GBM cell lines, which is in line with results

from other non-glial tumors (Multhoff, 2007). Heat shock proteins might have oncogene-like functions, for example, they could activate NF-κB which regulates several proteins that enhance tumor growth and tumor spreading (Sherman and Multhoff, 2007). Also, the regulation of heat shock proteins underlay oncogenic pathways such as TP53 pathway (Sherman and Multhoff, 2007). During transformation, genetic changes in cancers enhance the transcription of heat shock proteins and likewise stressed tumor cells must adapt to a hostile microenvironment for survival (Calderwood et al., 2006; Jego et al., 2010). This is thought to be reason why intracellular Hsp70 is translocated into plasma membrane and is secreted as exosomes in tumor cells (Gehrmann et al., 2008a; Vega et al., 2008).

On the other hand, in non-glial tumors, the expression of membrane Hsp70 could be enhanced by stress conditions as hypoxia and chemotherapeutic agents (Gehrmann et al., 2008a; Multhoff, 2007). For instance, in a leukemic cell line and colon carcinoma cell line, vincristine and paclitaxel compared to DNA-interacting antitumor agents (cytarabine, ifosfamid) increased not only the intracellular but also the membrane Hsp70 (Gehrmann et al., 2002). In other studies, the externalization of intracellular Hsp70 to the membrane-associated form under stress conditions serving as a “danger-signal” was investigated (Hightower and Guidon, 1989; Vega et al., 2008). So the effect of hypoxia on the expression of membrane Hsp70 was investigated in the current study.

The isolated subpopulations from primary GBM, which have positive membrane Hsp70 expression, were cultured in hypoxia for 72h and determined by FACS for the change of membrane Hsp70 expression. The results showed increased expressions of membrane Hsp70 were in primary tumor cell cultures and glioblastoma cell lines, but not in endothelial cells and mesenchymal stem-like cells,

which were recruited from the surrounding parenchyma or the host. The overexpression of membrane Hsp70 induced by hypoxia in *vitro* was confirmed by immunohistochemical staining of glioblastoma tissues. In consecutive immunostained slides, membrane Hsp70 expression was localized in the peri-necrotic hypoxic regions showing coexpression with the hypoxic marker Glut-1, but less in the well-vascularized areas.

In glioma tissues, several molecules such as HIF-1α, Glut-1, CAIX, LDH-5, MCT1 and MCT4 are expressed and regulated by hypoxia, Glut-1 was supposed to be one of the most sensitive marker for hypoxia in gliomas (Rademakers et al., 2011; Vaupel and Mayer, 2007) and was also analyzed in the current study. In WHO grade II and III gliomas, Glut-1 staining could also be detected in endothelium of vessels and also in erythrocytes as it was previously described (Stockhammer et al., 2008). In GBM, Glut-1-expression was found to be grade-dependently expressed and correlated with decreased survival in GBM patients (Flynn et al., 2008). As IDH might be involved in the oxidative decarboxylation of isocitrate to α-ketoglutarate which should result in an increased level of HIF-1α, the relationship between IDH mutation status and *in-vivo* hypoxic marker expression in WHO II and III gliomas was also analyzed (Metellus et al., 2011). But there was no correlation could be found between IDH-mutation status and in-*vivo* hypoxic markers as suspected, reflecting an adaptative behaviour of IDH-mutated glioma cells (Metellus et al., 2011; Williams et al., 2011). In addition, cancer cells were characterized by the "Warburg phenomenon"-producing energy predominantly by aerobic glycolysis in the cytosol, rather than by oxidation of pyruvate in mitochondria as in non-neoplastic cells (Warburg, 1956). IDH mutations in glioma cells could contribute to this phenomenon by changing metabolite pools and thereby facilitating glucose influx (Scott et al., 2011). During glioma progression, the glycolytic traits found in tumor cells might arise as adaptive

mechanisms to environmental constraints (Scott et al., 2011).

