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Expression of chemokines and their receptors in human meningiomas and schwannomas

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To My Wife and My Son

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Abbreviation

ABC	avidin-biotin-peroxidase complex
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
DAB	3,3'-diaminobenzidine-tetrahydrochloride
DNase	deoxyribonuclease
dNTP	deoxy-ribonucleoside triphosphate
EDTA	ethylene diamine tetraacetic acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IHC	immunohistochemistry
MPNST	malignant peripheral nerve sheath tumors
MRI	resonance imaging
mRNA	messenger ribonucleic acid
NCN	normal cranial nerves
NK	natural killer
NKT-cells	natural killer T-cells
NPC	nasopharyngeal carcinoma
NSN	normal spinal nerves
PFA	paraformaldehyde
Real time RT-PCR	real time reverse transcription-polymerase chain reaction
RNase	ribonuclease
RT	room temperature
SDF	stromal cell-derived factor
SPS	spinal schwannomas
TBS	Tris-buffered saline
VS	vestibular schwannomas
WHO	World Health Organization

1 Introduction

Origination, epidemiology and prognosis of meningiomas

Meningiomas are neoplasms derived from arachnoidal cells and they can be found anywhere in the dura mater. Meningiomas are considered to be the second most common intracranial tumors and account for 13%-26% of all primary brain tumors (Louis et al. 2000). Most (about 90%) of the meningiomas are slow growing benign tumors, classified as World Health Organization (WHO) grade I (Louis et al. 2007). However, atypical meningiomas (WHO grade II), which are characterized by increased cellularity and increased mitotic activity, account for approximately 8% of this tumor type. Anaplastic variants (WHO grade III), which make up approximately 2%, show a more aggressive biological behaviour and are associated with a high risk of local recurrence and a poor clinical outcome (Louis et al. 2000; Whittle et al. 2004). Meningiomas may invade adjacent structures, including dura mater, muscle and bone, and are considered to be anaplastic when invasion of the brain parenchyma is also present (Whittle et al. 2004). Compression or obstruction of cortical veins, venous sinuses, or ventricles is another complication and edema is a common occurrence in meningiomas, and may be related to the headaches and convulsions often seen in these patients (Pistolesi et al. 2002; Yoshioka et al. 1999; Lieu et al. 2000). Surgery is the mainstay of treatment, with the likelihood of recurrence inversely related to the extent of resection (Whittle et al. 2004; DeMonte. 1995). The Simpson scaling system describes the degree of surgical resection as ranging from grade 1 (complete tumor, dura mater, and bone removal) to grade 5 (tumor biopsy) (Simpson. 1957). The recurrence rate for meningiomas is on average 10% after a complete resection and up to 50% after a incomplete resection (partial resection or biopsy) (Simpson. 1957). In addition, recurrence rates are higher for the more aggressive histologic variants, with 5-year recurrence rates of 38% for atypical meningiomas and 78% for malignant meningiomas (DeMonte. 1995). When patients were grouped by histological type of tumors, those with benign meningiomas had an overall 5-year survival rate of 70%, whereas the overall 5-year survival rates in patients with atypical and anaplastic meningiomas were 75% and 55%, respectively (McCarthy et al. 1998). Furthermore, accepted alternative therapies for patients who have failed surgical intervention are currently limited to radiotherapy which has been shown to decrease of delay recurrence (Whittle et al. 2004;

Rogers et al. 2007). Currently, there are no pharmaceutical agents that are routinely used for adjuvant therapy.

Origination, epidemiology and prognosis of schwannomas

Schwannomas, benign peripheral nerve sheath neoplasm, usually encapsulated, are composed entirely of cells with the immunophenotype and ultrastructural features of Schwann cells. Synonyms include neurilemmomas and neurinomas (Scheithauer et al. 1999). Schwannomas occur in individuals of all ages, but show a peak incidence between the third and sixth decades (Scheithauer et al. 1999). No sex predilection is evident, although females are twice as often affected by central nervous system (CNS) schwannomas, while radiation-induced examples most often occur in males (Salvati et al. 1992). The tumor cells always stay on the outside of the nerve, but the tumor itself may either push the nerve aside and/or up against bony structure (thereby possibly causing damage). Schwannomas are relatively slow growing and can be removed surgically, but always can recur. Schwannomas can be conveniently subdivided according to location into intracranial (vestibular schwannomas, VS), intraspinal (spinal schwannomas, SPS), peripheral and visceral tumors (Scheithauer et al. 1999).

A vestibular schwannoma, often called an acoustic neuroma, is a benign primary intracranial tumor of the myelin-forming cells of the vestibulocochlear nerve (CN VIII). The term “vestibular schwannoma” is the correct one because the tumor involves the vestibular portion of the 8th cranial nerve and arises from Schwann cells, which are responsible for the myelin sheath in the peripheral nervous system. The vestibular schwannoma is the most frequent neoplasm of the lateral skull base. It accounts for 8% of all intracranial tumors (Tos et al. 1984). It may cause fatal situation by compressing vital brainstem structures. Clinically, it commonly appears as a sporadic tumor leading to either unilateral hearing loss, vertigo, or tinnitus. Depending on the patient’s age, associated symptoms, hearing function, tumor growth rate, and localization, several therapy options can be given to the patient, such as, monitoring of tumor growth by regular magnetic resonance imaging (MRI) scans, surgical removal of the lesion (Samii et al. 2001), or gamma knife therapy (Prasad et al. 2000).

Spinal schwannomas (SPS), account for about 25% of primary intradural spinal cord tumors in adults (Celli et al. 2005; Dorsi et al. 2004). Most are solitary schwannomas, which can occur throughout the spinal canal (Dorsi et al. 2004). In the literature, 70% to 80% of SPS are reported to be intradural in location, and those extending through the dural aperture as a dumbbell mass with both intradural and extradural components account for another 15% (Jinnai et al. 2005). Intramedullary schwannomas are extremely rare (Celli et al. 2005). The beginning symptoms are varied in accordance with the level of the tumor. The mild to severe back pain is most common symptoms. When the SPS grow large, they may compress the spinal cord completely and cause several obvious signs and symptoms. These signs and symptoms include, tingling sensation, weakness and fatigue, shooting pain, numbness of the back and spine, pain that diversifies towards the lower parts of the body, including legs. Surgery is supposed to be the best option for the treatment of SPS. The total resection of the lesion, which is the generally reachable goal of surgery, allows for good results in the cases in which the preoperative clinical findings are not particularly severe. The prognosis of SPS correlates to the size and type of the tumors. The outcome of this disease also depends on the preoperative neurological condition of patient (Jinnai et al. 2005).

Malignant peripheral nerve sheath tumor is a rare variety of soft tissue sarcoma of ectomesenchymal origin (Angelov et al. 2000). WHO originated the term “malignant peripheral nerve sheath tumor” replacing previous heterogeneous and often confusing terminology, such as malignant schwannoma, malignant neurilemmoma, and neurofibrosarcoma, for tumors of neurogenic origin and similar biological behavior (Wanebo et al. 1993). Malignant peripheral nerve sheath tumors (MPNST) may arise spontaneously in adult patients, although 5% to 42% of MPNST have an association with multiple neurofibromatosis Type-I (Evans et al. 2002). Examples originating from schwannomas are extremely rare (Woodruff et al. 1994). MPNST are usually observed in the extremities and trunk, in the deep soft tissue close to the distribution of sciatic nerve, branchial plexus and sacral plexus. Extremely rare examples occur in the head and neck region. Clinically, they present as an enlarging mass, often associated with pain and nerve deficit. MPNST are locally invasive lesions, frequently leading to multiple recurrences and eventual metastatic spread

(Patil et al. 2007). MPNST can spread with direct extension, hematogenous extension and through perineural spread. Lymph node metastasis is uncommon (Patil et al. 2007). Radical surgical resection is the treatment of choice in MPNST though they are biologically aggressive in nature (Ferner et al. 2002). However multimodality therapy, including surgical resection and adjuvant radiotherapy, is available, the prognosis remains poor and survival is found to be influenced by tumor location and size. Sex and cellular differentiation emerged as the new adverse prognostic factors for survival of the patients (Kar et al. 2006).

Characteristics of chemokines and their receptors

Chemokines are 8- to 12-kDa peptides that bind to specific G-protein-coupled, seven-span transmembrane receptors on the plasma membrane of target cells. Most chemokines bind to multiple receptors, and the same receptor may bind to more than one chemokine (Bajetto et al. 2002; Teicher et al. 2010). Chemokines are classified into four major subfamilies on the basis of the motif of the first two cysteine residues: CC, CXC, C and CX3C subfamilies (Zlotnik et al. 2000). The CXC-chemokines activate the CXC receptors (CXCR)1–6, CC-chemokines bind to the CC receptors (CCR) 1–10, and CX3CL1 exerts its effects through the CX3CR1 (Barbieri et al. 2010). Chemokines play an essential role in cellular migration and intracellular communication and were originally observed as inducible cytokines facilitating the recruitment of specific leukocyte subsets (Murphy. 2002). They are also involved in many other physiological and pathological processes, including neoplasia, in which they play an important role through multiple mechanisms. Chemokines affect tumor cell proliferation, regulate the angiogenic/angiostatic processes, control cell migration and metastasis, and regulate the recruitment of the immune cells into the tumor mass (Bajetto et al. 2002). Chemokine receptors can signal cell migration, altered cytokine production, cell proliferation, apoptosis or survival. Some chemokines are constitutively expressed (for example CXCL12/SDF-1; CX3CL1/fractalkine) while others are expressed only in responses to specific stimuli.

Characteristics of CX3CL1 and CX3CR1

CX3CL1 is the unique member of the CX3C class of chemokines. CX3CL1, originally

termed Fractalkine or Neurotactin, was cloned from activated endothelial cells and neurons respectively (Bazan et al. 1997). In contrast to most other chemokines, CX3CL1 is expressed as a transmembrane molecule, composed of a chemokine head tethered to the cell membrane by a mucine-stalk, followed by a membrane-spanning domain and a short cytoplasmic tail (Bazan et al. 1997). Unlike other chemokines, it exists in two forms, each mediating distinct biological actions (Imai et al. 1997; Fong et al. 1998). The membrane-anchored protein, which is primarily expressed on the inflamed endothelium, serves as an adhesion protein promoting the retention of monocytes and T cells in inflamed tissue. The soluble form resembles more a conventional chemokine and strongly induces chemotaxis. Both chemotaxis and adhesion are mediated by the G protein-coupled receptor CX3CR1. Unlike many promiscuous chemokines, it only signals through the CX3CR1 receptor (Ransohoff. 2009). The receptor CX3CR1 is predominantly expressed by hematopoietic cells, natural killer (NK) cells, Th1 lymphocytes, CD14⁺ monocytes and by microglia in the spinal cord and satellite cells in the dorsal root ganglia (Hughes et al. 2002; Verge et al. 2004). As a result of both the adhesion and chemoattractant activities of the chemokine, CX3CL1 has been thought to play an important role in inflammation, and indeed, accumulating evidence indicates that CX3CL1/CX3CR1 are involved in the pathogenesis of various inflammatory disorders such as glomerulonephritis, rheumatoid arthritis and systemic lupus erythematosus (Yajima et al. 2005; Ito et al. 2002; Sawai et al. 2005; Blaschke et al. 2003). CX3CL1 may be also involved in the pathogenesis of several diseases including atherosclerosis, cancer, and acquired immune deficiency syndrome (Braunersreuther et al. 2007; Zhang et al. 2007; Becker et al. 2007). Based on the chemotactic and adhesive properties, the CX3CL1/CX3CR1 complex may mediate either pro- or anti-tumor effects as well (D'Haese et al. 2010). However, it is still not clear whether CX3CL1/CX3CR1 are involved in tumorigenesis of human meningiomas and schwannomas.

Characteristics of CXCL16 and CXCR6

The CXC-chemokine CXCL16 is different from nearly all other known chemokines by its structure and function. CXCL16 is synthesized as transmembrane multi-domain molecule consisting of a chemokine domain followed by a glycosylated mucin-like stalk and a single

transmembrane helix. The only other transmembrane chemokine is CX3CL1/fractalkine as mentioned above. The chemokine CXCL16 was originally discovered as scavenger receptor for oxidized low-density lipoprotein (oxLDL) and therefore also termed SR-PSOX (Shimaoka et al. 2000) and independently as ligand for the CXC-chemokine receptor CXCR6 (also termed Bonzo, TYMSTR, STRL33). CXCL16 is the only ligand for the receptor CXCR6, and its soluble form induces the directional migration of CXCR6⁺ cells, such as CD4⁺ effector memory T cells and natural killer T-cells (NKT-cells) (Wilbanks et al. 2001; Matloubian et al. 2000). In its stalk-bound form, CXCL16 has also been shown to facilitate the adhesion of CXCR6⁺ cells via interactions with the chemokine domain (Shimaoka et al. 2004) similar to the interaction between CX3CL1 and CX3CR1 (Ludwig et al. 2007). The expression of the chemokine CXCL16 has been reported previously for monocytes/macrophages, B cells, dendritic cells keratinocytes and endothelial cells (Hase et al. 2006; Ludwig et al. 2005; Shimaoka et al. 2000) while the receptor CXCR6 has been found on activated T cells, NKT-cells, bone marrow plasma cells and smooth muscle cells (Chandrasekar et al. 2004; Hase et al. 2006; Matloubian et al. 2000). Furthermore, CXCL16 expression was discovered also within the human brain on endothelial cells, activated astroglial cells and glioma cells (Ludwig et al. 2005). However, the expression of CXCL16/CXCR6 in human meningiomas is still unknown.

Characteristics of CXCL12 and CXCR4

The stromal cell-derived factor (SDF)-1, recently renamed chemokine (CXC motif) ligand 12 (CXCL12) is a member of the chemokine family, which consists of low-molecular-weight proteins (8-15 kD) produced by a variety of cells involved in the allergic inflammation (Shirozu et al. 1995). CXCL12/SDF-1 is a chemokine with two isoforms: SDF-1a, the predominant 89-amino acid protein form of SDF-1, and SDF-1b, which contains an extension of four amino acids at the carboxyl terminus. A third form of SDF (SDF-1g) was identified in rat that is identical to SDF-1b, except for the insertion of 30 additional amino acids at the carboxyl terminus (Gleichmann et al. 2000). CXCL12 is a homeostatic chemokine. The major function of homeostatic chemokines is to regulate hematopoietic-cell trafficking and secondary lymphoid-tissue architecture. CXCL12 also has different regulatory roles in several

biologic processes, including cardiac and neuronal development, stem cell motility, neovascularization, angiogenesis, apoptosis, and tumorigenesis (Atluri et al. 2008; Chen et al. 2008; Hernández-López et al. 2008; Schönemeier et al. 2008; Zhang et al. 2008). The CXCL12 and its cognate receptor CXCR4 have recently taken on substantial interest because of their role in growth several human neoplasms (Balkwill. 2004). CXCL12/CXCR4 has been shown to be a key mediator of tumor spread, sitespecific metastasis, and patient survival (Burger et al. 2006). So, a study about the expression of CXCL12/CXCR4 in human schwannomas is required.

1.4 Aims of the study

Meningiomas and schwannomas are very common tumors in nervous system. Investigation in the molecular pathogenesis of meningiomas and schwannomas has the potential to improve diagnostic and therapeutic strategies, particularly for those with aggressive biological behaviour which always associated with a high risk of recurrence and a poor clinical outcome. As mentioned above, chemokines are involved in physiological and pathological processes of neoplasia. Chemokines and their receptors play a decisive role in tumor cell proliferation and survival. Little is known, however, about the role of chemokines in the pathogenesis of human meningiomas and schwannomas. So we decided to describe the expression of the CX3CL1/CX3CR1 and CXCL16/CXCR6 in human meningiomas, CX3CL1/CX3CR1 and CXCL12/CXCR4 in human schwannomas on mRNA and protein level, to figure the role of these chemokines and receptors in more detail. Elucidating the molecular mechanisms of these chemokine/receptor pairs in pathogenesis of human meningiomas and schwannomas we hope to get a deeper understanding of tumor development, and hope to identify possible targets for tumor therapy particularly for those with resistant to conventional therapy and poor prognosis.

2 Materials and Methods

2.1 Clinical materials and tumor tissue procession

Tumor tissue specimens used for our study were collected from 45 patients who were operated on between November 2000 and December 2010 at the Department of Neurosurgery, Kiel, Germany. The patients included 24 women and 21 men, aged 33 to 81 years at diagnosis, mean 58.6±9.4 years.

2.2 RNA isolation

Tissue samples including 10 for meningiomas WHO grade I, 10 for meningiomas WHO grade II, 7 for meningiomas WHO grade III, 6 for vestibular schwannomas (VS), 6 for spinal schwannomas (SPS), 6 for malignant peripheral nerve sheath tumors (MPNST), 5 for dura, 3 for normal spinal nerves (NSN) and 2 for normal 8th cranial nerves (NCN) were homogenized in 1 ml of TRIzol[®] Reagent per 50-100mg tissue by using a Polytron-homogenizer. After centrifugation at 12000 rpm for 10 minutes at 4°C, the cleared homogenate solution was transferred to a fresh tube. After addition of 0.2 ml of chloroform, the solution was shaken vigorously by hand for 15 seconds, and then incubated at 15°C to 30°C for 2-3 minutes. After a centrifugation step at no more than 12000 rpm for 15 minutes at 4°C, the mixtures were separated into a red low phenol-chloroform phase, an interphase and a colorless upper aqueous phase. The aqueous phase was transferred to a fresh tube and mixed with 0.5 ml isopropyl alcohol. After this, the solution was incubated at 25°C for 10 minutes and centrifugation at no more than 12000 rpm for 10 minutes at 4°C. By this, the RNA was precipitated from the aqueous phase and formed a gel-like white pellet on the bottom of tube. After removing the supernatant, the RNA pellet was washed with 1 ml 75% ethanol. After centrifugation at no more than 7500 rpm for 5 minutes at 4°C and removing the supernatant, the RNA pellet was briefly dried (air-dry for 5-10 minutes) and dissolved in RNase-free water. Then, the RNA was incubated for 10 minutes at 58°C, and chilled in an ice bath. Finally, RNA concentrations were quantified by a spectrophotometer (E=260 nm, A260/A280 ratio)

2.3 cDNA synthesis

To destroy the contaminating genomic DNA interfering with RT-PCR experiments, 2µg of

RNA were added to RNase-free water (diluted in RNase-free water to 7 μ l), 1 μ l RNase free DNase (1u/ μ l) and 1 μ l 10 \times reaction buffer for DNA denaturation for 15 minutes at 37 $^{\circ}$ C. 1 μ l EDTA (20mM) was added to the mixture and incubation at 65 $^{\circ}$ C for 10 minutes to stop function of RNase free DNase. For first strand synthesis, at first the mixture was added to 2 μ l random hexamer primer (100 μ g/ μ l), incubated at 70 $^{\circ}$ C for 5 minutes and chilled on ice. Then 4 μ l 5 \times reaction buffer, 2 μ l 10mM 4 dNTP mix, 1 μ l deionized water and 1 μ l RevertAidTM H Minus M-MuLV Reverse Transcriptase (200u/ μ l) was added to the mixture and all was incubated at 25 $^{\circ}$ C for 10 minutes and then at 42 $^{\circ}$ C for 60 minutes. The process was stopped by heating the solution at 70 $^{\circ}$ C for 10 minutes, and by quickly chilling it on ice.

