

Aus der Klinik für Neurochirurgie Kiel
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im Universitätsklinikum Schleswig-Holstein, Campus Kiel,
an der Christian-Albrechts-Universität zu Kiel

**The Expression and Significance of MGMT,
Ku80 and Ki67 Gene
in Different Grades of Spinal gliomas**

Inauguraldissertation
zur
Erlangung der Doktorwürde
der Medizinischen Fakultät
der Christian-Albrechts-Universität zu Kiel

vorgelegt von
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Kiel 2015

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Tag der mündlichen Prüfung: Kiel, den 13,04,2015

Zum Druck genehmigt, Kiel, den 10,04,2015

gez. : Prof.Dr. med. Andreas M.Stark

(Vorsitzender der Prüfungskommission)

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1. Introduction

1.1 Epidemiology, magnetic resonance imaging, pathological classification, treatment, prognostic immunophenotype and prognostic factors in spinal glioma

1.1.1 Epidemiology of spinal glioma

Primary tumors of the central nervous system (CNS) account for approximately 2-3% of all cancers. In Western countries, the annual incidence is approximately 15 patients per 100,000 populations and the prevalence has been estimated to approximately 69 patients per 100,000 populations (Ohgaki H, 2005). Primary CNS tumors comprise a heterogeneous group of benign and malignant neoplasms, the most common of which are tumors of glial cells, collectively referred to as gliomas. They are histologically classified according to the World Health Organization (WHO) classification of tumors of the nervous system. (Riemenschneider MJ, 2009). Gliomas are the most common primary human CNS tumors, that account for over 60% with 30-40% of them being glioblastoma multiforme (GBM), 10% being anaplastic astrocytoma (AA), and 10% being low grade gliomas (LGGs) (Ghosh A, 2004). But most CNS gliomas are brain glioma. Gliomas arising from the spinal cord are infrequent and comprise 5% of all primary central nervous system malignancies (Raco A, 2005; Santi M, 2003).

Spinal neoplasms in the adult population are mostly extradural (55%) and intradural extramedullary tumors (40%), whereas intramedullary tumors are rare (5%). In contrast, the rate of intramedullary tumors in children is higher with up to 35% of all spinal neoplasms. According to the literature, most spinal cord tumors in children are malignant gliomas, in more detail astrocytomas - in contrast to the adult population, where most spinal cord tumors are benign ependymoma (Stecco A, 2005; Costantini S, 1996; Koeller KK, 2000).

Spinal gliomas include astrocytomas and ependymomas. Spinal cord astrocytomas constitute approximately 1% of all primary central nervous system tumors, and 6 to 8% of all spinal cord tumors (Johnson DL, 1987). Few spinal cord astrocytomas are

anaplastic in nature; the majority of intramedullary spinal cord tumors are slow-growing low-grade ependymoma. Glioblastomas represent approximately 7.5% of all intramedullary gliomas and approximately 1.5% of all spinal cord tumors (Cohen AR, 1989). Unlike their intracranial counterpart, intramedullary glioblastomas have received scant attention in the literature, with fewer than 200 cases ever reported (Ciappetta P, 1991). Nonetheless, they are regarded as particularly threatening because of the densely packed essential fiber tracts and interneuronal networks within the cord's parenchyma.

The incidence of spinal glioma is increasing, but they are still regarded as rare tumors. The increase may be explained by improvement in diagnostic techniques especially due to the use of magnetic resonance imaging (MRI).

1.12 Magnetic Resonance Imaging characters of spinal glioma

MRI has become the best method for studying and identifying spinal cord neoplasm during the last 2 decades (Brotchi JF, 1999; Brotchi J, 1991).

Although MRI is not sufficiently specific to make a histologic diagnosis in glial tumors, typical imaging patterns can be identified for the more common tumor types. Ependymomas usually are enhanced brightly and homogeneously with contrast agents. They often have rostral and caudal cysts with a hemosiderin 'cap' at their poles, which is dark on T2-weighted images. On axial view, ependymomas are usually located in the center of the cord. Astrocytomas and gangliogliomas enhance less frequently, and if they do, enhancement is usually inhomogeneous. On axial views, they are eccentric and often asymmetrically enlarge the cord (Dillon WP, 1989; Patel U, 1998; Kothbauer KF, 2007).