Considering membrane Hsp70 can be secreted by membrane Hsp70 positive tumor cells in the form of exosomes (Multhoff and Hightower, 2011), membrane Hsp70 secretion in GBM was shown in this study for the first time. Conditioned media of subpopulations isolated from primary GBM were used to determine the concentration of membrane Hsp70 by ELISA with cmHsp70.1 antibody as capture antibody. In the line with FACS analysis results, only primary tumor cell cultures had high concentrations of membrane Hsp70. Three of four primary tumor cells cultures were detected to secrete more than 2000pg/ml membrane Hsp70 into conditioned medium. Endothelial cells and mesenchymal stem-like cells had significantly lower levels of membrane Hsp70, which were considered to be basic levels of membrane Hsp70 secretion. In order to find the clinical meanings of these in vitro results, serum samples of patients with primary and secondary GBM have been studied in this study with ELISA. Only in patients with primary GBM but not in secondary GBM or control epilepsy patients, membrane Hsp70 was highly secreted into bloodstream. Other groups combined extracellular Hsp70 with BMP20 and CXCL10 in a systematical research for indication of the presence of GBM (Elstner et al., 2011), but it was not possible to distinguish patients with primary GBM from secondary GBM by a single biomarker. In the current study, together with immunohistochemistry results, the results of membrane Hsp70 secreted into serum of patients with GBMs further suggested membrane Hsp70 might be a novel biomarker for the diagnosis of primary GBM.

So in the current study, in gliomas, we concluded that membrane Hsp70 might serve as tumor-specific marker in primary GBM and could be used to differentiate the *de-novo*-origin from secondary GBM. There is more work needed to be done to assess the prognostic and predictive value of membrane Hsp70 in primary GBM. For instance, more tumor specimens and serum samples from patients with different grade gliomas should be included into further analysis; membrane Hsp70 expression should be correlated with glioma progression, imaging findings, therapy responses, clinical outcome. On the other hand, although membrane Hsp70 expression on tumor cells can increase the immune response of natural killer cells or mediate the immunosuppressive function of myeloid-derived suppressive cells, the exact biological function of membrane Hsp70 has still to be elucidated. The process of Hsp70 translocate into plasma membrane during malignant transformation is not understood. Therefore, the biological function of membrane Hsp70 has to be further analyzed in order to tailor the treatment strategy of primary GBM.

5 Summary

Glioma is the most frequent primary central nervous system cancer. Despite multimodal therapeutic strategies, glioblastoma is one of the tumor entities with a dismal prognosis for the patient. On the basis of genetic and biologic differences, glioblastoma can be divided into two distinct subtypes: primary and secondary glioblastoma. Up to now, there is no unique molecular marker which can be used to characterize the most frequently existent primary glioblastoma. On the other hand, membrane Hsp70 is shown to be expressed in a variety of non-glial human and mouse tumor cells in *vitro* and in *vivo*. Membrane Hsp70 is considered to be a tumour-specific target structure.

In the current study, the expression of membrane Hsp70 in gliomas was investigated by using specifically targeting membrane Hsp70 antibody cmHsp70.1. In glioma tissues, membrane Hsp70 was shown to be expressed only in primary glioblastoma, but not in normal brain, WHO grade II, III astrocytmoas or secondary glioblastoma. In primary glioblastoma tissue, membrane Hsp70 expression was found to co-localize with hypoxia markers Glut-1 and HIF-1α. Moreover, membrane Hsp70 was shown to be expressed in primary tumor cell cultures and CD133 positive cells in a hypoxia-dependent manner, but not in endothelial cells or mesenchymal stem-like cells. On the clinical side, compared to patients with secondary glioblastoma, patients with primary glioblastoma showed higher concentrations of membrane Hsp70 in the serum.

So herein, the current study investigated the expression of membrane Hsp70 in gliomas and showed that membrane Hsp70 might serve as a novel, hypoxia-related marker for primary glioblastoma.

6 Zusammenfassung

Gliome sind die häufigsten primären hirneigenen Tumore. Trotz multimodalen therapeutischer Strategien ist das Glioblastom eine Tumorentität mit einer schlechten Prognose für den Patienten. Auf Grundlage der genetischen und biologischen Unterschiede können Glioblastome in zwei Subtypen unterteilt werden: primäre und sekundäre Glioblastome. Bis jetzt gibt es keinen eindeutigen molekularen Marker, der verwendet wird, um die am häufigsten vorhandenen primären Glioblastome zu charakterisieren. Auf der anderen Seite ist für membranständiges Hsp70 gezeigt worden, dass es in vielen nicht-Glia-Tumorzellen von Mensch und Maus *in vitro* und *in vivo* exprimiert wird. Membranständiges Hsp70 wird als tumor-spezifische Zielstruktur betrachtet.