2.4 Real-time RT-PCR

Real-time RT-PCR was performed in three replicates of each sample using a total reactive volume of 20 μ l, which contained 1 μ l of 20 \times Assays-on-DemandTM Gene Expression Assay Mix, 10 μ l of 2 \times TaqMan Universal PCR Master Mix and 100ng or 10ng of cDNA template (diluted in 9 μ l RNase-free water). After 2 minutes at 50 $^{\circ}$ C for uracil-N-glycosylase activation and 10 minutes at 95 $^{\circ}$ C for polymerase activation, 40 cycles of 15 seconds at 95 $^{\circ}$ C (denaturation) and 1 minutes at 60 $^{\circ}$ C (annealing and extension) were run. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each sample was tested as intrinsic positive control. Each plate included at least three "No Template Controls". The reaction was carried out with the MyiQTM Single Color Real-time PCR Detection System. Fluorescent data were converted into cycle threshold (C_T) measurements. C_T of each sample was averaged and then analyzed by a comparative C_T method for relative quantification of different gene expression. $\Delta C_T = [C_T(10ng) - 3.3 + C_T(100ng)] / 2 - C_T(GAPDH)$.

2.5 Immunohistochemistry

Based on the results of real-time RT-PCR, 3 samples of every subtype (meningiomas grade I, II, III, dura, SPS, VS, NSN) and 2 samples of MPNST, NCN were investigated by immunohistochemistry (IHC).

For IHC examination, fresh-frozen tumor tissues were cut in a freezing cryostat into 10 μ m slice sections. The sections were air dried and stored in -20 $^{\circ}$ C freezer until used.

IHC was performed using the avidin-biotin-peroxidase complex (ABC) method. The serum-control and isotype-control were performed for every primary antibody. The slices were post-fixation with 4% Para-formaldehyde (PFA) (4°C) in Tris-buffered saline (TBS) for 30 minutes at room temperature (RT). Then, the slices were rinsed in TBS 10 minutes for 2 times. To block endogenous peroxidase and non-specific binding, 3% H₂O₂ in 0.3% Triton X-100/TBS was used for 30 minutes at RT. After incubation of the slices in TBS 10 minutes for 2 times, diluted normal blocking serum (appropriate 10%) in TBS for 60 minutes at RT was used, respectively. The slides were directly transferred to an appropriate dilution of primary antibodies (see table 1). The antibodies were diluted in 0.3% Triton X-100/TBS and 2% normal blocking serum in TBS. Meanwhile, the isotype-control was directly transferred to an appropriate dilution of corresponding IgG without primary antibodies (see table 1), and the serum-control was directly transferred to the diluent of 2% normal serum and 0.3% Triton X-100/TBS. Then, sections were incubated at 4°C over night. After washing steps with TBS (10 minutes for 2 times), the slides were incubated with corresponding biotinylated second antibody (dilution 1:200) in 1.5% blocking serum in TBS for 60 minutes at RT (see table 1). After washing the slices in TBS 10 minutes for 2 times, amplification of the signal was carried out by ABC method with ABC Vectastain[®] kit. The signal was visualized by incubation with 0.06% 3,3'-diaminobenzidine-tetrahydrochloride (DAB) and 0.003% H₂O₂ in 0.1M Tris-HCl (pH 7.6) for about 3 minutes. Then, the slides were counterstained with Mayer's Hämalaun for 60 second. After washing with running tap water for 10 minutes, the slides were grads-dehydrated with ethanol (concentration from 70% → 80% → 95% → 95% → 100% → 100%, 1 minutes for each concentration). After cleared in RotiClear 10 minutes for 3 times, the slides were mounted with RotiMount[®] quick-harden mounting medium and coverslipped for investigation. Brightfield microscopy with digital photography was performed using a Zeiss microscope and Zeiss camera.

The immunostaining was assessed quantitatively as the percentage of positive cells in relation to negative ones. To evaluate immunohistochemical expression of chemokines markers in samples, five random fields around the center of the tissue were used for cell counting in each slide at 200×magnification, and the percentages of immunoreactive cells per field were averaged.

Table 1 Antibodies used for detection of tumor cell markers

	Antibody	Original Concentration	Diluent's Concentration	Source
Primary Antibody	CX3CL1	500µg/ml	1:200	Mouse Monoclonal
	CX3CR1	200µg/ml	1:200	Rabbit Polyclonal
	CXCL16	100µg/ml	1:200	Goat Polyclonal
	CXCR6	500µg/ml	1:50	Mouse Monoclonal
	CXCL12	200µg/ml	1:100	Rabbit Polyclonal
	CXCR4	1000µg/ml	1:300	Rabbit Polyclonal
	Isotype control for CX3CL1	500µg/ml	1:200	Normal Mouse IgG
	Isotype control for CX3CR1	1000µg/ml	1:1000	Normal Rabbit IgG
	Isotype control for CXCL16	1000µg/ml	1:2000	Normal Goat IgG
	Isotype control for CXCR6	500µg/ml	1:50	Normal Mouse IgG
	Isotype control for CXCL12	1000µg/ml	1:500	Normal Rabbit IgG
	Isotype control for CXCR4	1000µg/ml	1:300	Normal Rabbit IgG
Secondary Antibody	Biotimylated anti-Mouse	1.5mg/ml	1:200	Horse
	Biotimylated anti-Rabbit	1.7mg/ml	1:200	Donkey
	Biotimylated anti-Goat	1.5mg/ml	1:200	Rabbit

2.6 Statistical analysis

Statistical analysis were investigated for the following factors: 1) Comparison about the ΔC_T values of real-time RT-PCR between meningiomas of different WHO grades and dura; 2) Comparison about the ΔC_T values of real-time RT-PCR between VS and NCN; 3) Comparison about the ΔC_T values of real-time RT-PCR between MPNST, SPS and NSN; 4) Comparison about the percentages of immunostained cells between meningiomas of different WHO grades and dura; 5) Comparison about the percentages of immunostained cells between VS and NCN; 6) Comparison about the percentages of immunostained cells between MPNST, SPS and NSN; 7) Correlation between the mRNA expression (ΔC_T values of real-time RT-PCR) of

chemokines and its receptors in meningiomas of different WHO grades; 8) Correlation between the mRNA expression (ΔC_T values of real-time RT-PCR) of chemokines and its receptors in schwannomas (including VS and SPS) and MPNST. The statistical analysis was carried out with SPSS 10.0 for windows (SPSS Inc, Chicago, IL, USA). Student's t-test with independent samples and bivariate correlation analysis (Pearson correlation coefficients) were used. Significance level in all tests was $P=0.05$.

3 Results

3.1 Patients' materials

A total amount of 45 fresh frozen tumor tissue specimens were analyzed in the study. The group included 24 women and 21 men, with a mean age of 58.6 ± 9.4 years at diagnosis. Tumor tissue consisted of 10 meningiomas WHO grade I, 10 meningiomas WHO grade II, 7 meningiomas WHO grade III, 6 spinal schwannomas (SPS), 6 vestibular schwannomas (VS) and 6 malignant peripheral nerve sheath tumors (MPNST). Only one tumor tissue specimen obtained from a recurrent meningioma WHO grade III, while the others were not from the recurrent tumors. First of all, we wanted to know whether chemokines and their receptors were expressed in these tumor tissues on mRNA level. By real-time RT-PCR, the mRNA levels of chemokines and their receptors were measured for all these samples. Of these materials, 3 samples of every subtype (meningiomas WHO grade I, II, III, SPS, VS) and 2 samples of MPNST were investigated by IHC. The clinical data of patients are given in appendix 1. Meanwhile, 5 dura, 3 normal spinal nerves (NSN) and 2 normal 8th cranial nerves (NCN) served as normal control samples, which were obtained from the Department of General Pathology, Kiel, Germany.

3.2 CX3CL1 expression in meningiomas

First, we evaluate CX3CL1 expression in different meningiomas on mRNA level by real-time RT-PCR. In general, compared to the normal dura a down-regulated mRNA expression of CX3CL1 was found in meningiomas of all different WHO grades (see figure 1-I). Thereby, the mean normalized ΔC_T values were 5.16 in meningiomas of all WHO grades and 2.52 in normal dura, which means that CX3CL1 mRNA expression in meningiomas decreased about 6 folds over that in normal dura. However, CX3CL1 mRNA expression varied in different WHO grades of meningiomas. The average ΔC_T values were 5.43, 4.84 and 5.23 in meningiomas grade I, II and III, respectively. Meanwhile, within the same subtype, CX3CL1 mRNA expression also varied in different tissue samples (for instance, the ΔC_T values differed from 0.76 to 4.86 in different dura samples). Compared to dura, a statistical significant difference of CX3CL1 mRNA expression could be found in meningiomas grade I ($P<0.01$), II ($P<0.05$) and III ($P<0.01$).

Next, IHC was performed to evaluate CX3CL1 expression on the protein level in meningiomas of different WHO grades and normal dura. 3 samples of every subtype (meningiomas WHO grade I, II, III, dura) were chosen for IHC. Immunostaining of CX3CL1 were found in all meningiomas and normal dura, and CX3CL1 positive cells were all stained brown compare to the serum-control and isotype-control (figure 1-II and 1-III). Overall, CX3CL1 positive cells varied in different WHO grades of meningiomas, the average percentages were 11.04%, 9.95% and 7.45% in meningiomas grade I, II and III, respectively (see figure 1-II). In contrast, 18.24% CX3CL1 positive cells were detected in normal dura (see figure 1-II). Meanwhile, within the same subtype, CX3CL1 positive cells also varied in different tissue samples. For example, the percentages of positive cells differed from 11.02% to 22.70% in different dura samples (see figure 1-II). Nevertheless, a statistical significant difference of the amounts of CX3CL1 positive cells was noted in meningiomas grade III compared to normal dura ($P<0.05$). There was a tendency that CX3CL1 was lower expressed in meningiomas grade I and II than in normal dura, though the difference was not significant.

CX3CL1 expression in meningiomas

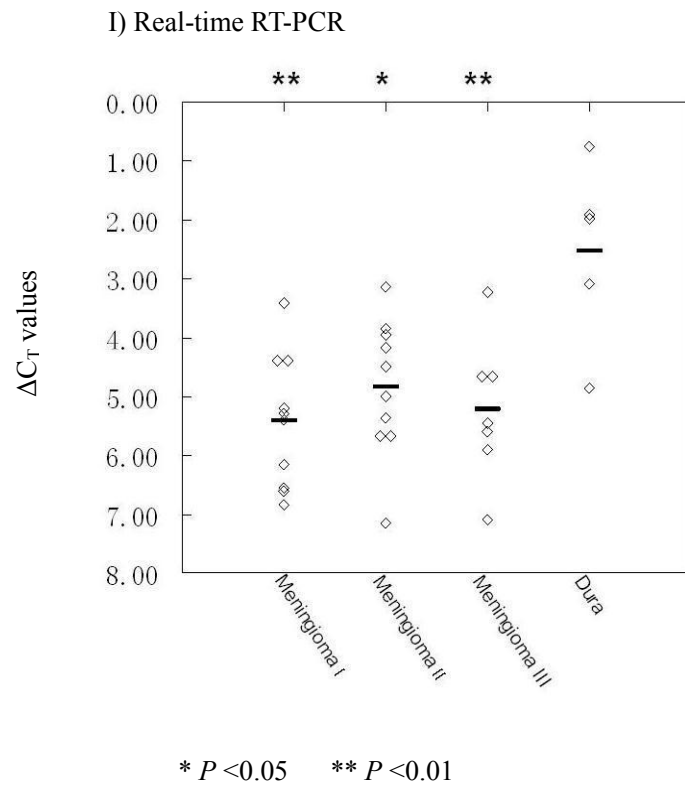


Figure 1-I CX3CL1 mRNA expression in meningiomas and dura measured by real-time RT-PCR: Low ΔC_T values indicate a high expression [logarithmic scale, a $\Delta C_T = 3.3$ corresponds to a 10-fold difference, $\Delta C_T = [C_{T(10ng)} - 3.3 + C_{T(100ng)}]/2 - C_{T(GAPDH)}$]. Individual points demonstrate normalized ΔC_T values from individual samples. The mean normalized ΔC_T values were 5.43, 4.84 and 5.23 in meningiomas grade I, II and III, respectively and 2.52 in normal dura. Compared to dura, a statistical significant difference of CX3CL1 mRNA expression could be found in meningiomas grade I ($P < 0.01$), II ($P < 0.05$) and III ($P < 0.01$).

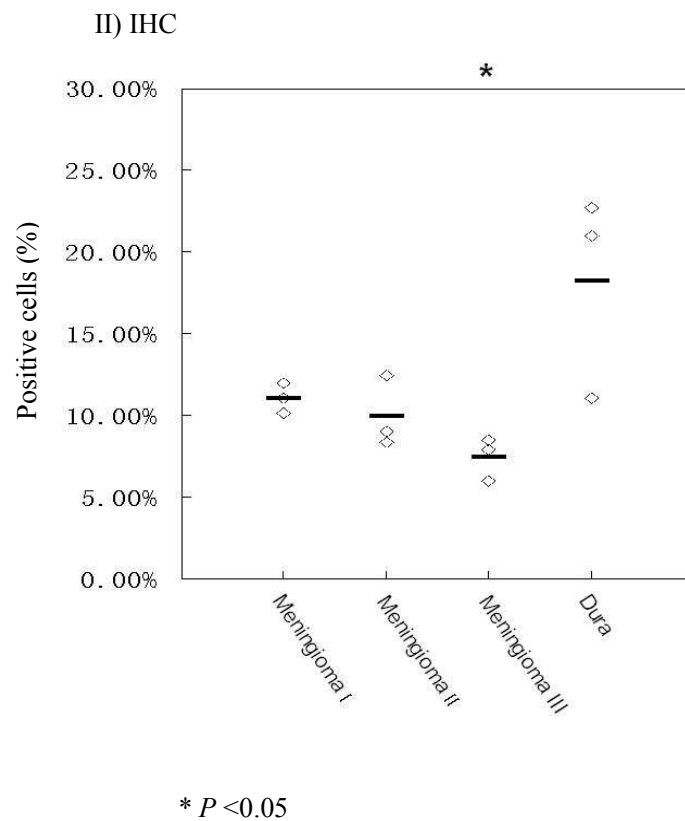


Figure 1-II Expression of CX3CL1 on the protein level in meningiomas of different WHO grades and dura: Individual points represent the percentages of CX3CL1 positive cells obtained by IHC. The average percentages of CX3CL1 positive cells were 11.04%, 9.95% and 7.45% in meningiomas grade I, II and III, respectively and 18.24% in normal dura. The lowest amounts of CX3CL1 positive cells were found in meningiomas grade III, and showed a statistical significant difference compared to normal dura ($P < 0.05$).

CX3CL1 expression in meningiomas

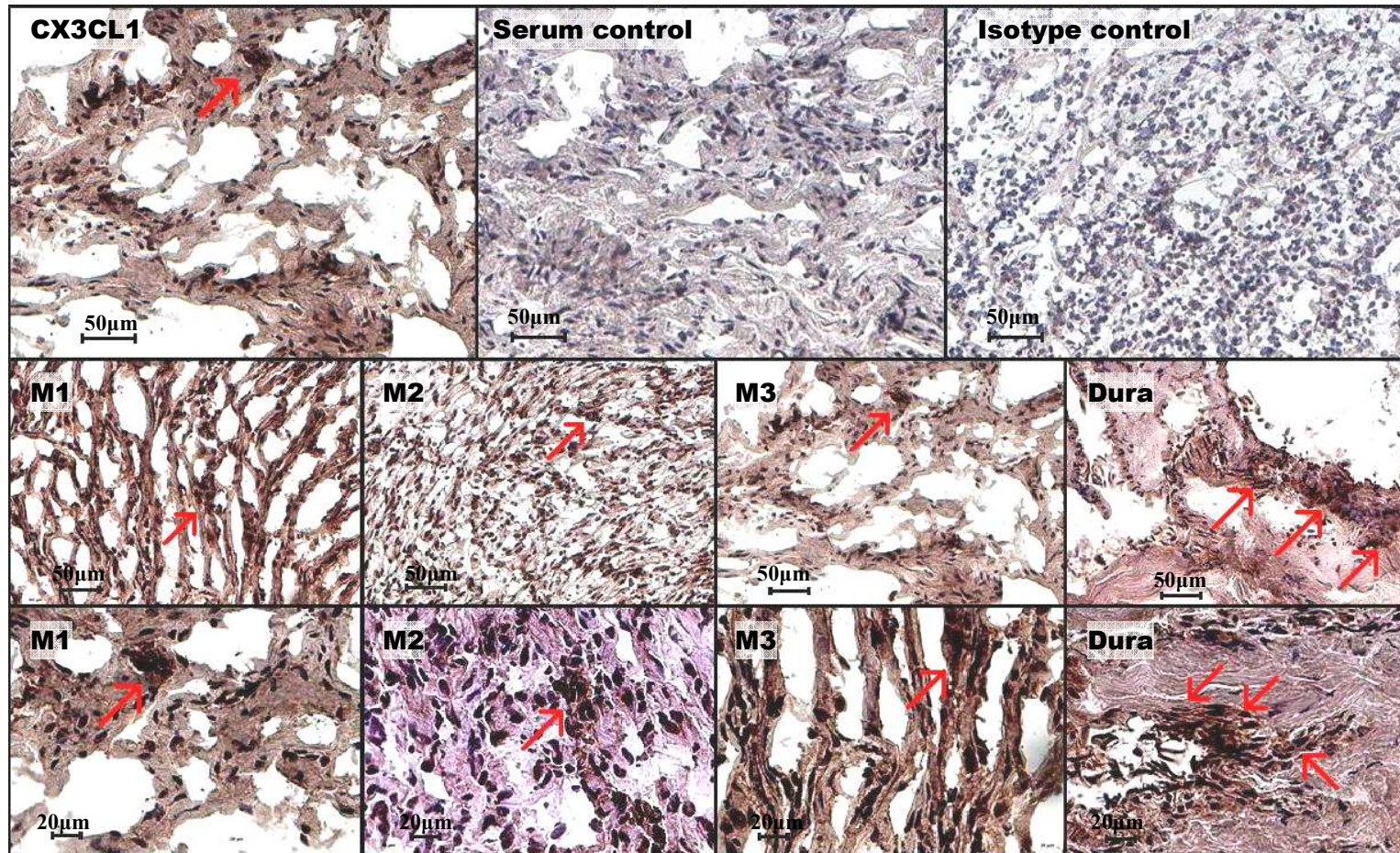


Figure 1-III Immunohistochemical staining of CX3CL1 (original magnification $\times 200$ of the first two lines, $\times 400$ of the third line) in meningiomas of different WHO grades and dura. Immunostaining of CX3CL1 was found in all meningiomas and normal dura, and CX3CL1 positive cells were all stained brown compare to the serum-control and isotype-control (red arrow). The lowest amounts of CX3CL1 positive cells were found in meningiomas grade III, and the highest amounts were found in normal dura. (M1: Meningioma grade I; M2: Meningioma grade II; M3: Meningioma grade III)

3.3 CX3CR1 expression in meningiomas

In meningiomas of different WHO grades, CX3CR1 expression was observed by real-time RT-PCR and IHC as well (figure 2-I, II and III).