1.13 Pathological classification of spinal glioma

Tumor resection and not only tumor biopsy is the standard in spinal glioma surgery to harvest tissue for histological diagnosis.

Surgery is indicated for any newly diagnosed intramedullary tumor in a child or in an adult for three reasons: first to receive the histological diagnosis, second to reduce the

cell load for further treatments, and third to decompress the cord in order to prevent short- and long-term neurologic dysfunctions (Kothbauer, 2007).

Spinal gliomas can occur throughout the entire spinal axis from medulla oblongata to sacral myelon. According to the latest World Health Organization (WHO) classification of tumors of the nervous system (Louis DN, 2007; Kleihues and Webster, 2000), four histological malignancy grades have been defined for spinal glioma that reach from benign tumors of WHO grade I to highly malignant tumors of WHO grade IV. Pilocytic astrocytoma, the most common glioma in childhood, is the prototype of a WHO grade I lesion. On the other end of the spectrum, WHO grade IV is assigned to glioblastoma, the most common and most malignant type of glioma, which preferentially manifests in adults with a peak incidence between 50 and 60 years. The histological diagnosis and grading is: (i) grade I (ependymoma I and pilocytic astrocytoma I), (ii) grade II (ependymoma II, ganglioglioma II and astrocytoma II), (iii) grade III (ependymoma III and astrocytoma III) and (iiii) grade IV (glioblastoma IV).

Pilocytic astrocytoma I or, less frequently, fibrillary astrocytomas I are the benign spinal tumors. The latter are, like in the brain, poorly demarcated from normal tissue. The pilocytic tumors frequently have large areas of good demarcation as well as cystic components and smaller areas of diffuse growth next to the normal cord tissue (Kothbauer, 2007).

Gangliogliomas are also low-grade tumors and occur primarily in children and young adults. Histologically, they are characterized by well-differentiated neuronal and astrocytic cells. The neurons have characteristic nuclear and nucleolar features, abundant cytoplasm, and argyrophilic neuritic processes. They express neuronal markers like synaptophysin and neurofilament proteins. Most frequently, intramedullary gangliogliomas grow slowly and thus have an indolent course (Lang FF, 1993).

Ependymomas are glial tumors derived from the primitive ependymal or subependymal cells of the cerebral ventricles and the central canal of the spinal cord (Fakhrai N, 2004). They constitute 5% of all neuroepithelial tumors. Concerning

malignancies in the pediatric age, ependymomas are the third most common tumor of the central nervous system, usually located intracranially with a high recurrence rate. In adults, however, more than 50% of all ependymomas are intraspinal and the frequency of recurrence is rare (Fakhrai N, 2004). According to the WHO, ependymomas are divided into 3 groups: (i) subependymomas and myxopapillary ependymomas, WHO grade I (indolent subtypes) (ii) atypical ependymomas, WHO grade II, and (iii) anaplastic tumors, WHO grade III (N. Fakhrai et al, 2004). Ependymomas are the most common intramedullary neoplasm in adults, while in children they account for only 12% of all intramedullary tumors. Ependymomas typically have a central location in the spinal cord. Most often they are well circumscribed and clearly delineated from the surrounding spinal cord tissue. Frequently, they have rostral and caudal capping cysts at the tumor poles. Practically all of them are histologically benign. Myxopapillary ependymomas are a subgroup of ependymomas with characteristic microcystic histologic features. They are typically located in the conus-cauda region (Schweitzer JS, 1992). They may grossly enlarge the filum and displace the nerve roots laterally and anteriorly. In spite of their benign histology, a small percentage of them tend to disseminate in the subarachnoid space. Spinal cord astrocytomas account for approximately 3% of all central nervous system astrocytomas. (Roonprapunt C, 2006). Few spinal cord astrocytomas are anaplastic (grade III) in nature; most are slow-growing lesions. Glioblastomas (grade IV) represent approximately 7.5% of all intramedullary gliomas and approximately 1.5% of all spinal cord tumors. In adults, spinal cord astrocytomas are typically low grade (WHO Grade I or II), with high-grade lesions accounting for 10 to 30% of the cases. Patients with WHO Grade III or IV lesions have a median survival of 10 months (Roonprapunt C, 2006; Santi M, 2003).