In der aktuellen Studie wurde die Expression von membranständigem Hsp70 in Gliomen durch einen spezifischen anti-membranständiges Hsp70 Antikörper cmHsp70.1 untersucht. In Gliomgewebe wurde membranständiges Hsp70 nur im primären Glioblastom, aber nicht in normalem Gehirn, WHO-Grad II, III oder sekundären Glioblastomen exprimiert. Die Expression von membranständigem Hsp70 wurde in primären Glioblastomgewebe gefunden und co-lokalisierte mit Hypoxie Markern wie Glut-1 und HIF-1α. Darüber hinaus wurde gezeigt, dass membranständiges Hsp70 in Primärkulturen und CD133-positiven Zellen, die aus primären Glioblastomen isoliert wurden, exprimiert wird und durch Hypoxsie stimulier bar ist, aber nicht in Endothelzellen oder mesenchymalen Stammzell-artigen Zellen nachgewiesen wird. Auf der klinischen Seite, konnten wir zudem nachweisen, dass Patienten mit primärem Glioblastom höchere Konzentration von membranständigem Hsp70 im Serum als Patienten mit sekundären Glioblastomen.

In der aktuellen Studie wurde die Expression von membranständigem Hsp70 in Gliomen untersucht. Es wurde gezeigt, dass membranständiges Hsp70 als neuartiger, Hypoxie-bezogener Marker für primäre Glioblastom dienen könnte.

7 Specific materials

7.1 Chemicals and materials

Chemical/material	Company
Aceton	Sigma-Aldrich, Germany
Bovine serum albumin(BSA)	Sigma-Aldrich, Germany
Dako immunohistochemistry kit	Dako, Germany
ELISA kit	R&D systems, USA
Entellan	Merck, Germany
Ficoll-paque gradient medium	Biochrom, Germany
Matrigel	BD Biosciences, Germany
Haematoxylin	Merck, Germany
Isopropanol	Merck, Germany
Propidium iodide	Applichem, Germany
Tween20	Applichem, Germany
Trypsin/EDTA	Sigma-Aldrich, Germany
Oil Red S	Sigma-Aldrich, Germany

7.2 Cell culture

Component	Company
B27	Invitrogen, USA
Beta-glycerophosphate	Fluka, Switzerland
Dexamethason	Sigma-Aldrich, Germany

DMEM	Biochrom, Germany
DMEM/F12	Invitrogen, USA
FCS	Biochrom, Germany
FGF	R&D systems, Germnay
Human EGF	R&D systems, Germany
N2	Invitrogen, USA
Non-essential amino acids	Invitrogen, USA
Penicillin/Streptomycin/Glutamine	Invitrogen, USA
L-ascorbic acid	Sigma-Aldrich, Germany
Isobutylmethylxanthine	Sigma-Aldrich, Germany
Indomethacin	Sigma-Aldrich, Germany
Insulin	Sigma-Aldrich, Germany
Microvascular endothelial cell growth medium	Provitro, Germany

7.3 Equipments and buffers

Equipment	Company
Dynabeads magnetic beads	Dynabeads, Germany
FACS Calibur Flowcytometer	BD Biosciences, USA
MACS column	Miltenyi Biotech, Germany
Hypoxia incubator	Binder Brutschrank, Germany
Ibidi angiogenesis slides	ibidi GmbH, Germany
Plastic flasks	Nunc, Germany

PBS puffer 10x

NaCl	87.7g
Na2HPO4	11.7g
KCL	2g
KH2PO4	2.4g

ddH20 to 1000ml

Adjust PH to 7.4

TBS puffer

Tris	50mM
NaCl	150Mm

Adjust PH to 7.6

FACS puffer

1x PBS

10% FCS

8. References

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