CX3CR1 mRNA expression could be detected in all specimens of meningiomas and normal dura (see figure 2-I). The average ΔC_T values were 5.71 in meningiomas of all WHO grades and 3.77 in normal dura. Meanwhile, CX3CR1 mRNA expression varied in different WHO grades of meningiomas. The mean normalized ΔC_T values were 5.23, 4.54 and 8.06 in meningiomas grade I, II and III, respectively. Furthermore, within the same subtype, the normalized ΔC_T values also varied in different tissue samples. For example, the normalized ΔC_T values differed from 4.53 to 11.22 in different meningiomas WHO grade III samples (see figure 2-I). Compared to dura, a statistical significant difference of CX3CR1 mRNA expression could be found only in meningiomas grade III ($P < 0.01$).

For IHC, immunostaining of CX3CR1 was found in all meningiomas and normal dura samples. Like CX3CL1, CX3CR1 positive cells were all stained brown compare to the serum-control and isotype-control (figure 2-II and III). The average percentages of CX3CR1 positive cells were 11.37%, 14.04% and 7.52% in meningiomas grade I, II and III, respectively and 19.45% in normal dura (see figure 2-II). As same as real-time RT-PCR, a statistical significant difference of the amounts of CX3CR1 positive cells was noted only in meningiomas grade III compared to normal dura ($P < 0.05$). The amounts of CX3CR1 positive cells were a little bit lower in meningiomas grade I and II than in normal dura, but the difference was not significant.

CX3CR1 expression in meningiomas

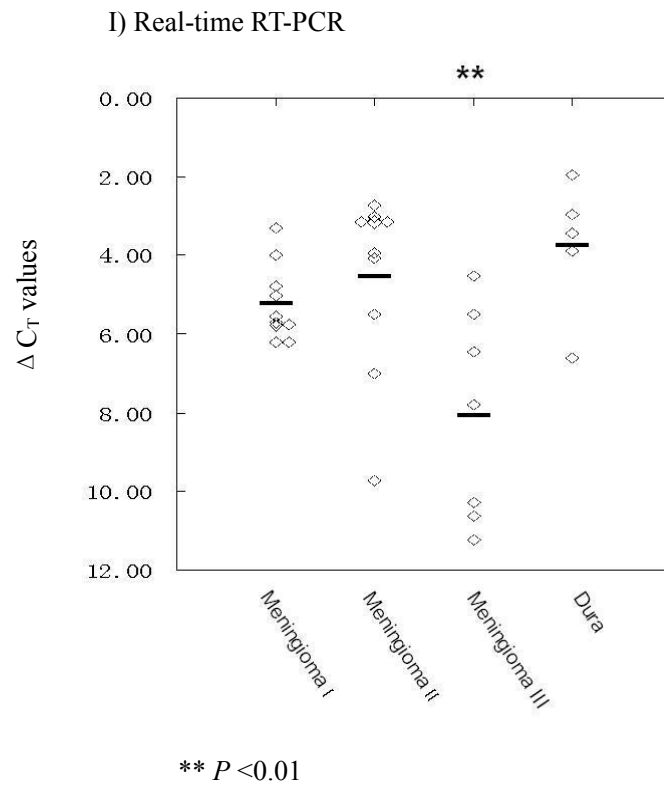


Figure 2-I CX3CR1 mRNA expression in meningiomas and dura measured by real-time RT-PCR: Low ΔC_T values indicate a high expression [logarithmic scale, a $\Delta C_T = 3.3$ corresponds to a 10-fold difference, $\Delta C_T = [CT_{(10ng)} - 3.3 + CT_{(100ng)}]/2 - CT_{(GAPDH)}$]. Individual points demonstrate normalized ΔC_T values from individual samples. The mean normalized ΔC_T values were 5.23, 4.54 and 8.06 in meningiomas grade I, II and III, respectively and 3.77 in normal dura. Compared to dura, a statistical significant difference of CX3CR1 mRNA expression could be found in meningiomas grade III ($P < 0.01$).

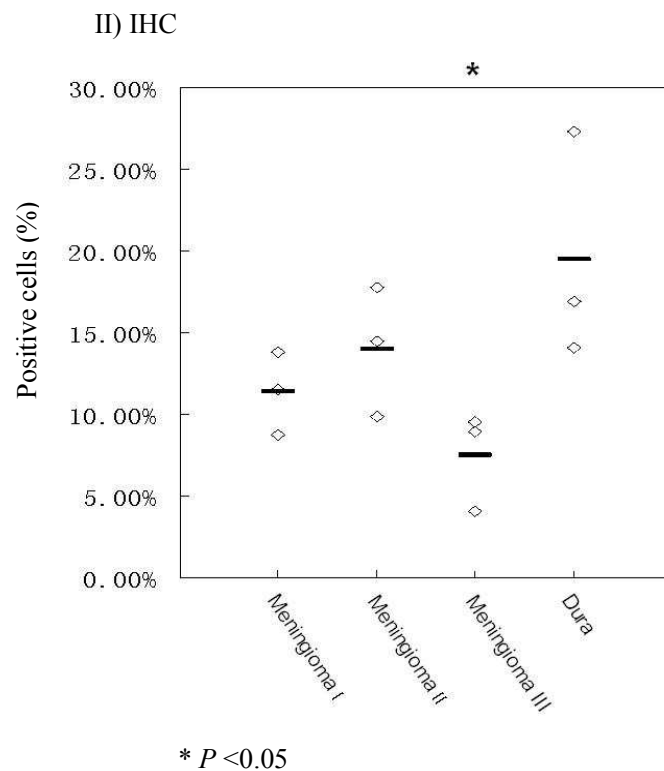


Figure 2-II Expression of CX3CR1 on the protein level in meningiomas of different WHO grades and dura: Individual points represent the percentages of CX3CR1 positive cells obtained by IHC. The average percentages of CX3CR1 positive cells were 11.37%, 14.04% and 7.52% in meningiomas grade I, II and III, respectively and 19.45% in normal dura. The lowest amounts of CX3CR1 positive cells were found in meningiomas grade III, and showed a statistical significant difference compared to normal dura ($P < 0.05$).

CX3CR1 expression in meningiomas

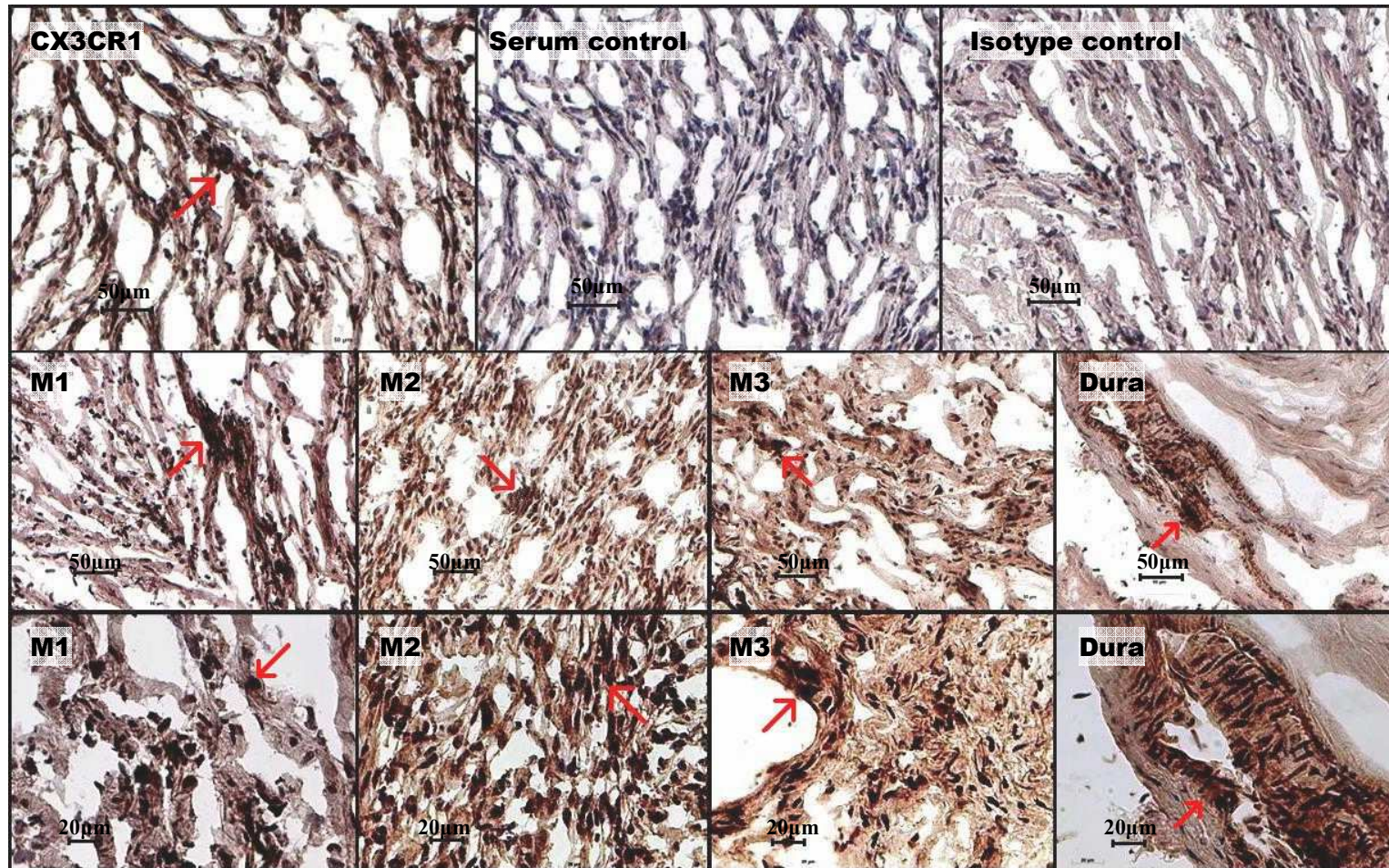


Figure 2-III Immunohistochemical staining of CX3CR1 (original magnification $\times 200$ of the first two lines, $\times 400$ of the third line) in meningiomas of different WHO grades and dura. Immunostaining of CX3CR1 was found in all meningiomas and normal dura, and CX3CR1 positive cells were all stained brown compare to the serum-control and isotype-control (red arrow). The lowest amounts of CX3CR1 positive cells were found in meningiomas grade III, and the highest amounts were found in normal dura. (M1: Meningioma grade I; M2: Meningioma grade II; M3: Meningioma grade III)

3.4 CXCL16 expression in meningiomas

Subsequently, CXCL16 mRNA expression in meningiomas of different WHO grades was confirmed by using real-time RT-PCR (figure 3-I). In general, compared to the normal dura a down-regulated mRNA expression of CXCL16 was found in meningiomas of all different WHO grades. The mean normalized ΔC_T values were 4.28, 3.90 and 5.18 in meningiomas grade I, II and III, respectively and 2.84 in normal dura. Compared to dura, a statistical significant difference of CXCL16 mRNA expression could be found in meningiomas grade I ($P<0.05$) and III ($P<0.01$). Although CXCL16 was a little bit lower expressed in meningiomas grade II than in normal dura, the difference was not significant.

Based on the results of real-time RT-PCR, the protein levels of CXCL16 were measured by IHC. Immunostaining of CXCL16 was found in all meningiomas and normal dura. Meanwhile, CXCL16 immunoreactive cells were all stained brown compare to the serum-control and isotype-control (figure 3-II and III). The average percentages of CXCL16 immunoreactive cells were 16.19%, 18.13% and 11.78% in meningiomas grade I, II and III, respectively and 18.56% in normal dura (see figure 3-II). The amounts of CXCL16 positive cells in meningiomas grade III was lower than that in normal dura with a statistical significant difference ($P<0.05$). There was no statistical significant difference between meningiomas grade I and II compared to normal dura.

CXCL16 expression in meningiomas

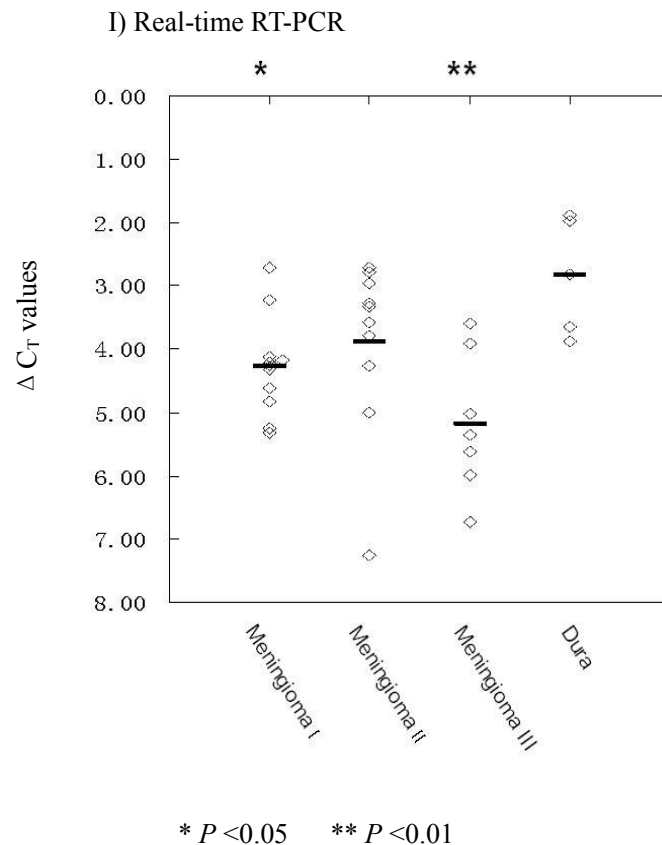


Figure 3-I CXCL16 mRNA expression in meningiomas and dura measured by real-time RT-PCR: Low ΔC_T values indicate a high expression [logarithmic scale, a $\Delta C_T = 3.3$ corresponds to a 10-fold difference, $\Delta C_T = [CT_{(10ng)} - 3.3 + CT_{(100ng)}] / 2 - CT_{(GAPDH)}$]. Individual points demonstrate normalized ΔC_T values from individual samples. The mean normalized ΔC_T values were 4.28, 3.90 and 5.18 in meningiomas grade I, II and III, respectively and 2.84 in normal dura. Compared to dura, a statistical significant difference of CXCL16 mRNA expression could be found in meningiomas grade I ($P < 0.05$) and III ($P < 0.01$).

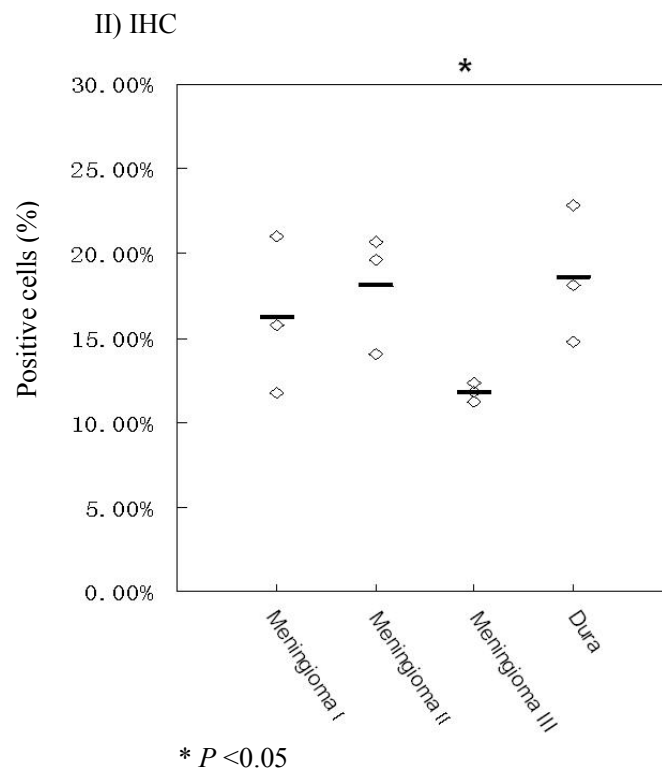


Figure 3-II Expression of CXCL16 on the protein level in meningiomas of different WHO grades and dura: Individual points represent the percentages of CXCL16 positive cells obtained by IHC. The average percentages of CXCL16 positive cells were 16.19%, 18.13% and 11.78% in meningiomas grade I, II and III, respectively and 18.56% in normal dura. The lowest amounts of CXCL16 positive cells were found in meningiomas grade III, and showed a statistical significant difference compared to normal dura ($P < 0.05$).

CXCL16 expression in meningiomas

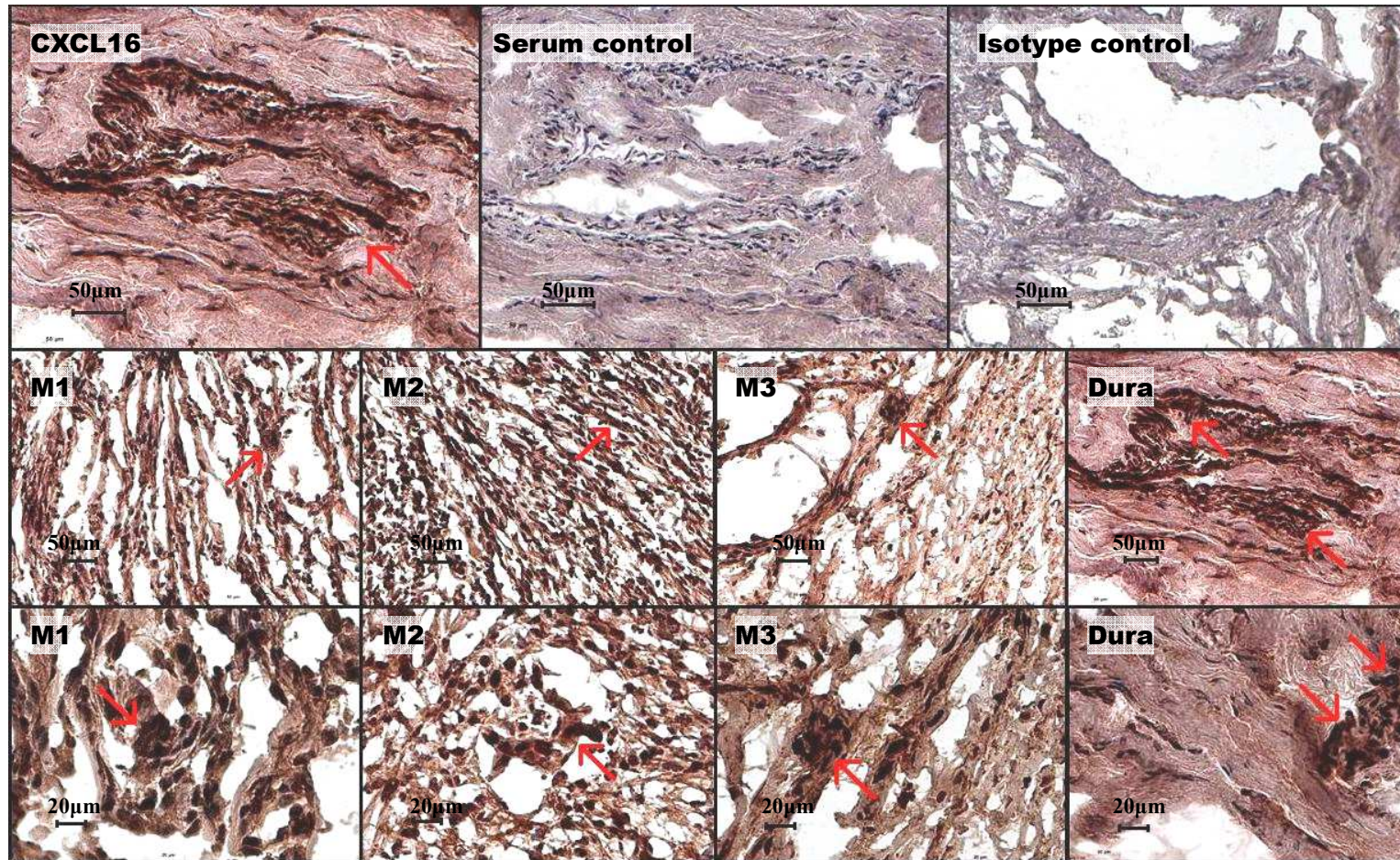


Figure 3-III Immunohistochemical staining of CXCL16 (original magnification $\times 200$ of the first two lines, $\times 400$ of the third line) in meningiomas of different WHO grades and dura. Immunostaining of CXCL16 were found in all meningiomas and normal dura, and CXCL16 positive cells were all stained brown compare to the serum-control and isotype-control (red arrow). The lowest amounts of CXCL16 positive cells were found in meningiomas grade III, and the highest amounts were found in normal dura. (M1: Meningioma grade I; M2: Meningioma grade II; M3: Meningioma grade III)

3.5 CXCR6 expression in meningiomas

Then, we investigated CXCR6 mRNA expression in meningiomas of different WHO grades and normal dura by real-time RT-PCR as well (figure 4-I). In contrast to the normal dura, CXCR6 mRNA expression was relatively low in meningiomas. The mean normalized ΔC_T values were 12.18, 10.91 and 12.15 in meningiomas grade I, II and III, respectively and 9.7 in normal dura. Based on high ΔC_T values indicating low expression, the expression levels of CXCR6 were relatively lower in all grade of meningiomas and normal dura than that of other molecules investigated in this study. However, a statistical significant difference of CXCR6 mRNA expression could be found in meningiomas grade I compared to normal dura ($P < 0.05$).