1.14 Prognostic immunophenotype

Gliomas are the most common primary human brain tumors. Over the past 20 years the cytogenetic and molecular genetic alterations associated with brain glioma formation and progression have been intensely studied. Genetic profiles in order to

define immunophenotypic profiles that better identify risk groups and prognostic assessment, as additional aids to the definition of brain tumors have been incorporated in the WHO classification. MGMT promoter hypermethylation in glioblastomas or detection of losses of chromosome arms 1p and 19q in oligodendroglial tumors have been identified as prognostic markers.

The most common chromosomal abnormality has been studied in diffuse astrocytomas of WHO grade II: trisomy 7 or at least a gain of 7q, which has been detected by comparative genomic hybridization in 50% of the cases (Nishizaki T, 1998; Schrock, E 1996). Very popular molecular alterations are mutations of the TP53 tumor suppressor gene at 17q13.1, which can be found in about 60% of cases as well as the just recently identified codon 132 mutations of the isocitrate dehydrogenase 1 (IDH1) gene in about 70% of diffuse astrocytomas (Balss J, 2008; Parsons DW, 2008). In addition, about 25% of anaplastic astrocytomas carry mutations in the retinoblastoma gene (RB1). In contrast to glioblastomas, allelic losses on 10q and mutation of the PTEN tumor suppressor gene on 10q23 are rare in anaplastic astrocytomas (<10% of cases) (Smith JS, 2000). Glioblastomas usually carry multiple chromosomal and genetic aberrations, for example, TP53 mutation is found in 30% of primary glioblastomas. In contrast, secondary glioblastomas arise from a lower-grade precursor lesion and carry TP53 and IDH1 mutations in more than two thirds of the cases (Ohgaki H, 2007; Parsons DW, 2008). Also epigenetic silencing of various genes has been described as overrepresented in either primary (NDRG2) or secondary (MGMT and EMP3) glioblastomas (Tepel M, 2007; Mueller W, 2008), and this epigenetic silencing of MGMT by means of promoter hypermethylation is present in about 40% of primary glioblastomas and has been identified as the main mechanism reducing MGMT expression and thereby diminishing its DNA repair activity.

Suri VS et al analyzed the expression of p53 and Ki67 and could show in 4 out of 11 cases of clear cell ependymomas in brain higher Ki67 indices as compared to classical grade II ependymomas, thus further highlighting the importance of differentiating the various subtypes (Suri VS, 2004). Benadiba M et al (2009) found that Ku80 expression, a critical protein involved in DNA repair as a heterodimer with Ku70, was

decreased by 71% after using Gamma-linolenic acid (GLA) in C6 glioma cells in vitro. It is indicated that reduced Ku80 is responsible for the increase in micronucleus formation in GLA-treated cells in a similar manner to that found in Ku80 null cells exposed to radiation. The decreased expression of Ku80 and E2F1 could make cells more susceptible to radiotherapy and chemotherapy (Benadiba M, 2009).

Integrative analysis of the different platforms will not only expand our knowledge of the molecular basis of brain gliomas but also pave the way to a molecularly refined brain glioma classification and facilitate the detection of “genes in context”, i.e. pathways of gliomagenesis that can be specifically targeted with individualized therapies (Riemenschneider MJ, 2009).

Although there have been many molecular and genetic research efforts on brain glioma for the past decades as mentioned above, similar studies for the spinal glioma are scarce. Richard A. R and Robert W (2007) have reported one case of a 28-year-old woman with a high-grade spinal cord astrocytoma. They treated the patient using temozolomide (TMZ) that led to functional recovery and regression of the residual tumor as demonstrated on serial magnetic resonance images, in the same time, genetic testing revealed that this tumor did not express the DNA repair enzyme MGMT. This is the first and only one case report in the literature correlating MGMT expression with the clinical response of a spinal glioma treated using TMZ until now . Another correlation molecular and genetic studies of spinal glioma was on selected candidate genes revealed frequent NF2 gene mutations in intramedullary spinal ependymomas, while deletions of the CDKN2A gene were frequent in intracranial supratentorial ependymomas but rare in ependymomas from spinal or other locations (Taylor MD, 2005). These immunophenotypes in spinal glioma on the other hand were seldom studied maybe due to its rare incidence. There is still uncertainty with regard to many proteins expressions in spinal glioma.