After that, CXCR6 protein expression was investigated by IHC. CXCR6 immunoreactive cells were found in all meningiomas and normal dura which were stained brown compare to the serum-control and isotype-control (figure 4-II and III). In meningiomas grade I, II and III, the mean percentages of CXCR6 positive cells were 5.84%, 6.73% and 6.75%, respectively. Normal dura expressed 8.75% CXCR6 positive cells (figure 4-II). No statistical significant differences could be found between meningiomas of different WHO grades and normal dura.

CXCR6 expression in meningiomas

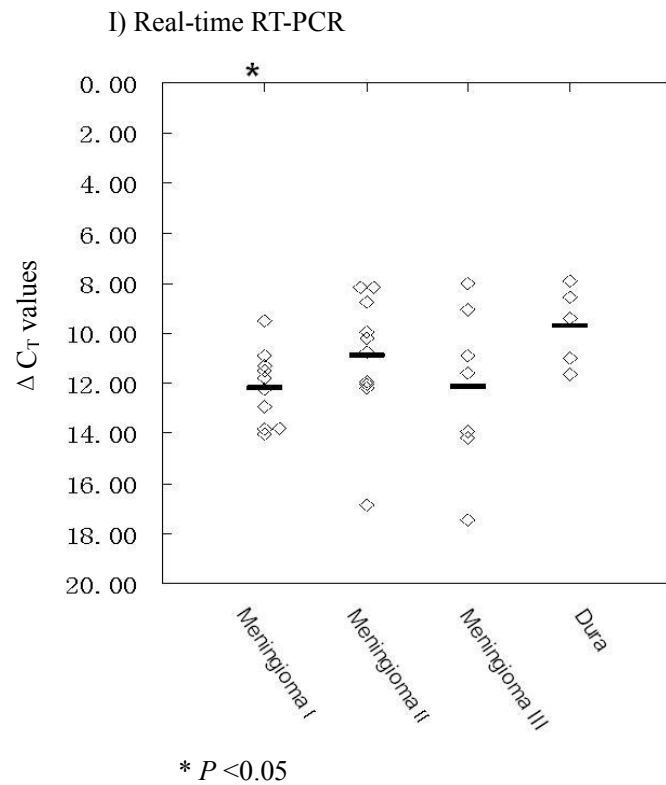


Figure 4-I CXCR6 mRNA expression in meningiomas and dura measured by real-time RT-PCR: Low ΔC_T values indicate a high expression [logarithmic scale, a $\Delta C_T = 3.3$ corresponds to a 10-fold difference, $\Delta C_T = [CT_{(10ng)} - 3.3 + CT_{(100ng)}]/2 - CT_{(GAPDH)}$]. Individual points demonstrate normalized ΔC_T values from individual samples. The mean normalized ΔC_T values were 12.18, 10.91 and 12.15 in meningiomas grade I, II and III, respectively and 9.7 in normal dura. Compared to dura, a statistical significant difference of CXCR6 mRNA expression could be found only in meningiomas grade I ($P < 0.05$).

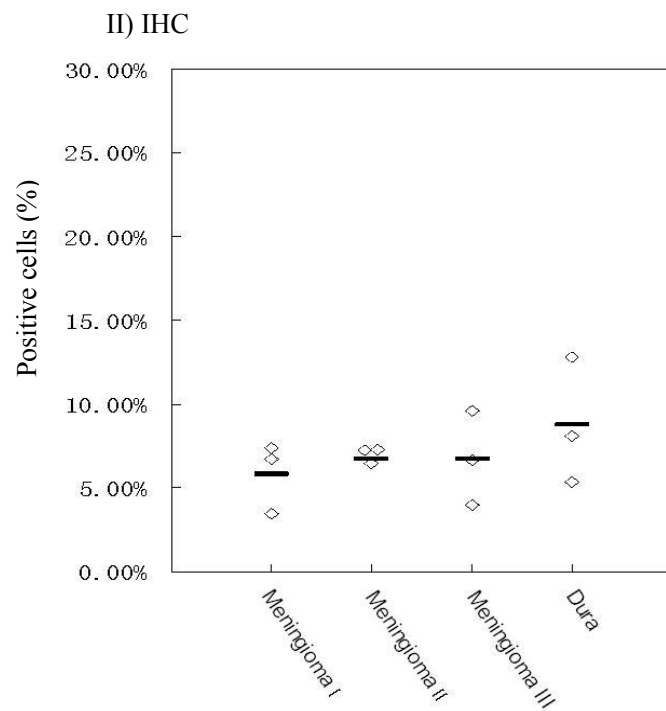


Figure 4-II Expression of CXCR6 on the protein level in meningiomas of different WHO grades and dura: Individual points represent the percentages of CXCR6 positive cells obtained by IHC. The average percentages of CXCR6 positive cells were 5.84%, 6.73% and 6.75% in meningiomas grade I, II and III, respectively and 8.75% in normal dura. The amounts of CXCR6 positive cells were similar in all meningiomas and normal dura. No statistical significant differences could be found between meningiomas of different WHO grades and normal dura.

CXCR6 expression in meningiomas

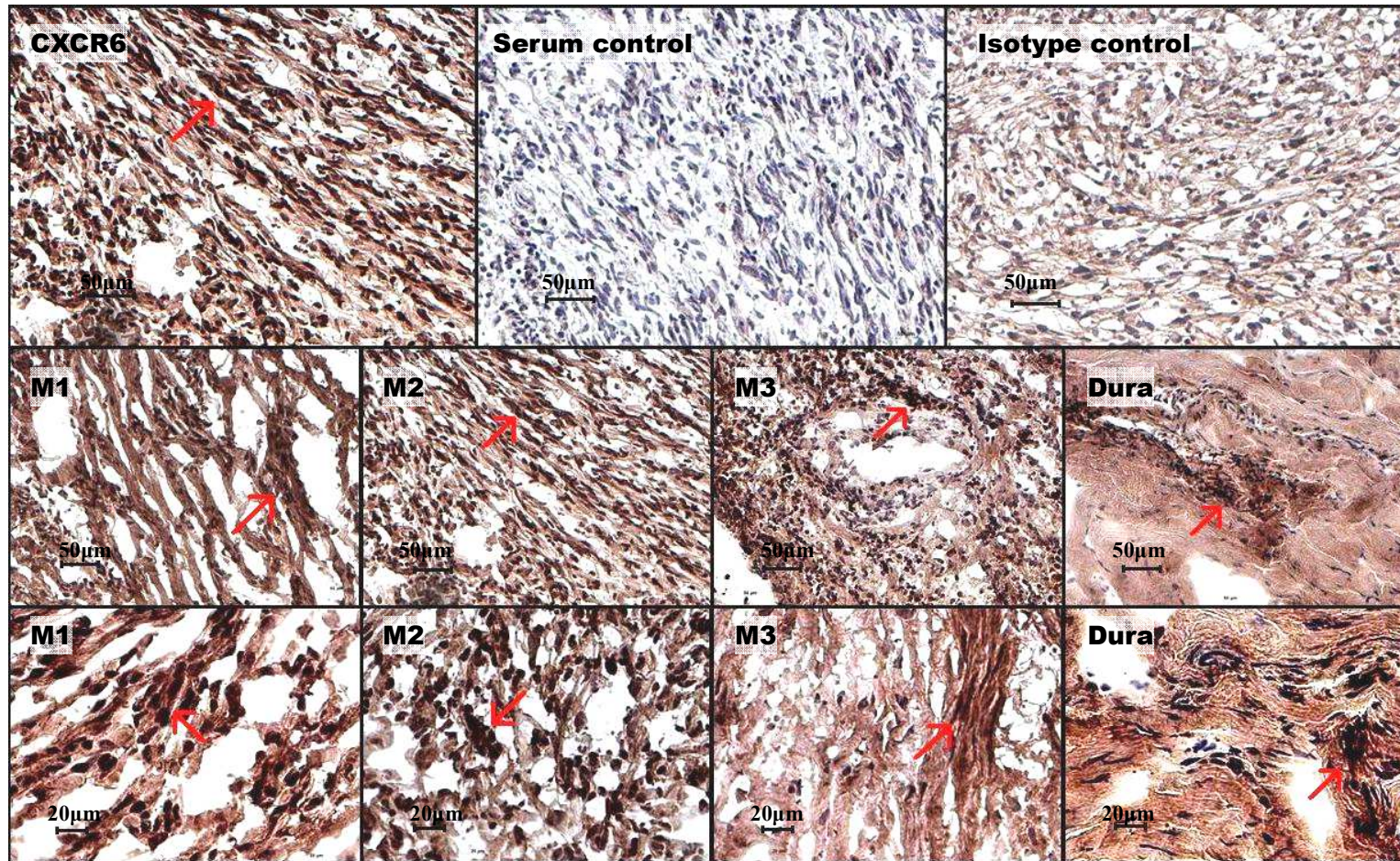


Figure 4-III Immunohistochemical staining of CXCR6 (original magnification $\times 200$ of the first two lines, $\times 400$ of the third line) in meningiomas of different WHO grades and dura. Immunostaining of CXCR6 was found in all meningiomas and normal dura, and CXCR6 positive cells were all stained brown compare to the serum-control and isotype-control (red arrow). The amounts of CXCR6 positive cells were similar in meningiomas of all WHO grades and normal dura. (M1: Meningioma grade I; M2: Meningioma grade II; M3: Meningioma grade III)

3.6 CX3CL1 expression in schwannomas

To assess the potential role of CX3CL1 in different schwannomas, the expression of this chemokine on mRNA and protein level was examined by real-time RT-PCR and IHC.

In general, CX3CL1 mRNA expression could be detected in all specimens of schwannomas and normal nerves. Compared to NCN and NSN, a down-regulated mRNA expression of CX3CL1 was found in VS, SPS and MPNST (see figure 5-I). Thereby, the mean normalized ΔC_T values were 7.15, 6.15 and 3.60 in VS, SPS and MPNST, respectively. Intriguingly, a significant difference of CX3CL1 mRNA expression could be detected between NCN and NSN. The mean normalized ΔC_T values were 4.16 in NCN and -0.26 in NSN. Compared to NCN, a statistical significant difference of CX3CL1 mRNA expression could be found in VS ($P<0.05$). Compared to NSN, a statistical significant difference also could be found in SPS ($P<0.01$).

For IHC, immunostaining of CX3CL1 was found in all schwannomas, malignant peripheral nerve sheath tumors and normal nerves. CX3CL1 positive cells were all stained brown compare to the serum-control and isotype-control (figure 5-II and 5-III). The average percentages of CX3CL1 positive cells were 5.78%, 6.86% and 11.07% in VS, SPS and MPNST, respectively (see figure 5-II). Like real-time RT-PCR, a difference of CX3CL1 protein expression also could be found between NCN and NSN. The average percentages of CX3CL1 positive cells were 10.85% in NCN and 15.65% in NSN. Compared to NCN, no statistical significant difference of the amounts of CX3CL1 positive cells could be found in VS. Compared to NSN, a statistical significant difference could be found only in SPS ($P<0.05$), but there was no statistical significant difference between MPNST and NSN.

CX3CL1 expression in schwannomas

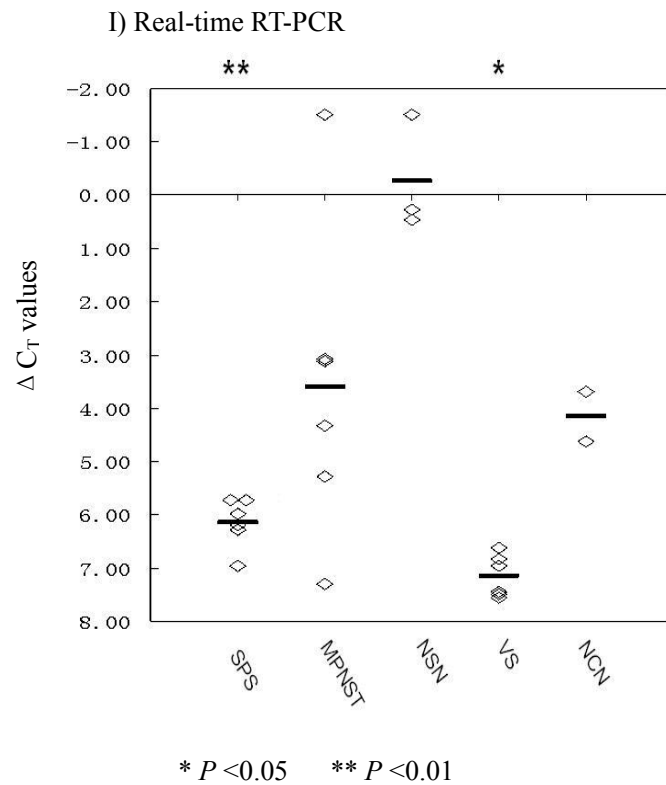


Figure 5-I CX3CL1 mRNA expression in schwannomas and normal nerves measured by real-time RT-PCR: Low ΔC_T values indicate a high expression [logarithmic scale, a $\Delta C_T = 3.3$ corresponds to a 10-fold difference, $\Delta C_T = [CT_{(10ng)} - 3.3 + CT_{(100ng)}] / 2 - CT_{(GAPDH)}$]. Individual points demonstrate normalized ΔC_T values from individual samples. The mean normalized ΔC_T values were 6.15, 3.60, 7.15, -0.26 and 4.16 in SPS, MPNST, VS, NSN and NCN, respectively. Compared to NSN, a statistical significant difference of CX3CL1 mRNA expression could be found in SPS ($P < 0.01$), and compared to NCN, a statistical significant difference could be found in VS ($P < 0.05$).

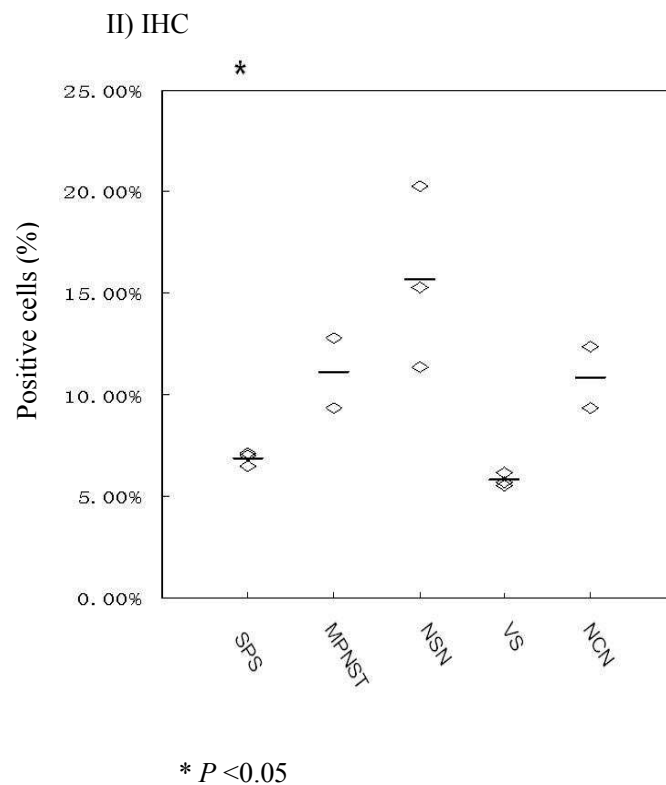


Figure 5-II Expression of CX3CL1 on the protein level in schwannomas and normal nerves: Individual points represent the percentages of CX3CL1 positive cells obtained by IHC. The average percentages of CX3CL1 positive cells were 6.86%, 11.07%, 5.78%, 15.65% and 10.85% in SPS, MPNST, VS, NSN and NCN, respectively. Lower amounts of CX3CL1 positive cells were found in SPS and showed a statistical significant difference compared to NSN ($P < 0.05$).

(SPS: spinal schwannomas; MPNST: malignant peripheral nerve sheath tumors; VS: vestibular schwannomas; NCN: normal cranial nerves; NSN: normal spinal nerves)

CX3CL1 expression in schwannomas

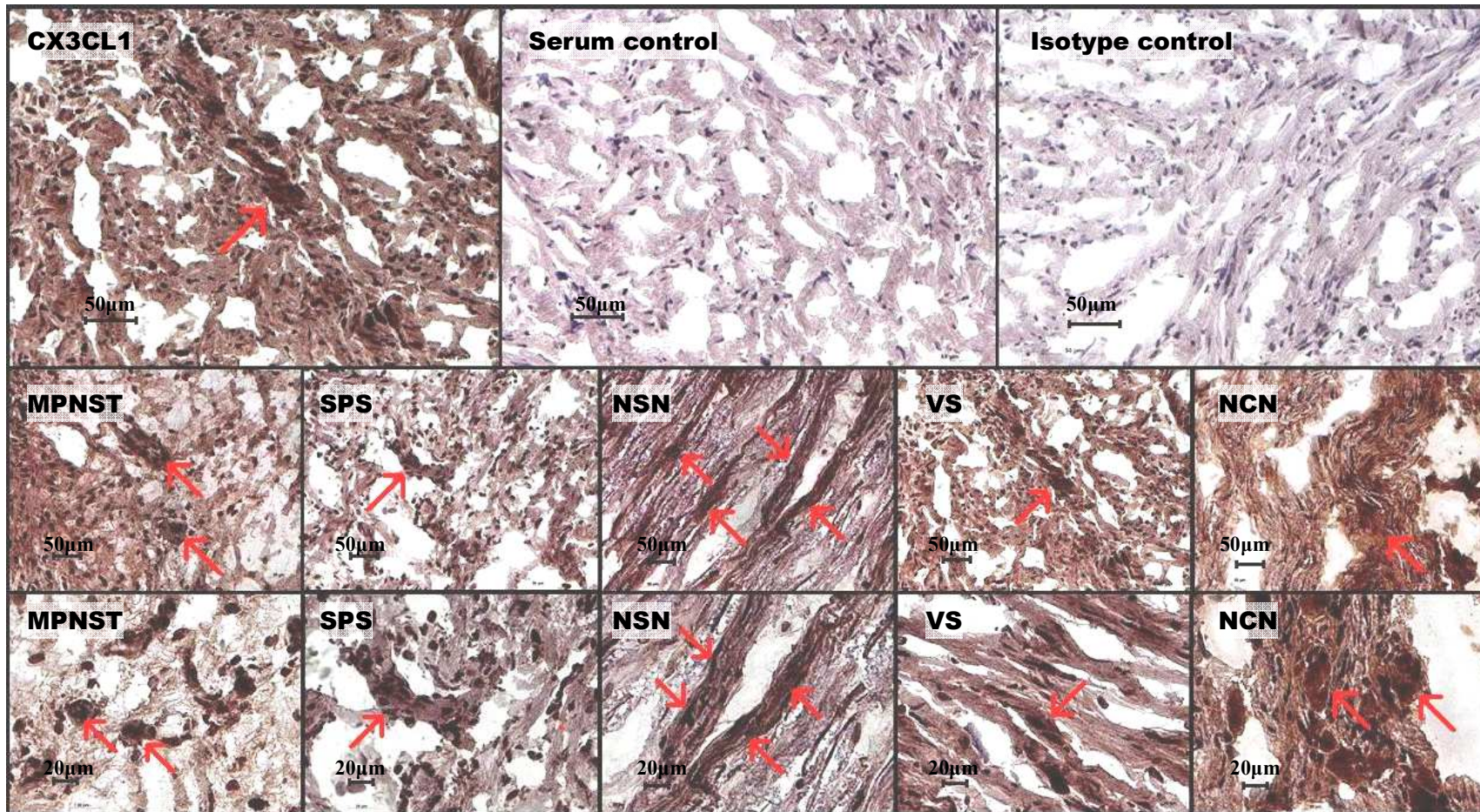


Figure 5-III Immunohistochemical staining of CX3CL1 (original magnification $\times 200$ of the first two lines, $\times 400$ of the third line) in schwannomas and normal nerves. Immunostaining of CX3CL1 was found in MPNST, SPS, VS, NSN and NCN. CX3CL1 positive cells were all stained brown compare to the serum-control and isotype-control (red arrow). Low amounts of CX3CL1 positive cells were found in MPNST and SPS compared to NSN. Meanwhile low amounts of CX3CL1 positive cells were also found in VS compared to NCN. (MPNST: malignant peripheral nerve sheath tumors; SPS: spinal schwannomas; VS: vestibular schwannomas; NSN: normal spinal nerves; NCN: normal cranial nerves)

3.7 CX3CR1 expression in schwannomas

The mRNA levels of CX3CR1 were measured by real-time RT-PCR and determined in relation to that of GAPDH. CX3CR1 mRNA expression could be measured in all cases of the investigated samples except for one case in MPNST and another in NSN. Compared to NCN, remarkably up-regulated mRNA expression of CX3CR1 mRNA was found in VS. The mean normalized ΔC_T values were 1.56 in VS and 5.01 in NCN with a statistical significant difference ($P < 0.01$). However, the mean normalized ΔC_T values were 1.04, 5.59 and 2.49 in SPS, MPNST and NSN, respectively. So, a slightly up-regulated expression of CX3CR1 mRNA could be detected in SPS compared to NSN. In contrast to this, a down-regulated expression of CX3CR1 mRNA could be detected in MPNST compared to NSN. No statistical significant differences could be found between SPS, MPNST and NSN (see figure 6-I).

Based on the results of real-time RT-PCR, protein level of CX3CR1 was detected by IHC. CX3CR1 positive cells were all stained brown compare to the serum-control and isotype-control (figure 6-II and 6-III). In VS and NCN, the mean percentages of CX3CR1 positive cells were 18.95% and 10.32%, respectively (see figure 6-II). As same as real-time RT-PCR, the amounts of CX3CR1 positive cells in VS were higher than that in NCN with a statistical significant difference ($P < 0.05$). In SPS, MPNST and NSN, the mean percentages of CX3CR1 positive cells were 15.69%, 11.10% and 12.71%, respectively. The amounts of CX3CR1 positive cells in SPS were also higher than that in NSN with a statistical significant difference ($P < 0.05$). No statistical significant difference could be found between MPNST and NSN.

CX3CR1 expression in schwannomas

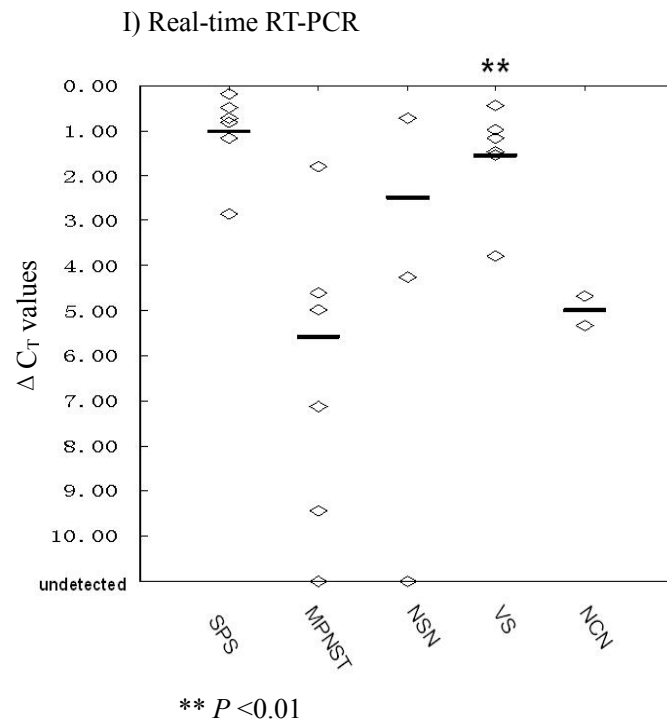


Figure 6-I CX3CR1 mRNA expression in schwannomas and normal nerves measured by real-time RT-PCR: Low ΔC_T values indicate a high expression [logarithmic scale, a $\Delta C_T = 3.3$ corresponds to a 10-fold difference, $\Delta C_T = [CT_{(10ng)} - 3.3 + CT_{(100ng)}] / 2 - CT_{(GAPDH)}$]. Individual points demonstrate normalized ΔC_T values from individual samples. CX3CR1 mRNA expression was undetectable in one MPNST sample and another NSN sample. The mean normalized ΔC_T values were 1.04, 5.59, 1.56, 2.49 and 5.01 in SPS, MPNST, VS, NSN and NCN, respectively. Compared to NCN, a statistical significant difference of CX3CR1 mRNA expression could be found in VS ($P < 0.05$). No statistical significant differences could be found between SPS, MPNST and NSN.

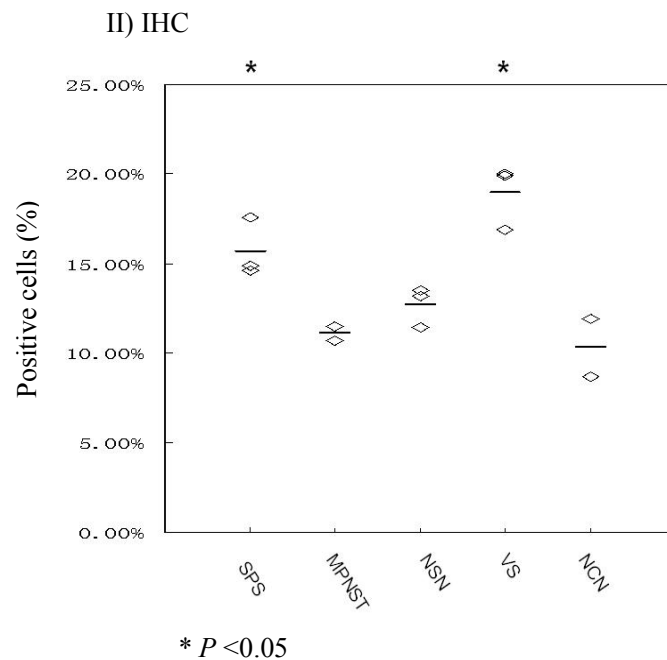


Figure 6-II Expression of CX3CR1 on the protein level in schwannomas and normal nerves: Individual points represent the percentages of CX3CR1 positive cells obtained by IHC. The average percentages of CX3CR1 positive cells were 15.69%, 11.10%, 18.95%, 12.71% and 10.32% in SPS, MPNST, VS, NSN and NCN, respectively. Low amounts of CX3CR1 positive cells were found in SPS, and showed a statistical significant difference with NSN ($P < 0.05$). Compared to NCN, high amounts of CX3CR1 positive cells were found in VS ($P < 0.05$).

(SPS: spinal schwannomas; MPNST: malignant peripheral nerve sheath tumors; VS: vestibular schwannomas; NCN: normal cranial nerves; NSN: normal spinal nerves)

CX3CR1 expression in schwannomas

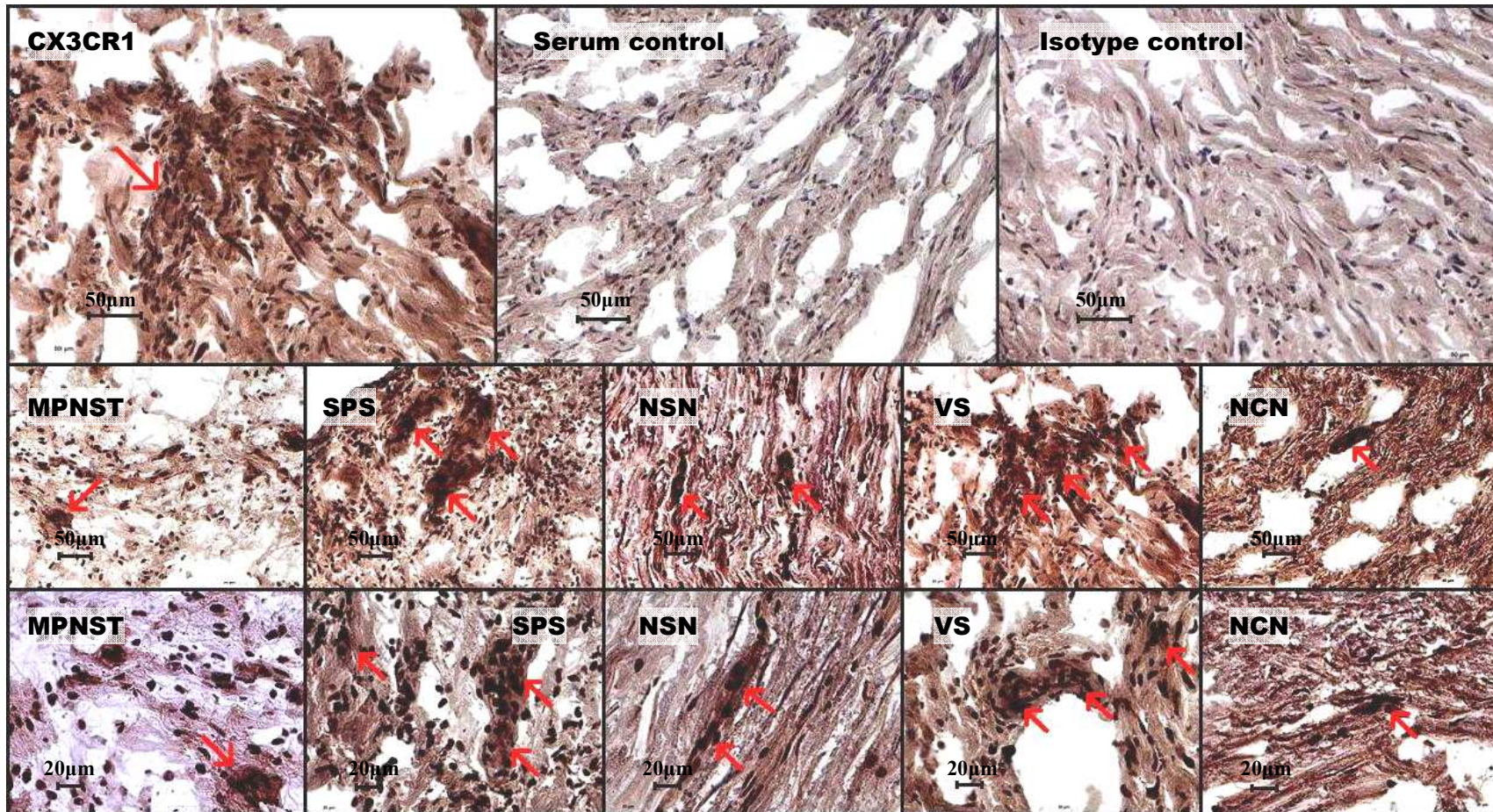


Figure 6-III Immunohistochemical staining of CX3CR1 (original magnification $\times 200$ of the first two lines, $\times 400$ of the third line) in schwannomas and normal nerves. Immunostaining of CX3CR1 was found in MPNST, SPS, VS and normal nerves (NSN and NCN), and CX3CR1 positive cells were all stained brown compare to the serum-control and isotype-control (red arrow). Low amounts of CX3CR1 positive cells were found in MPNST compared to NSN and high amounts were found in SPS compared to NSN. Meanwhile, high amounts of CX3CR1 positive cells were found in VS compared to NCN. (MPNST: malignant peripheral nerve sheath tumors; SPS: spinal schwannomas; VS: vestibular schwannomas; NSN: normal spinal nerves; NCN: normal cranial nerves)

3.8 CXCL12 expression in schwannomas

Then, we measured CXCL12 mRNA expression in VS, SPS and MPNST by real-time RT-PCR (see figure 7-I). CXCL12 mRNA expression could be detected in all schwannomas, MPNST and normal nerves. We found that the expression level of CXCL12 mRNA in NSN was remarkably higher than that in NCN. The mean normalized ΔC_T values were 3.96 in NCN and -3.99 in NSN. Because of low ΔC_T values indicating high expression, the expression levels of CXCL12 were relatively higher in schwannomas and MPNST than that of other molecules measured in this study. Thereby, the mean normalized ΔC_T values were 1.93, 1.16 and -2.21 in VS, SPS and MPNST, respectively. A up-regulated mRNA expression of CXCL12 was found in VS compared to NCN, but the difference was not significant. A down-regulated mRNA expression of CXCL12 was found in SPS and MPNST compared to NSN. A statistical significant difference of CXCL12 mRNA expression could be found only in SPS ($P < 0.01$), but there was no statistical significant difference in MPNST compared to NSN.

After that, CXCL12 protein expression in different schwannomas was confirmed by using IHC. CXCL12 positive cells were all stained brown compare to the serum-control and isotype-control (figure 7-II and 7-III). The average percentages of CXCL12 positive cells were 14.69%, 15.17% and 21.12% in VS, SPS and MPNST, respectively. A difference of CXCL12 protein expression also could be found between NCN and NSN. The average percentage of CXCL12 positive cells were 8.64% in NCN and 21.97% in NSN. No statistical significant difference of the amounts of CXCL12 positive cells could be found in VS compared to NCN. Compared to NSN, a statistical significant difference could be found only in SPS ($P < 0.05$). No statistical significant difference could be found between MPNST and NSN.

CXCL12 expression in schwannomas

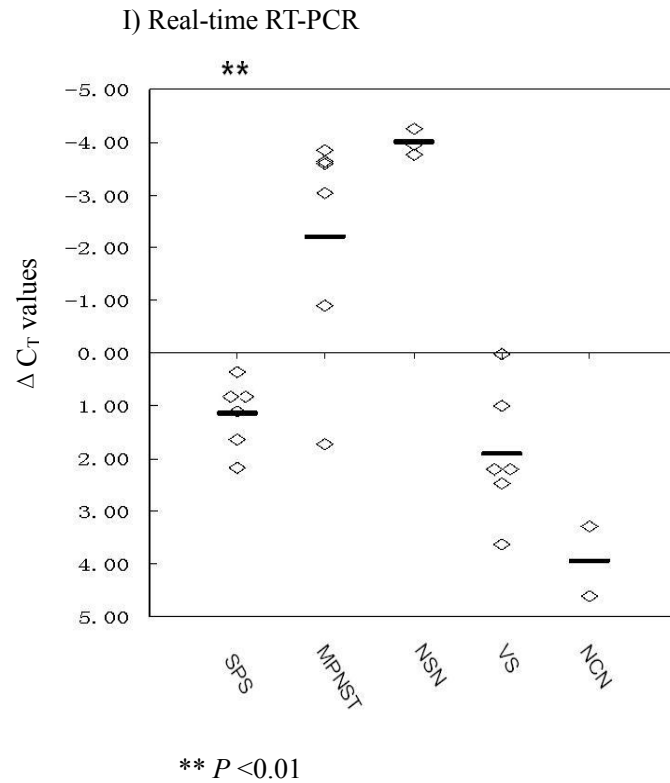


Figure 7-I CXCL12 mRNA expression in schwannomas and normal nerves measured by real-time RT-PCR: Low ΔC_T values indicate a high expression [logarithmic scale, a $\Delta C_T = 3.3$ corresponds to a 10-fold difference, $\Delta C_T = [CT_{(10ng)} - 3.3 + CT_{(100ng)}] / 2 - CT_{(GAPDH)}$]. Individual points demonstrate normalized ΔC_T values from individual samples. The mean normalized ΔC_T values were 1.16, -2.21, 1.93, -3.99 and 3.96 in SPS, MPNST, VS, NSN and NCN respectively. Compared to NSN, a statistical significant difference of CXCL12 mRNA expression could be found in SPS ($P < 0.01$).

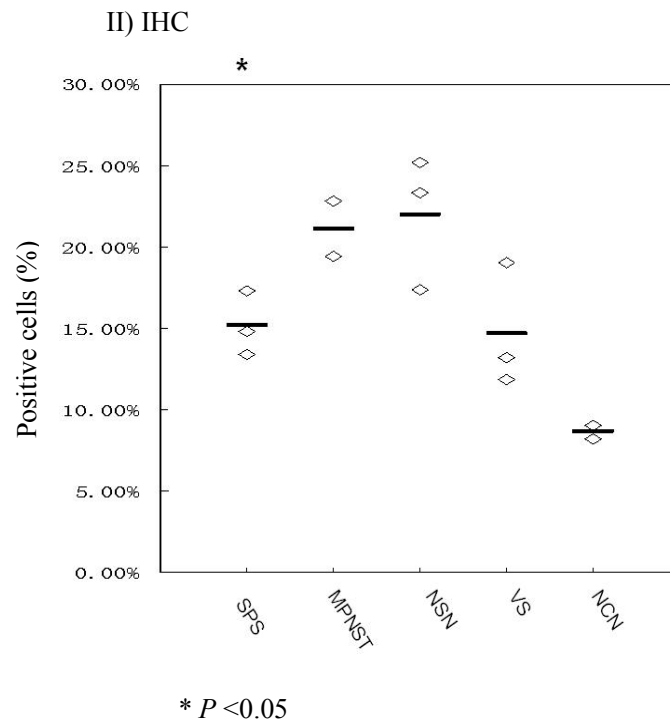


Figure 7-II Expression of CXCL12 on the protein level in schwannomas and normal nerves: Individual points represent the percentages of CXCL12 positive cells obtained by IHC. The average percentages of CXCL12 positive cells were 15.17%, 21.12%, 14.69%, 21.97% and 8.64% in SPS, MPNST, VS, NSN and NCN, respectively. Low amounts of CXCL12 positive cells were found in SPS, and showed a statistical significant difference compared to NSN ($P < 0.05$).