1.15 Prognostic factors in spinal glioma

Spinal gliomas are uncommon tumors; and malignant spinal cord gliomas grades III-IV (astrocytoma III, ependymoma III and glioblastoma IV) are even rarer. As such,

there is no consensus regarding the treatment of most glioma (Richard AR, 2007). Prognostic factors are important not only for discrimination of patients with spinal glioma into specific risk groups, for the identification and assessment of appropriate therapies and to predict survival, but also for comparing results of clinical trials. Since traditional prognostic markers in brain glioma, such as KPS (Karnofsky performance score), p53, PTEN (Phosphatase and Tensin homolog) (Umesh S, 2009), 1p/19q codeletion (Ducray F, 2009) and MGMT (Richard AR, 2007; Ducray F, 2009) have not been described before and are not applicable to spinal glioma several studies were initiated to search for new markers predicting survival and selecting an adequate therapy (Taylor MD, 2005; Richard AR, 2007).

Because spinal gliomas are rare tumors, it is difficult to identify prognostic factors. Prognostic pathobiological biomarkers in spinal glioma have been described clinically by Rudà R (Rudà R, 2008): If compared with intracranial ependymomas, spinal ependymomas are less frequent and exhibit a better prognosis. There is evidence that intracranial and spinal ependymomas share some similar molecular profiles with the radial glia of their corresponding locations. The majority of studies have shown a major impact of the extent of resection; thus, a complete resection must be achieved, whenever possible, at first surgery or at reoperation. Involved field radiotherapy is recommended for anaplastic or incompletely resected grade II tumors. Craniospinal irradiation is reserved for metastatic disease. Chemotherapy is not advocated as primary treatment, but utilized as salvage treatment for patients failing surgery and radiotherapy. The extent of resection and radiotherapy is considered as statistically significant prognostic factors for spinal ependymoma (Rudà R, 2008).

1.16 Treatment of spinal glioma

Because primary spinal cord gliomas are infrequent, there is no consensus regarding the management of malignant spinal cord glioma. Surgery, radiation therapy, and chemotherapy have been used in various combinations; however, the extent of resection and use of adjuvant chemotherapy or radiation therapy have not been clearly identified to be associated with a survival advantage (Jallo GI, 2003).

Before the advent of microsurgery and MRI, most surgeons and neurosurgeons were reluctant to attempt many spinal tumor surgeries due to the technical difficulties. Under these conditions, the neurological risk of resecting intramedullary neoplasms was considered unacceptably high, and this led to the development of a conservative treatment concept with biopsy, dural decompression and subsequent radiation therapy regardless of the histological diagnosis. Thus, the treatment strategy for intramedullary tumors gradually developed, and still continues to evolve, to an aggressive surgical concept. This biopsy or subtotal surgical resection only, involved field radiotherapy strategy for intramedullary tumor is administered even till nowadays in some developing areas. Since most intramedullary tumors are low-grade neoplasms, complete or even near-complete resection results in a good long-term progression-free survival with acceptable neurological morbidity (Constantini S et al, 2000). The use of MRI has dramatically improved the anatomical understanding of the morphological pathology; and the use of intraoperative neurophysiological monitoring has tremendously increased the understanding of the functional integrity of the spinal cord pathways during surgery and the process of neurological recovery thereafter. Nevertheless, differences of opinion still exist about patients suffering from an intramedullary tumor and their optimal individualized treatment. In spite of the advances in surgical treatment, the old concept of ‘biopsy and radiation’ is apparently not extincted (O’Sullivan C, 1994; Houten JK, 2000). Today a total or near-total resection should be attempted with surgery for all spinal gliomas for an optimal prognosis and to confirm the histological diagnosis. (Karl FK, 2007).

Despite this lack of consensus, surgery followed by radiation therapy, especially for incompletely resected lesions and recurrent tumors, is a common treatment paradigm. Several studies indicate that the use of postoperative radiation therapy modestly improves both local control and survival in spinal cord ependymomas and astrocytomas. Modern treatment planning and imaging allow more accurate target definition and respect for related normal tissue tolerances (Isaacson SR, 2000; Minehan KJ, 1995; Houten JK, 2000). But no study has convincingly demonstrated a beneficial effect of radiation therapy on survival or neurological function for

low-grade tumors so far.