(SPS: spinal schwannomas; MPNST: malignant peripheral nerve sheath tumors; VS: vestibular schwannomas; NCN: normal cranial nerves; NSN: normal spinal nerves)

CXCL12 expression in schwannomas

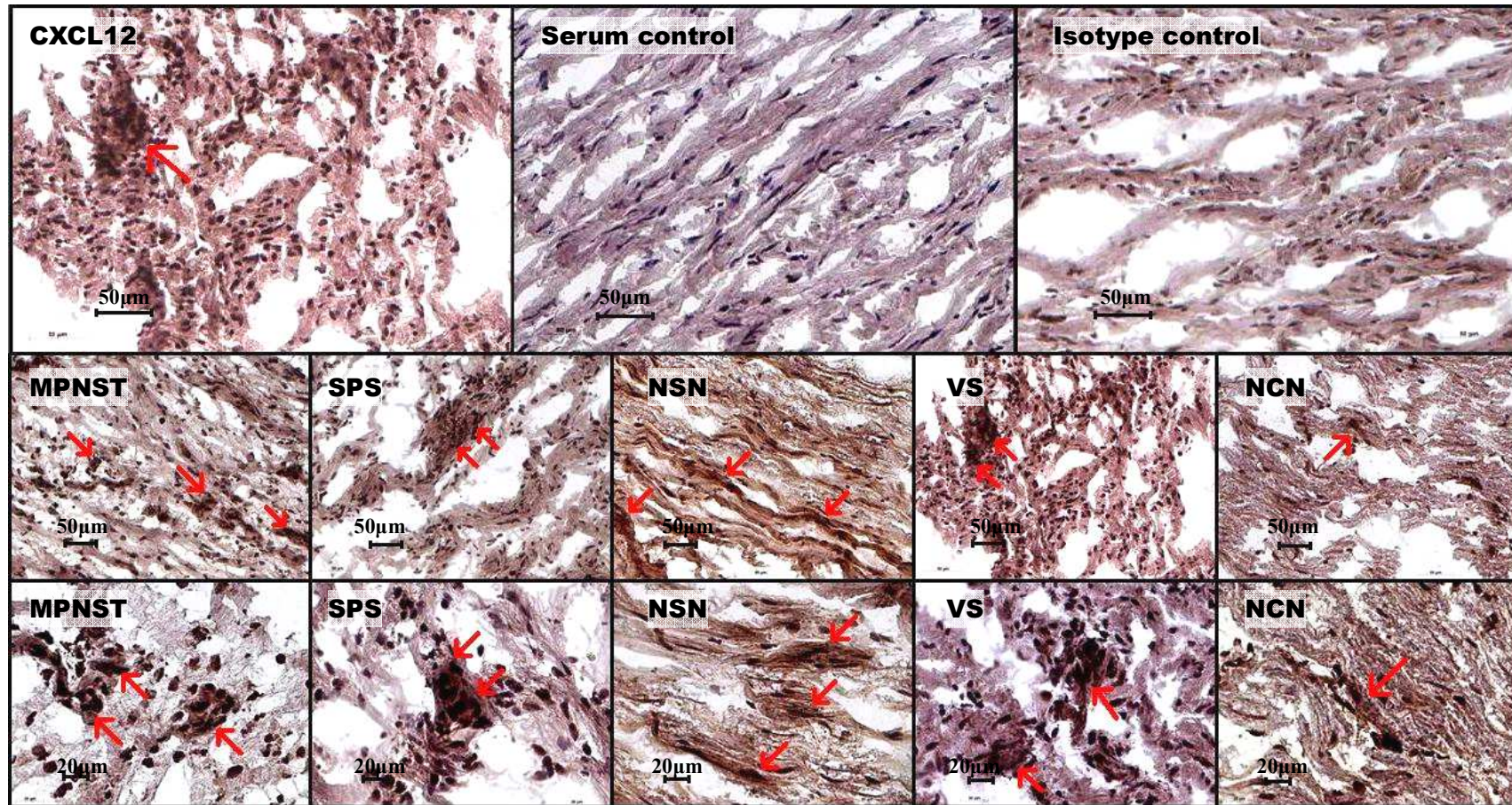


Figure 7-III Immunohistochemical staining of CXCL12 (original magnification $\times 200$ of the first two lines, $\times 400$ of the third line) in schwannomas and normal nerves. Immunostaining of CXCL12 was found in MPNST, SPS, VS and normal nerves (NSN and NCN), and CXCL12 positive cells were all stained brown compare to the serum-control and isotype-control (red arrow). Low amounts of CXCL12 positive cells were found in SPS compared to NSN and the amounts of CXCL12 positive cells were similar between MPNST and NSN. Meanwhile, high amounts of CXCL12 positive cells were found in VS compared to NCN. (MPNST: malignant peripheral nerve sheath tumors; SPS: spinal schwannomas; VS: vestibular schwannomas; NSN: normal spinal nerves; NCN: normal cranial nerves)

3.9 CXCR4 expression in schwannomas

CXCR4 mRNA expression in different schwannomas was analyzed using real-time RT-PCR. And as same as CX3CR1, CXCR4 mRNA expression could be measured in all cases of the investigated samples except for one case in MPNST and another in NSN (figure 8-I). The mean normalized ΔC_T values were 5.29 in VS and 6.84 in NCN. The CXCR4 mRNA expression in VS was slightly up-regulated, but no statistical significant difference could be obtained compared to NCN. However, the mean normalized ΔC_T values were 4.74, 5.48 and 4.01 in SPS, MPNST and NSN, respectively. A slightly down-regulated expression of CXCR4 mRNA could be detected in SPS and MPNST compared to NSN. No statistical significant differences could be found between SPS, MPNST and NSN.

Finally, CXCR4 protein expression in VS, SPS and MPNST were investigated by IHC. As same as CXCL12, CXCR4 positive cells were all stained brown compare to the serum-control and isotype-control (figure 8-II and 8-III). In VS and NCN, the mean percentages of CXCR4 positive cells were 11.21% and 4.94%, respectively. The amounts of CXCR4 positive cells in VS were higher than that in NCN with a statistical significant difference ($P < 0.05$). In SPS, MPNST and NSN, the mean percentages of CXCR4 positive cells were 11.54%, 10.59% and 10.08%, respectively. There were no statistical significant differences between SPS, MPNST and NSN.

CXCR4 expression in schwannomas

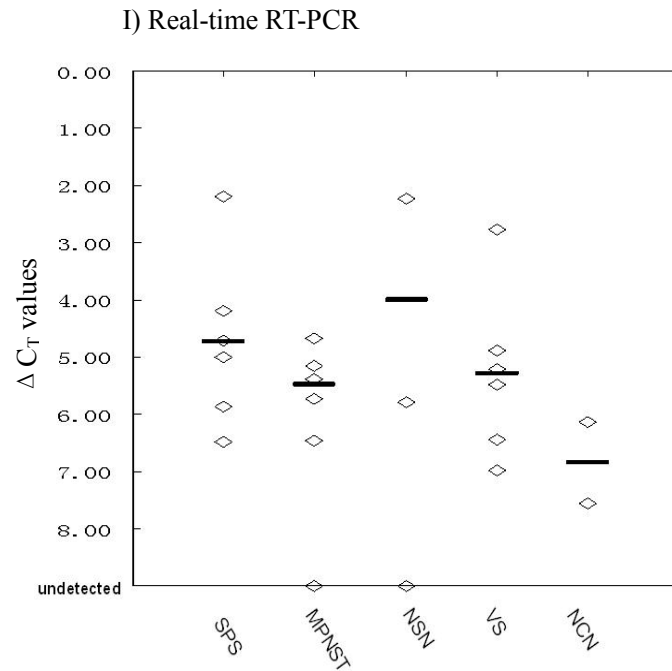


Figure 8-I CXCR4 mRNA expression in schwannomas and normal nerves measured by real-time RT-PCR: Low ΔC_T values indicate a high expression [logarithmic scale, a $\Delta C_T = 3.3$ corresponds to a 10-fold difference, $\Delta C_T = [CT_{(10ng)} - 3.3 + CT_{(100ng)}] / 2 - CT_{(GAPDH)}$]. Individual points demonstrate normalized ΔC_T values from individual samples. CXCR4 mRNA expression was undetectable in one MPNST sample and another NSN sample. The mean normalized ΔC_T values were 4.74, 5.48, 5.29, 4.01 and 6.84 in SPS, MPNST, VS, NSN and NCN, respectively. No statistical significant differences of CXCR4 mRNA expression could be found between SPS, MPNST and NSN. Meanwhile, no statistical significant difference could be found in VS compared to NCN.

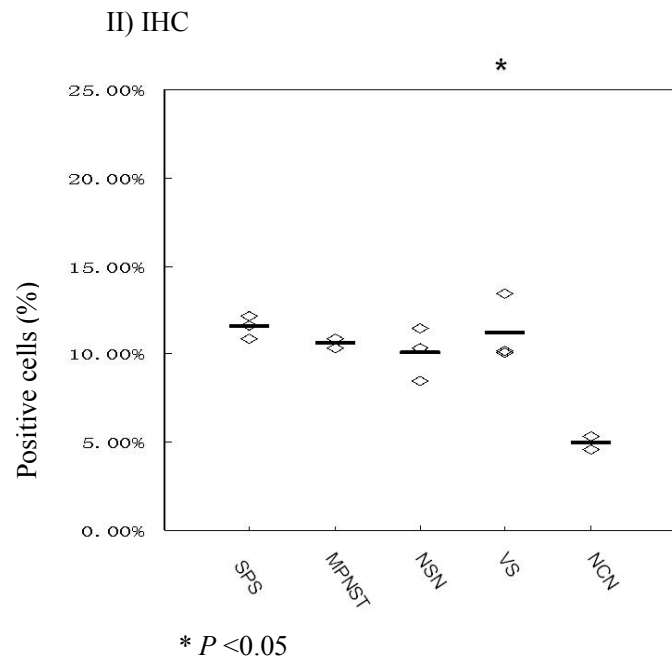


Figure 8-II Expression of CXCR4 on the protein level in schwannomas and normal nerves: Individual points represent the percentages of CXCR4 positive cells obtained by IHC. The average percentages of CXCR4 positive cells were 11.54%, 10.59%, 10.08% and 4.94% in SPS, MPNST, VS, NSN and NCN, respectively. High amounts of CXCR4 positive cells were found in VS, and showed a statistical significant difference compared to NCN ($P < 0.05$). The amounts of CXCR4 positive cells were similar between SPS, MPNST and NSN, and showed no statistical significant difference.

(SPS: spinal schwannomas; MPNST: malignant peripheral nerve sheath tumors; VS: vestibular schwannomas; NCN: normal cranial nerves; NSN: normal spinal nerves)

CXCR4 expression in schwannomas

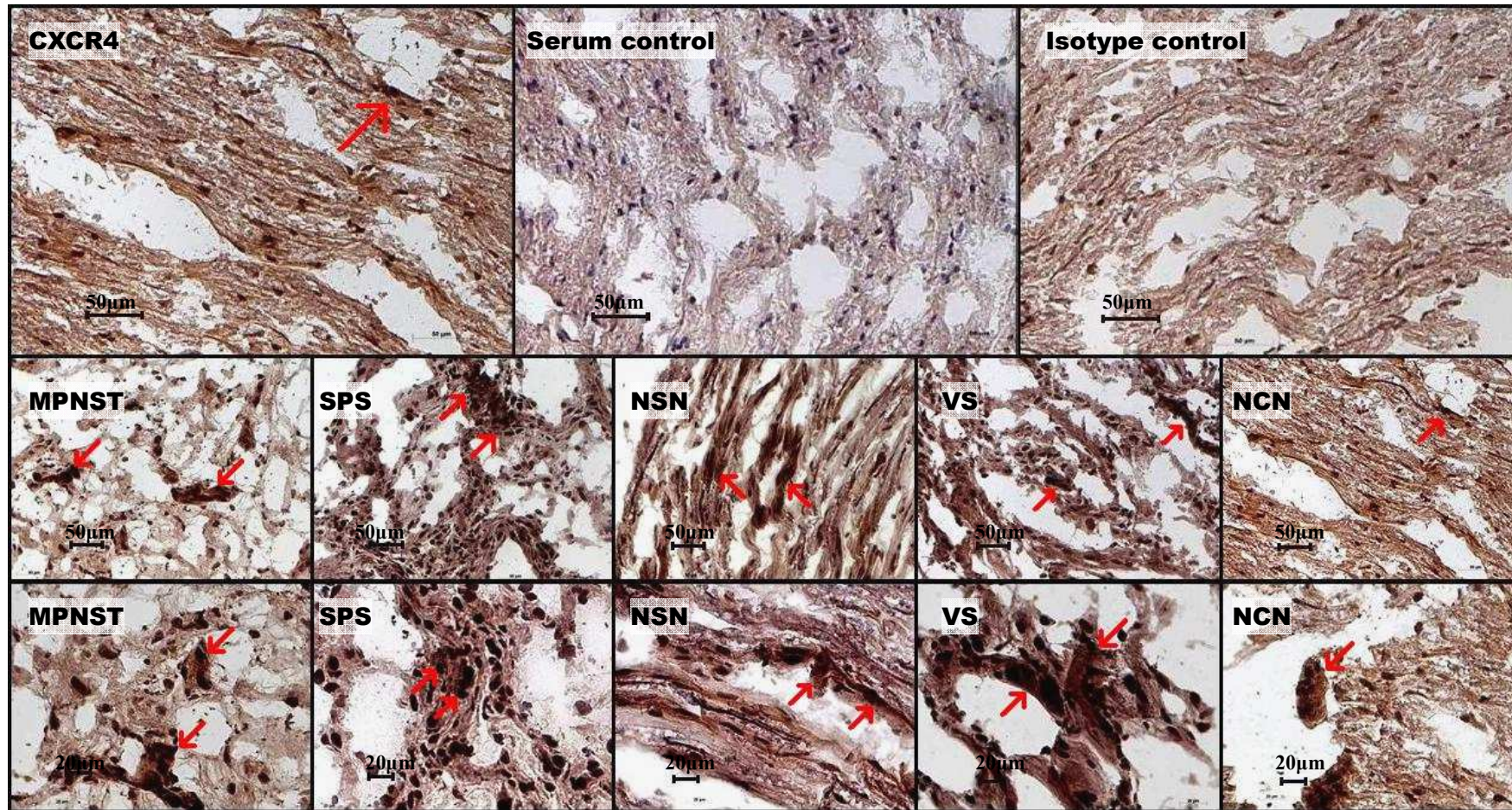


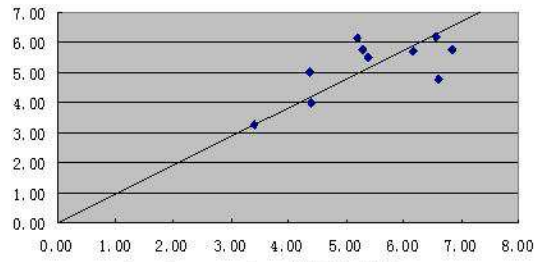
Figure 8-III Immunohistochemical staining of CXCR4 (original magnification $\times 200$ of the first two lines, $\times 400$ of the third line) in schwannomas and normal nerves. Immunostaining of CXCR4 was found in MPNST, SPS, VS and normal nerves (NSN and NCN), and CXCR4 positive cells were all stained brown compare to the serum-control and isotype-control (red arrow). The amounts of CXCR4 positive cells were found to be similar between SPS, MPNST and NSN. Meanwhile, high amounts of CXCR4 positive cells were found in VS compared to NCN. (MPNST: malignant peripheral nerve sheath tumors; SPS: spinal schwannomas; VS: vestibular schwannomas; NSN: normal spinal nerves; NCN: normal cranial nerves)

3.10 Correlations between chemokines (CX3CL1, CXCL16) and their receptors (CX3CR1, CXCR6) mRNA levels in meningiomas

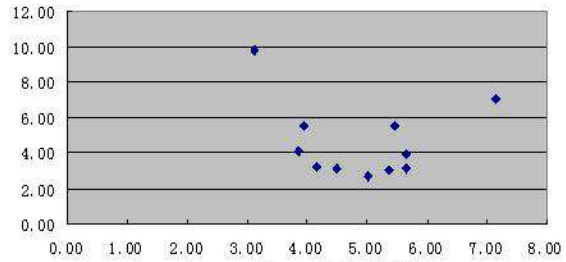
The chemokines and their respective receptors are co-expressed and associated among them at the mRNA level. So, in order to analyze the possible correlation between the expression levels of chemokines (CX3CL1, CXCL16) and their receptors (CX3CR1, CXCR6) in meningiomas, Pearson correlation analysis was used with SPSS 10.0 for windows (see figure 9-I and II). In all meningiomas samples, a positive correlation between the expression of CXCL16 and CXCR6 could be observed ($P < 0.01$). On the contrary, no correlation between CX3CL1 and CX3CR1 expression could be found. Subsequently, the possible correlations were analyzed in different WHO grade meningiomas respectively. A positive correlation between the expression of CX3CL1 and CX3CR1 could be observed in WHO grade I ($P < 0.05$), meanwhile a positive correlation between the expression of CXCL16 and CXCR6 could be observed in WHO grade II ($P < 0.01$) and grade III ($P < 0.05$).

Correlations between chemokines and their receptors in meningiomas

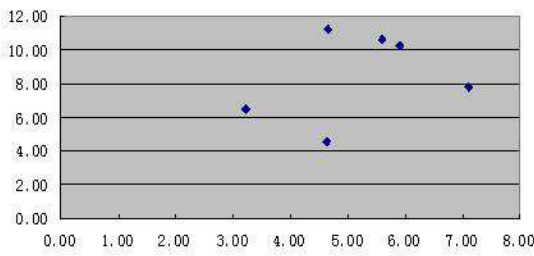
CX3CL1



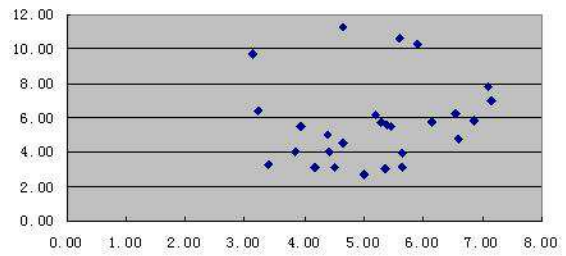
meningioma I: $P < 0.05$



meningioma II: $P > 0.05$



meningioma III: $P > 0.05$

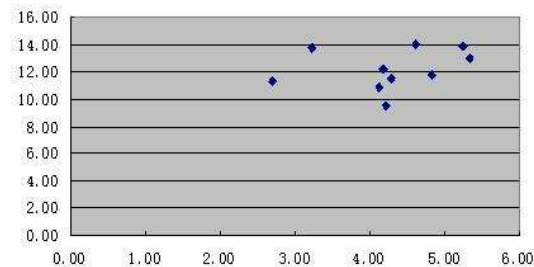


I+II+III: $P > 0.05$

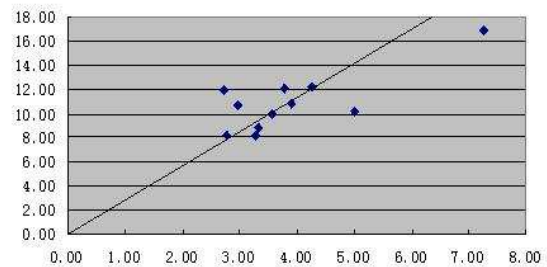
CX3CR1

Figure 9-I Correlation graphs between CX3CL1 and CX3CR1 mRNA expression: Each data point represents the value for an individual tumor. A significant positive correlation was observed in meningiomas grade I by the Pearson correlation analysis ($P < 0.05$).

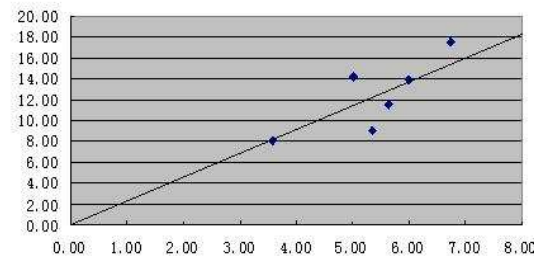
CXCL16



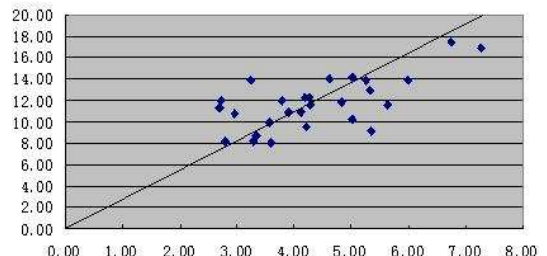
meningioma I: $P > 0.05$



meningioma II: $P < 0.01$



meningioma III: $P < 0.05$



I+II+III: $P < 0.01$

CXCR6

Figure 9-II Correlation graphs between CXCL16 and CXCR6 mRNA expression: Each data point represents the value for an individual tumor. By the Pearson correlation analysis, significant positive correlation was observed in all meningiomas samples ($P < 0.01$) and meningiomas grade II ($P < 0.01$), grade III ($P < 0.05$), respectively.

3.11 Correlations between chemokines (CX3CL1, CXCL12) and their receptors (CX3CR1, CXCR4) mRNA levels in schwannomas

As same as meningiomas, Pearson correlation analysis was used to analyze the possible correlation between the expression levels of chemokines (CX3CL1, CXCL12) and their receptors (CX3CR1, CXCR4) in schwannomas (including SPS and VS) and MPNST (see figure 10-I and II). In all schwannomas samples, a positive correlation between the expression of CXCL12 and CXCR4 could be observed ($P < 0.05$). On the contrary, no correlation between CX3CL1 and CX3CR1 expression could be found. In MPNST, no positive correlation could be observed between the expression of chemokines (CX3CL1, CXCL12) and their receptors (CX3CR1, CXCR4).

Correlations between chemokines and their receptors in schwannomas

CX3CL1

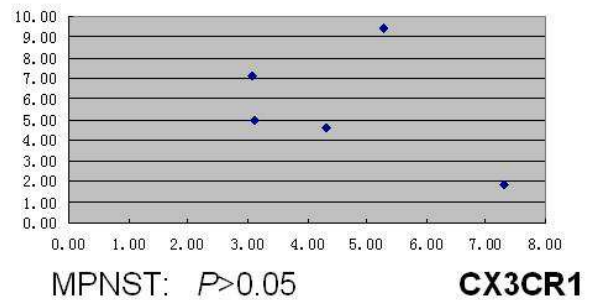
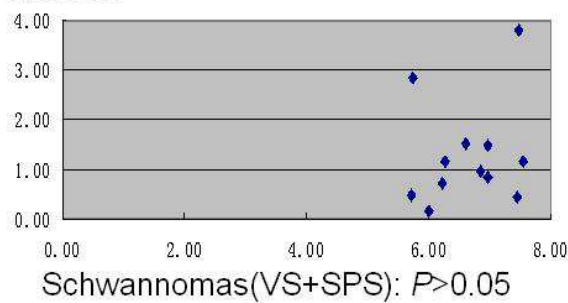


Figure 10-I Correlation graphs between CX3CL1 and CX3CR1 mRNA expression: Each data point represents the value for an individual tumor. No significant positive correlation was observed in schwannomas and MPNST by the Pearson correlation analysis.

CXCL12

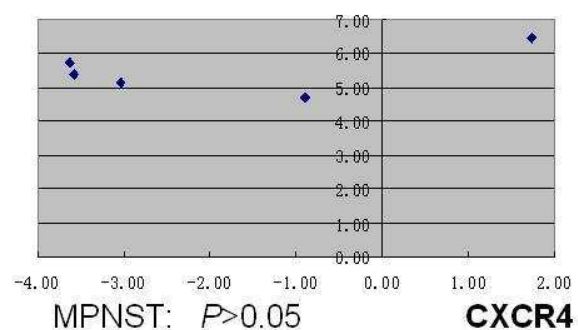
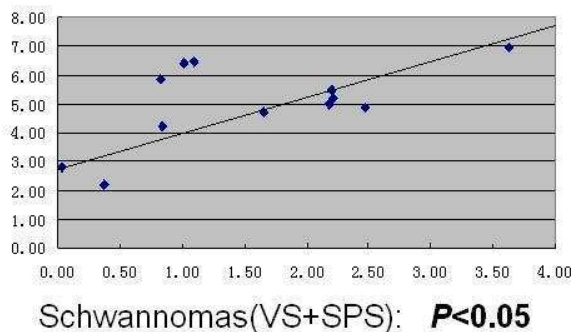


Figure 10-II Correlation graphs between CXCL12 and CXCR4 mRNA expression: Each data point represents the value for an individual tumor. By the Pearson correlation analysis, a significant positive correlation was observed in schwannomas ($P < 0.05$).

4 Discussion

Most meningiomas and schwannomas are slowly growing benign tumors, however, many of them, as well as anaplastic meningiomas and malignant peripheral nerve sheath tumors (MPNST), show an aggressive biological and clinical behavior associated with high rates of recurrence and unfavorable prognosis. The molecular mechanisms involved in initiation, proliferation and progression of meningiomas and schwannomas are not yet fully understood. Chemokines are the largest family of cytokines in human immunophysiology. They are a family of peptide mediators that play an essential role in cell activation, differentiation, and trafficking. In addition to chemoattraction, recent investigations have suggested a possible role of chemokines in tumor growth and metastasis and in host-tumor response (Homey et al. 2002). Additionally, several ligands activate the angiogenic switch in neoplastic tissues (Strieter et al. 2006; Müller et al. 2001; Balkwill et al. 2004; Menten et al. 2002). However, gene modification of chemokines was reported to play an important role in the induction of antitumor immunity in some animal neoplasms (Luster et al. 1993; Sgadari et al. 1997).

In the brain, chemokines are expressed by astroglial, microglial, neuronal, meningeal and endothelial cells, and they may form – alongside with neurotransmitters and neuropeptides– a third major messenger system (Ambrosini et al. 2004; de Haas et al. 2007). Functionally, chemokines are linked in the physiological context with neuronal patterning (Reiss et al. 2002), neuron–glia and glia–glia interactions as well as under pathological conditions with infections, chronic inflammatory diseases (e.g. multiple sclerosis), neurodegenerative diseases (e.g. Alzheimer's disease), ischemia and progression of brain tumors (Zhou et al. 2002). To learn more about the role of chemokines in meningiomas and schwannomas, the aims of the study were to investigate the expression of three chemokine/receptor pairs (CX3CL1/CX3CR1, CXCL12/CXCR4 and CXCL16/CXCR6) on mRNA and protein levels.

4.1 CX3CL1 and CX3CR1 expression in meningiomas

CX3CL1 is a unique CX3C chemokine acting as an adhesion molecule in its membrane form and behaving as a true chemokine in its soluble form. It is one of the most expressed chemokines in the brain (Cardona et al. 2006; Harrison et al. 1998; Hatori et al. 2002). Neurons and astrocytes are major producers of CX3CL1 and microglial cells express the

receptor (Miller et al. 2008; Mizuno et al. 2003); however, CX3CL1 expression has been detected also in astrocytes/glial cells (Mizuno et al. 2003; Ludwig et al. 2008), as well as few studies documented expression of CX3CR1 in neurons (Meucci et al. 2000). CX3CR1 expression by tumor cells has been investigated in prostate (Shulby et al. 2004), breast (Andre et al. 2006), pancreatic adenocarcinomas (Marchesi et al. 2008) and gliomas (Held-Feindt et al. 2011). But limited data are available on the expression of the CX3CL1/CX3CR1 in meningiomas.

In this study, first, the expressions of CX3CL1 and CX3CR1 mRNA were examined by real-time RT-PCR in solid tumor materials. Meningiomas originate from arachnoid cap cells and have close relationship to the dura. It was very difficult to collect arachnoid cap cells, so we chose normal dura tissues, which include also arachnoid cap cells, as controls. On the whole, the CX3CL1 mRNA expression level was decreased in meningiomas of all WHO grades compared to normal dura. Furthermore, CX3CL1 mRNA expression varied in different WHO grades of meningiomas. Compared to normal dura, a statistical significant difference of CX3CL1 mRNA expression could be found in meningiomas grade I ($P<0.01$), II ($P<0.05$) and III ($P<0.01$). These results were confirmed by IHC, by which lower percentages of CX3CL1 positive cells were found in meningiomas grade I, II and III in relation to normal dura, though statistical significant difference only noted in grade III. Down-regulation of CX3CL1 could be found for the first time in meningiomas, especially in WHO grade III. So, lower expression of CX3CL1 might be involved in tumorigenesis of human anaplastic meningiomas. Meanwhile, as same as the CX3CL1, lower CX3CR1 mRNA expression were found in meningiomas of all WHO grades compare to normal dura, and a statistical significant difference could be found in meningiomas grade III ($P<0.01$). The same results were obtained by IHC. Lowest percentage of CX3CR1 positive cells was found in meningiomas grade III, and showed a statistical difference compared to normal dura ($P<0.05$). Therefore, the expression of CX3CR1 mRNA and protein in poorly differentiated anaplastic meningiomas was obviously lower than that in the well differentiated low-grade meningiomas, and, this down-regulated CX3CR1 expression indicated the possibility that CX3CR1 was also involved in malignant transformation from low-grade meningiomas to high-grade meningiomas. Furthermore, a statistical positive correlation between the expression of CX3CL1 and CX3CR1 could be observed in

meningiomas grade I, but not in grade II and III. So, CX3CL1 and CX3CR1 were possibly co-expressed in benign meningiomas (WHO grade I), and perhaps were unbalanced expressed in atypical and anaplastic meningiomas (WHO grade II and III). In conclusion, we could demonstrate for the first time, that CX3CL1/CX3CR1 mRNA and protein amounts were down-regulated in meningiomas, especially in anaplastic variants, which means lower expression of CX3CL1/CX3CR1 axis might plays a pivotal role in the development of human anaplastic meningiomas. In addition, unbalanced expression of CX3CL1/CX3CR1 might be associated with a malignant tendency of high-grade meningiomas.

As a matter of fact, regulation of CX3CL1 cleavage has a direct impact on neuronal survival both *in vitro* and *in vivo* (Cardona et al. 2006). And CX3CR1 mediates protective anti-inflammatory effects against neurotoxicity in the brain (Cardona et al. 2006). Experimental evidence established that the CX3CL1/CX3CR1 axis plays a major role in the neuron/microglia cross-talk, and in neuroprotection under conditions of inflammation/injury (Cardona et al. 2006; Harrison et al. 1998; Miller et al. 2008; Mizuno et al. 2003). Additionally, animal models of cancer vaccinations based on the overexpression of CX3CL1 in tumor cells revealed a strong antitumor response (Guo et al. 2003A). The CX3CL1 gene was transduced into murine lung carcinoma cells and could induce antitumor immunity through chemoattraction and activation of T cells, dendritic cells and natural killer (NK) cells (Guo et al. 2003A; Guo et al. 2003B). Murine colon adenocarcinoma cells C26 and melanoma B16F10 cells showed that antitumor immune response induced by CX3CL1 gene transfer was depended on NK and T cells activation (Xin et al. 2005). However, Lavergne et al (2003) reported that in a EL-4 lymphoma model, CX3CL1-mediated antitumor effects occurred only via NK cells but not T cells because this antitumor effect was observed only in T cell- and B cell-deficient Rag1^{-/-} mice, but was ablated in NK cell-deficient beige mice. So, transfer of the CX3CL1 gene into tumor cells could elicit a specific antitumor immunity capable of inhibiting tumor growth which leads to increased survival of tumor-bearing hosts (Tang et al. 2007). In hepatocellular carcinoma patients, high expression of CX3CL1/CX3CR1 correlates with better prognosis and fewer recurrences, both local and distant (Matsubara et al. 2007). In view of these observations and our results, CX3CL1/CX3CR1 axis would be considered as a potentially suitable molecule for immunoprevention or gene therapy in human meningiomas,

especially in anaplastic variants.

4.2 CXCL16 and CXCR6 expression in meningiomas

CXCL16 is one of the two known transmembrane chemokines, which is not only found on immune cells, but constitutive expression has also been described on fibroblasts, keratinocytes, endothelial cells and cancer cells of different tumour origin (Hojo et al. 2007; Hundhausen et al. 2007; Ludwig et al. 2005; Scholz et al. 2007; Wågsäter et al. 2004; Held-Feindt et al. 2008; Ludwig et al. 2008). And its receptor CXCR6 was reported to be expressed not only on immune cells, but also on bone marrow plasma cells, smooth muscle cells and different tumor cells (Shimaoka et al. 2004; Chandrasekar et al. 2004; Hase et al. 2006; Matloubian et al. 2000; Hattermann et al. 2008). In a previous study, it was found that the ligand CXCL16 was expressed in the normal human brain at capillary endothelial cells and abundantly in the human brain in malignant glioma cells and reactive astrocytes *in situ* and *in vitro* (Ludwig et al. 2005). Furthermore, CXCL16 has been detected in human cerebrospinal fluid of patients with inflammatory brain diseases (le Blanc et al. 2006). In human endothelial and glioma cells, CXCL16 is induced by IFN- γ and TNF- α and shed to its soluble form by the cell-surface proteases ADAM10 and ADAM17 (Abel et al. 2004; Ludwig et al. 2005). Nevertheless, nothing is known about the expression and role of CXCL16/CXCR6 in human meningiomas.

Our present study provides that a expression of CXCL16 and CXCR6 were identified in normal dura. Compared to normal dura, a down-regulated mRNA expression of CXCL16 was found in meningiomas of all different WHO grades. Statistical significant differences could be found in meningiomas grade I ($P<0.05$) and III ($P<0.01$). Similar results were found by IHC, though a statistical significant difference was only noted in grade III ($P<0.05$). However, we could draw the conclusion that a lower expression of CXCL16 mRNA and protein might be involved in tumorigenesis of high-grade meningiomas. A statistical significant difference of CXCR6 mRNA expression could be found only in meningiomas grade I compared to normal dura ($P<0.05$). With investigation by IHC, CXCR6 protein expression in meningiomas grade I, II, III and normal dura were found to be similar, and no statistical significant differences could be found between them. Meanwhile, CXCR6 expression was in general low in all investigated

samples. So we could not directly draw the conclusion that CXCR6 is involved in the development of meningiomas. Nevertheless, a positive correlation between the expression of CXCL16 and CXCR6 could be observed in all meningiomas samples ($P < 0.01$). This result further support the notion that CXCL16 and CXCR6 are associated with each other in human meningiomas. On the whole, these results support the hypothesis that the down-regulated expression of CXCL16 might associated with the pathogenesis of human anaplastic meningiomas.

The expression and functional role of CXCL16 and CXCR6 have been investigated in different types of tumor. CXCL16 and CXCR6 are always over-expressed in tumor cells, such as breast cancer tissues (Ludwig et al. 2005; Matsumura et al. 2008), prostate cancer tissues (Hu et al. 2008), colorectal cancer tissues (Hojo et al. 2007), schwannomas (Held-Feindt et al. 2008) and pancreatic ductal adenocarcinoma (Wente et al. 2008; Gaida et al. 2008). CXCL16 induces migration and invasion of glial precursor cells via its receptor CXCR6 (Hattermann et al. 2008). Furthermore, a tumor promoting role of the CXCL16-CXCR6 axis has been reported in schwannomas (Held-Feindt et al. 2008). 82.5% of pancreatic ductal adenocarcinoma patients displayed up-regulation of CXCL16 in sera. Additionally, CXCR6 interacted with trans-membrane CXCL16 to inhibit proliferation in pancreatic carcinoma *in vitro* and *in vivo* (Wente et al. 2008; Gaida et al. 2008). CXCR6 was also expressed in nasopharyngeal carcinoma (NPC) cell lines. For this, soluble CXCL16 attracted migration of NPC cells in a dose-dependent manner, and CXCR6 was detected in metastatic tumor tissues, but not in primary NPC tumors (Ou et al. 2006). High CXCL16 expression correlated with a good prognosis and increased levels of tumor-infiltrating lymphocytes (Hojo et al. 2007). In addition, an anti-migratory function of CXCL16 in renal cancer cells was demonstrated with migration assay. High CXCL16 expression in patients showed significantly longer overall survival times (Gutwein et al. 2009). In contrast, in human rectal cancer, downregulation of CXCL16 has been reported, but the correlation between CXCL16 expression and clinical outcome of the patients has not been analyzed (Wågsäter et al. 2004).

Recently, a number of studies have tested the antitumor effects of chemokines. The concept is that the expression of specific chemokines at the tumor site may attract T cells, NK cells, and dendritic cells bearing relevant chemokine receptors, which possibly leads to the induction of

antitumor immunity. Significant tumor suppressive activity was reported for chemokines such as CX3CL1 (also called Fractalkine) by transducing their genes into a variety of experimental tumors as we mentioned above. It remains to be tested whether CXCL16 has any strong antitumor effect in such experimental tumors.

We here provide the first evidence that the CXCL16 may play only a modest role in the pathogenesis of meningiomas. Further experiments have to be done to explore the function of CXCL16/CXCR6 axis in human meningiomas.

4.3 CX3CL1 and CXCL12 expression in normal peripheral nerves

As same as meningiomas, in relation to normal control samples, we decided to investigate the expression of chemokines and their receptors on mRNA and protein levels in human schwannomas and MPNST as well. Intriguingly, a significant difference of CX3CL1 mRNA expression could be found between 2 normal 8th cranial nerves (NCN) and 3 normal spinal nerves (NSN) at first. The same results also could be found for CXCL12 mRNA expression measured by real-time RT-PCR. So, we demonstrate at first, that a significant expression difference of CX3CL1 and CXCL12 between NCN and NSN exist, although the physiological role of these chemokines in normal peripheral nerves remains unknown. In view of this, we analyze the results in vestibular schwannomas (VS) and spinal schwannomas (SPS) separately, compared to NCN and NSN as normal controls, respectively.

4.4 CX3CL1 and CX3CR1 expression in schwannomas

One chemokine/receptor pair that has been gaining attention in recent years is CX3CL1 and CX3CR1. Several reports showed that CX3CL1 is abundantly expressed by the nervous system, and also is constitutively expressed by spinal neurons and tethered to the membrane of primary afferents, whereas its receptor, CX3CR1, is predominantly expressed by microglia (Lindia et al. 2005; Verge et al. 2004; Zhuang et al. 2007). However, little is known about the expression and function of CX3CL1 and CX3CR1 in human schwannomas and MPNST. In this study, we detected the expression of CX3CL1 and CX3CR1 in VS, SPS and MPNST on mRNA and protein level simultaneously for the first time. The VS is generally regarded as a benign tumor that originates from the Schwann cells of the vestibular part of the 8th cranial

nerve. So, we chose 2 NCN tissues (also included Schwann cells and parts of axons) as controls for VS. As a result, lower expression of CX3CL1 mRNA was found in VS compared to NCN with a statistical significant difference ($P<0.05$). Similar results were also found by IHC, though the difference was not significant. In contrast, higher expression of CX3CR1 mRNA and higher percentage of CX3CR1 positive cells were found in VS compared to NCN with statistical significant differences ($P<0.05$).