With new drugs widely used for chemotherapeutic management of central nervous system tumors, some have been tried for intramedullary tumors. Only the higher-grade glial tumors have been treated on a larger scale with a combination treatment which includes chemotherapy (Allen JC, 1998). Only recurrent low-grade tumors have been treated with chemotherapy. Temozolomide demonstrated a modest efficacy with acceptable toxicity in a group of 22 patients (Chamberlain MC, 2008). Besides that, there is only little experience regarding the effect of chemotherapy on spinal gliomas (Chamoun RB, 2006). Given the recent advances in the chemotherapeutic treatment of cranial malignant gliomas (Stupp R, 2005) and with the guide of tumor molecular prognostic factors, a standard chemotherapy regimen could possibly be started before the time of tumor progression or with tumor relapse of spinal gliomas in the foreseeable future (Taylor, MD, 2005; Richard AR, 2007).

1.2 O⁶-Methylguanine DNA methyltransferase (MGMT)

1.2.1 MGMT protein molecular structure and function

The MGMT gene on chromosome band 10q26 encodes a DNA repair protein that removes alkyl groups from the O⁶ position of guanine, an important site of DNA alkylation (Gerson SL, 2004). It contains five exons and four introns (length > 170 kb). The promoter region is CpG-rich, lacks TATA and CAAT boxes and has ten Sp1 transcription factor binding sites and two glucocorticoid response elements (GREs). MGMT protein, as a DNA repair protein, is a small enzyme-like substance of 207 amino acids (MW of 23 kDa). In several ways, MGMT protects the cellular genome from the mutagenic effects of alkylating agents (Kaina C, 2007).

MGMT can contribute to alkylating agent resistance (Middlemas DS, 2000). It exerts its protective effect by removing cytotoxic chloroethyl and methyl adducts from the O⁶ position of guanine to an internal cysteine, yielding guanine and S-alkylcysteine (Pegg AE, 1995). The MGMT-mediated repair process is unique and differs from other DNA repair pathways because MGMT is not part of a repair complex but acts

alone. This repair mechanism mediated by MGMT involves the transfer of the alkyl group from the alkylation site of the DNA to an internal active site represented by a cysteine (Cys) residue in the amino acid sequence of the MGMT protein. Because the alkyl receptor site is not regenerated, the number of O6-alkylguanine adducts that can be removed from DNA *in vivo* is limited by the number of MGMT molecules and the rate of synthesis of the protein. There is evidence that MGMT limits the cytotoxicity of cyclophosphamide (Friedman HS, 1999). MGMT is ubiquitously expressed in normal human tissues, although at variable extent in selected tissues and on individual bases (Gerson T, 1986), but is overexpressed in all types of human tumors, including colon cancer, glioma, lung cancer, breast cancer, leukemia, lymphomas, and myeloma. MGMT activity of normal and neoplastic human brain specimens has been reported (Citron M, 1991). The values for normal brain and primary brain tumors are similar and vary up to 40-fold for normal tissue and 100-fold for tumors (Citron M, 1991). To date, there has been no paired comparison of the MGMT content of normal and neoplastic brain from the same patient. The level of MGMT expression is protean in various tumor tissues because of epigenetic inactivation of the MGMT gene. In particular, hypermethylated CpG islands in the MGMT promoter seem to be the most important mechanism for MGMT gene silencing and for the down-regulation of the expression. Several studies have reported transcriptional silencing of this gene in up to 50% (Shen K, 2005). MGMT silencing is also often observed in tumors in which a number of other genes are suppressed by methylation.