Then, we analyzed the CX3CL1/CX3CR1 expression in SPS and MPNST, 3 NSN samples served as normal controls. As same as VS, lower expression of CX3CL1 mRNA and lower percentages of CX3CL1 positive cells were found in SPS compared to NSN. The differences were statistical significant. Meanwhile, higher expression of CX3CR1 mRNA was obtained in SPS compared to NSN, although no statistical significant difference could be measured. Similar results were also obtained by IHC. To sum up the above results, we may draw the conclusion that lower expression of CX3CL1 and higher expression of CX3CR1 might be involved in the pathogenesis of human schwannomas (including VS and SPS). In regard to MPNST, the malignant form of schwannomas, we found that both the expression of CX3CL1/CX3CR1 mRNA and percentages of CX3CL1/CX3CR1 positive cells in MPNST were a little bit lower than those in NSN, but no statistical significant difference could be found. Moreover, we analyze the possible correlation between the expression levels of CX3CL1 and CX3CR1 in VS, SPS and MPNST, but no positive correlation could be observed. On the whole, these results support the hypothesis that a down-regulated expression of CX3CL1 and a up-regulated expression of CX3CR1 might be associated with the pathogenesis of human schwannomas.

As mentioned above, CX3CL1 acts as an adhesion molecule in its membranous form and behaves as a true chemokine in its soluble form. Pancreatic ductal adenocarcinoma cells bearing CX3CR1 specifically adhere to CX3CL1-expressing cells of neural origin and migrate in response to CX3CL1 produced by neurons and nerve fibers, contributing to perineural dissemination in pancreatic cancer (Marchesi et al. 2008). Prostate cancer cells that express CX3CR1 adhere to human bone marrow endothelial cells and migrate toward a medium conditioned by osteoblasts, which secrete the soluble form of the chemokine contributing to the high likelihood of prostate cancer cells metastasizing to the skeleton

(Jamieson et al. 2008; Shulby et al. 2004). CX3CL1 also was shown to have tumor suppressive activity, following CX3CL1 gene transfer into murine lung carcinoma, colon adenocarcinoma, lymphoma and hepatocellular carcinoma cell lines (Guo et al, 2003A; Guo et al, 2003B; Xin et al. 2005; Lavergne et al. 2003; Tang et al. 2007). The expression of CX3CR1 on NK cells and some T cell subpopulations has been the rationale to exploit CX3CL1 in cancer immunotherapy. The dual function of CX3CL1, as chemoattractant for leukocytes potentially displaying an antitumor response and as an adhesion molecule for tumor cells expressing the receptor may explain clinical discrepancies reported on the role of this chemokine/receptor pair in tumors. Further studies are needed to elucidate the role of CX3CL1/CX3CR1 in human schwannomas in more detail, also to provide the rationale to evaluate them as potential targets for therapeutics.

4.5 CXCL12 and CXCR4 expression in schwannomas

The last aim of this study was to describe the expression of CXCL12 and CXCR4 in human schwannomas on mRNA and protein levels. CXCL12 has been documented to be expressed by cells in several different tissues, including endothelial cells in the bone marrow (Ponomaryov et al. 2000), distal tubular cells of the kidney (Tögel et al. 2005), and dendritic cells in the skin (Pablos et al. 1999). Indeed, in contrast to many chemokines whose expression is strongly upregulated during inflammatory responses, both CXCL12 and CXCR4 are constitutively expressed at high levels in many tissues, including the developing and adult nervous systems (Stumm et al. 2002; Stumm et al. 2003). With respect to nervous systems, a recent report has shown that meningeal CXCL12 and its receptor CXCR4 are critically involved in two distinct aspects of precerebellar neuron migration: (1) confining the migrating cells to marginal streams, (2) and promoting the anterior migration of anterior extramural stream cells (Zhu et al, 2009). Expression of CXCR4 in the CNS was shown in a variety of cell types including astrocytes, microglia, cerebellar granule cells, and neurons (Bajetto et al. 2002). Recently, CXCR4 expression was also demonstrated on mouse oligodendrocyte precursor cells (Dziembowska et al. 2005). In addition, CXCL12 also has been found in glioma (Rempel et al. 2000; Zhou et al. 2002; Barbero et al. 2003), medulloblastoma (Rubin et al. 2003), lymphoma (Corcione et al. 2000), ovarian (Porcile et al. 2005) and pancreatic

cancer (Koshiba et al. 2000). As CXCR4 is expressed on several cancer cells, these CXCR4-positive cancer cells may metastasize to organs that secrete/express CXCL12. Regarding gliomas, recent data demonstrate that glioma tumor stem-like cells promote tumor angiogenesis and vasculogenesis via a CXCL12/CXCR4 pathway (Folkins et al. 2009; Hattermann K et al. 2010). In addition, CXCL12/CXCR4 have recently been shown to be expressed on primary central nervous system lymphomas, and a role for chemokines in the pathogenesis of primary central nervous system lymphomas has been suggested (Brunn et al. 2007; Smith et al. 2007; Fischer et al. 2009).

We evaluated CXCL12 and CXCR4 expression in schwannomas (using real-time RT-PCR, confirmed by IHC) in a series of surgical schwannomas specimens. As mentioned above, we chose 2 NCN tissues as controls for VS and 3 NSN tissues as controls for SPS and MPNST. The expression of CXCL12 was identified in all specimens, and its expression levels were relatively higher than other chemokines we have measured. Lower expression of CXCL12 mRNA and lower percentages of CXCL12 positive cells were found in SPS compared to NSN. The difference was statistical significant ($P<0.01$). On the contrary, higher expression of CXCL12 mRNA and higher percentages of CXCL12 positive cells were found in VS compared to NCN, but no statistical significant difference could be measured. As a matter of fact, the expression levels of CXCL12 in VS and SPS were similar. Thus, the significant difference of CXCL12 expression between NCN and NSN was the main reason due to this opposite results. So, to better understand the physiological role of CXCL12 in normal peripheral nerves (including cranial nerve and spinal nerve), further study will be necessary. However, as a result of this study, we could draw the conclusion that a lower expression of CXCL12 mRNA and protein might be involved in tumorigenesis of human spinal schwannomas. As same as CXCL12, CXCR4 mRNA expression in VS was slightly up-regulated compared to NCN. In accordance with this, CXCR4 protein expression in VS was significant higher than that in NCN and the difference was statistical significant ($P<0.05$). CXCR4 mRNA expression in SPS was a little bit lower than that in NSN, meanwhile, CXCR4 protein expression in SPS was a little bit higher than that in NSN. No statistical significant differences could be found. Furthermore, in MPNST, the expression levels of CXCL12 and CXCR4 were similar to those in NSN, with no statistical significant differences.

Moreover, we analyze the possible correlation between the expression levels of CXCL12 and CXCR4 in schwannomas (including VS and SPS) and MPNST. A positive correlation between the expression of CXCL12 and CXCR4 could be observed in schwannomas ($P < 0.05$), but not in MPNST. At least, this result indicated that CXCL12 and CXCR4 were co-expressed and associated with each other in human schwannomas at mRNA levels.

To our knowledge, this is the first report demonstrating the expression of CXCL12 and its receptor CXCR4 in schwannomas and MPNST, although CXCL12/CXCR4 was previously shown to be involved in the progression of different human tumors, including those developing in the brain (Salmaggi et al. 2004; Maderna et al. 2007; Oh et al. 2009; Balkwill. 2004). CXCL12 and CXCR4 have been detected in adult glioblastoma multiforme, where their expression was reported to increase with tumor grade (Rempel et al. 2000). Additionally, CXCR4 was found on glioma stem-like cells and was diminished upon differentiation. (Hattermann K et al. 2010). *In vitro*, CXCL12 acts as a growth factor for glioblastoma cell lines and normal astrocytes, increasing their proliferation (Bajetto et al. 2001; Barbero et al. 2003). It has been reported that CXCL12 stimulates chemotaxis, survival, and cell proliferation in glioblastoma multiforme, medulloblastoma cell lines and xenografted tumors (Bajetto et al. 2006; Rubin et al. 2003). In addition, CXCL12 and CXCR4 were shown to be involved in the proliferation of meningeal tumor cells, contributing to the biological features of this neoplasm, such as the ability to survive and to grow autonomously (Barbieri et al. 2006). It is also now clear that CXCR4 signaling regulates the migration and development of neural stem cells that form numerous structures in the brain and peripheral nervous systems (Belmadani et al. 2005; Li et al. 2008). Interestingly, CXCR4 signaling not only regulates the migration and proliferation of neural stem cells but also regulates the growth of axons once these cells start to develop into neurons (Lieberam et al. 2005; Pujol et al. 2005). Collectively, these studies indicate that CXCL12/CXCR4 may have different regulatory roles in several biologic processes.

Indeed, it has been proposed that CXCL12 or CXCR4 could be a potential target for therapeutic intervention. Numerous pharmacological agents exist that can modulate CXCL12/CXCR4-induced responses both *in vitro* and *in vivo* (Coscia et al. 2004). Inhibitors of CXCR4 are already available and currently under investigation in preclinical studies of

human cancers (Rubin et al. 2003). Antagonists of CXCR4 receptors such as the drug AMD3100 are used clinically to release hematopoietic stem cells into the circulation for transplantation purposes (Lemoli et al. 2008). A better understanding of the role of CXCL12 and CXCR4 in schwannomas will enable a greater manipulation of this important biological axis to affect the outcome of the disease.

5 Summary

Meningiomas and schwannomas are common tumors of the nervous system. Most patients with meningiomas or schwannomas have a good quality of life. However, reviews of large patient groups reveal that this optimistic view is not always satisfying. Accepted alternative therapies for patients who have failed surgical intervention are currently limited.

It is known from large clinical studies that chemokines and their receptors are involved in physiological and pathological processes of many human tumors. Limited data are available on the role of the chemokine/receptor axis in meningiomas and schwannomas.

In our study, for the first time, the mRNA and protein expression levels of three chemokine/receptor pairs (CX3CL1/CX3CR1, CXCL12/CXCR4 and CXCL16/CXCR6) were investigated in human meningiomas and schwannomas by real-time RT-PCR and immunohistochemistry. First, lower expressions of CX3CL1, CX3CR1 and CXCL16 were found in meningiomas compare to normal dura, especially in anaplastic variants. Lower CXCR6 mRNA expression could be found only in meningiomas grade I compared to normal dura. Additionally, lower expression of CX3CL1 and higher expression of CX3CR1 were found in human schwannomas than that in normal nerves. Lower expression of CXCL12 was found in spinal schwannomas compared to normal spinal nerves, with a statistical significant difference. Meanwhile, CXCR4 protein expression in vestibular schwannomas was significant higher than that in normal cranial nerves. Intriguingly, significant differences of CX3CL1 and CXCL12 mRNA expression could be found between 2 normal cranial nerves and 3 normal spinal nerves. Finally, correlation analysis showed significant positive correlation between the expression of CXCL16 and CXCR6 in all meningiomas. Meanwhile, a positive correlation between the expression of CX3CL1 and CX3CR1 could be observed only in meningiomas WHO grade I. In regard to schwannomas, a positive correlation between the expression of CXCL12 and CXCR4 could be observed. No positive correlation could be observed between the expression levels of CX3CL1 and CX3CR1 in all meningiomas and schwannomas.

Taken together these results showed that chemokines and their receptors are involved in the pathogenesis of human meningiomas and schwannomas. In this view, our results provide an interesting basis for further investigations that should be performed to characterize the roles of chemokines and their receptors in human meningiomas and schwannomas.

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7 Appendix

7.1 Appendix 1 The clinical data of patients

Case No.	Gender M / F	Age at diagnosis	Histology	Recurrence Yes / No	Detection
1	F	51	Meningothelial meningioma WHO I	N	Re, I
2	M	36	Fibroblastic meningioma WHO I	N	Re
3	F	65	Meningothelial meningioma WHO I	N	Re
4	F	45	Meningothelial meningioma WHO I	N	Re, I
5	F	71	Meningothelial meningioma WHO I	N	Re, I
6	F	54	Meningothelial meningioma WHO I	N	Re
7	F	79	Meningothelial meningioma WHO I	N	Re
8	F	65	Meningothelial meningioma WHO I	N	Re
9	M	59	Meningothelial meningioma WHO I	N	Re
10	F	73	Meningothelial meningioma WHO I	N	Re
11	M	56	Atypical meningioma WHO II	N	Re
12	M	57	Atypical meningioma WHO II	N	Re, I
13	F	59	Atypical meningioma WHO II	N	Re
14	F	63	Atypical meningioma WHO II	N	Re
15	M	81	Atypical meningioma WHO II	N	Re
16	F	57	Atypical meningioma WHO II	N	Re
17	F	59	Atypical meningioma WHO II	N	Re, I
18	F	49	Atypical meningioma WHO II	N	Re
19	F	69	Atypical meningioma WHO II	N	Re, I

20	M	50	Atypical meningioma WHO II	N	Re
21	F	67	Anaplastic (malignant) meningioma WHO III	N	Re
22	F	56	Anaplastic (malignant) meningioma WHO III	N	Re
23	M	46	Anaplastic (malignant) meningioma WHO III	N	Re, I
24	M	52	Anaplastic (malignant) meningioma WHO III	N	Re
25	F	60	Anaplastic (malignant) meningioma WHO III	N	Re, I
26	M	70	Anaplastic (malignant) meningioma WHO III	N	Re, I
27	M	78	Anaplastic (malignant) meningioma WHO III	Y	Re
28	M	70	Malignant peripheral nerve sheath tumor	N	Re, I
29	M	47	Malignant peripheral nerve sheath tumor	N	Re, I
30	F	58	Malignant peripheral nerve sheath tumor	N	Re
31	M	69	Malignant peripheral nerve sheath tumor	N	Re
32	M	71	Malignant peripheral nerve sheath tumor	N	Re
33	M	57	Malignant peripheral nerve sheath tumor	N	Re
34	M	33	Vestibular schwannoma	N	Re, I
35	F	43	Vestibular schwannoma	N	Re
36	M	57	Vestibular schwannoma	N	Re
37	M	71	Vestibular schwannoma	N	Re, I
38	F	46	Vestibular schwannoma	N	Re, I
39	F	54	Vestibular schwannoma	N	Re
40	M	73	Spinal schwannoma C5/6	N	Re
41	M	60	Spinal schwannoma T11/12	N	Re, I
42	M	36	Spinal schwannoma T5	N	Re, I
43	F	38	Spinal schwannoma T12/L1	N	Re, I
44	F	69	Spinal schwannoma L3	N	Re
45	F	60	Spinal schwannoma T6/7	N	Re

7.2 Appendix 2 Reagents, kits and instruments

RNA Isolation

Polytron-homogenizer (ULTRA-TURRAX[®] T25 basic, Germany)

Centrifuge (Eppendorf 5417R, Germany)

UV/Visible Spectrophotometer (Ultrospeco 3100 pro, Amersham Biosciences, USA)

TRIZOL[®] Reagent (Invitrogen[™] Life Technologies, Germany, No.15596-018)

Chloroform (Carl Roth GmbH, Germany, Lot. 6340.1)

Isopropyl alcohol (Merck kGaA, Germany, No.1.09634.2500)

75% ethanol (J. T. Baker, Holland, No.8006)

cDNA Synthesis

10×Reaction buffer (Promega, USA, No.M198A)

RNase free DNase (1u/μl, Promega, USA, No.M6101)

EDTA (Carl Roth GmbH, Germany, Lot. 80431.1)

Random hexamer primer (100μg/μl, Amersham Biosciences, USA, No.27-2166-01)

5×Buffer for M-MuLV RT (Fermentas, USA, No.00013612)

10mM dNTP mix (1.0mM-final concentration, Fermentas, USA, No.R0192)

RevertAid[™] H Minus M-MuLV Reverse Transcriptase (200u/μl, Fermentas, USA, No.EP0452)

Real-time RT-PCR

2×TaqMan Universal PCR Master Mix (Applied Biosystems, USA, No.4326708)

20×Assays-on-Demand[™] Gene Expression Assay Mix

GAPDH primer (Applied Biosystems, USA, No.Hs99999905)

CX3CL1 primer (Applied Biosystems, USA, No.Hs00171086)

CX3CR1 primer (Applied Biosystems, USA, No.Hs00365842)

CXCL16 primer (Applied Biosystems, USA, No.Hs00222859)

CXCR6 primer (Applied Biosystems, USA, No.Hs00174843)

CXCL12 primer (Applied Biosystems, USA, No.Hs00171022)

CXCR4 primer (Applied Biosystems, USA, No.Hs00237052)

MyiQ[™] Single Color Real-time PCR Detection System (BIO-RAD, USA)

Immunohistochemistry

Slicing machine (Jung CM3000, Leica Instruments, Nussloch, Germany)

Para-formaldehyde (Merck, Germany, Lot.1.04005.1000)

Triton X-100 (Merck, Germany, Lot.1.08603.1000)

Primary antibody

CX3CL1 (Mouse mAb, R&D Systems, No.MAB3651)

CX3CR1 (Rabbit pAb, Santa Cruz Biotechnology, Inc, No.Sc-30030)

CXCL16 (Goat Ab, R&D Systems, No.AF976)

CXCR6 (Mouse mAb, R&D Systems, No.MAB699)

CXCL12 (Rabbit pAb, Santa Cruz Biotechnology, Inc, No.Sc-28876)

CXCR4 (Rabbit pAb, Abcam, No.ab7199)

Second antibody

Biotinylated Horse anti Mouse IgG (Vector Laboratories, IHC. Burlingame, CA, No.BA-2000)

Biotinylated Rabbit anti Goat IgG (Vector Laboratories, IHC. Burlingame, CA, No.BA-5000)

Biotin-SP-conjugated AffiniPure F(ab')₂ Fragment Donkey Anti-Rabbit IgG (Jackson Immuno Reasearch Laboratories, IHC. No.711-066-152)

Normal blocking serum

Donkey (Jackson Immuno Reasearch Laboratories, IHC. No.017-000-121)

Horse (Jackson Immuno Reasearch Laboratories, IHC. No.008-000-121)

Rabbit (Jackson Immuno Reasearch Laboratories, IHC. No.011-000-120)

Normal Goat IgG (R&D Systems, No.AB-108-C)

Normal Mouse IgG (R&D Systems, No.MAB002)

Normal Rabbit IgG (R&D Systems, No.AB-105-C)

ABC Vectastain[®] Kit (Vector Laboratories INC, Burlingane, USA. No.PK-6100)

3,3'-diaminobenzidine-tetrahydrochloride (DAB) (Roche Diagnostics GmbH, Germany, No.11718096001)

Mayer's Hämalaun (Carl Roth GmbH, Germany, No.T865.2)

RotiClear (Carl Roth GmbH, Germany, No.A538.5)

RotiMount[®] (Carl Roth GmbH, Germany, Art.HP68.1)

Microscope (Axiovert 2000, Carl Zeiss, Germany)

Camera Zeiss (AxioCam MRc5, Carl Zeiss, Germany)

Software (AxioVs 40 V4.5.00, Carl Zeiss Imaging Solutions GmbH, Germany)

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