1.22 MGMT and chemosensitivity

MGMT is implicated in the removal of DNA alkyl adducts from the O6 position of guanine, one of the targets of alkylating drugs. Methylation of the MGMT promoter results in gene inactivation, thus potentially leading to increased sensitivity to treatment with alkylating agents. It has been observed that MGMT gene expression seems to be related to the methylation of the MGMT promoter, MGMT enzyme

activity, protein expression and cell resistance to anti-tumor alkylating agents by a series of experiments, which could predict a possible chemosensitivity. Therefore the MGMT methylation status is of therapeutic and prognostic interest (Kaina B, 2007). Failure of nitrosoureas against CNS tumors results from tumor cell resistance to DNA damage impacted by these drugs (Demetrius M, 1999). It has been found that those tumors with high MGMT activity and abundance of MGMT protein were resistant to alkylating chemotherapeutics, while those with low MGMT activity and little MGMT protein expression were sensitive. Hence, MGMT behaves as a predictor of response to chemotherapy and also may be a prognostic biomarker. Given MGMT is one of the most important factors determining drug resistance to alkylating therapy, strategies have been developed to inhibit MGMT expression in tumors with the aid of MGMT inhibitors, and further enhance the anti-neoplastic efficiency of alkylating agents.

1.23 MGMT and primary central nervous system tumor

Because brain gliomas are the most common primary central nervous system tumor, most studies assess the MGMT status in brain tumors focused on serial patients with glioma. The level of MGMT protein in cranial malignant glioma varies widely ranging from almost undetectable to very high level. Esteller et al investigated the relationship between MGMT promoter methylation and response to carmustine in 47 patients with gliomas. In this study, MGMT gene promoter methylation was associated with a better response to chemotherapy, greater overall survival and longer time to progression (Esteller, 2000). Several studies reported similar results recently (Nakasu, 2007; Brandes, 2008). The low MGMT protein expression was further identified as an independent favorable prognostic factor in terms of OS of brain glioma (Nagane, 2007). These results were consistent with the findings in the large phase III EORTC/NCIC trial conducted for patients with newly diagnosed GBM (Hegi, 2005).

Although there are many research efforts on MGMT with brain glioma, similar studies for the spinal glioma are rare. Richard et al have reported one case of a 28-year-old woman with a recurrent high-grade spinal cord astrocytoma (Richard AR,

2007), where the genetic testing revealed that this tumor did not express MGMT, so they treated the patient with temozolomide (TMZ), which led to functional recovery and regression of the residual tumor. This is the first and only case reported in the literature correlating MGMT expression with the clinical response of a spinal glioma treated with alkylating chemotherapy. The systemic status of MGMT in spinal glioma and its correlation to clinical outcome is still unknown.

1.3 Ku80 introduction

1.31 Ku80 protein molecular structure and function

Ku is a heterodimeric protein of 70 and 80 kDa polypeptides that also is abundant in nuclei of cultured human cells. Ku binds DNA ends and targets DNA-PKcs to DNA strand breaks (Moll U, 1999). The kinase is presumed to activate or regulate the components that repair Double-strand breaks (DSBs). Ku80 is derived from the XRCC5 gene localized to chromosome 2q33`q35 in human cells. Ku80 binds to DNA ends, nicks, gaps, and hairpins. In vitro, Ku forms a complex called DNA-dependent protein kinase (DNA-PK) by associating with a 450-kDa catalytic subunit and DNA-PKcs. So, DNA-PKcs and Ku80 expression was usually parallel. The highest levels were observed in spermatogenic cells, and in neurons and glial cells of the central and autonomic nervous system. Ku80, Ku70, DNA-PKcs, Xrcc4, and DNA ligase IV are critical for the repair of DNA ends by nonhomologous end joining (NHEJ). The Ku plays a key role in multiple nuclear processes, e.g., DNA repair, chromosome maintenance, transcription regulation and VJ recombination (Skorokhod OM, 2006). Although both Ku proteins and DNA-PKcs bind independently to the DNA ends, the greater part of this function is performed by the Ku70/Ku80 heterodimer, rather than DNA-PKcs itself (Hammarsten O, 1998).

Ionizing radiation directly produces DSBs, and it is believed that a single unrepaired DSBs will result in a broken chromosome and be responsible for cell death. Mice deleted for Ku70 or Ku80 exhibit hypersensitivity to γ -radiation, defective V(D)J recombination, genomic instability and early aging with low-cancer levels (Holcomb

VB, 2006). The similar phenotype suggests Ku70 and Ku80 function is restricted to the Ku heterodimer. However, there is reason to believe Ku70 or Ku80 may function independent of the Ku heterodimer. Each subunit enters the nucleus through a different nuclear localization signal (Lim JW, 2008) and Ku70 levels increase in response to γ -radiation without Ku80 (Brown KD, 2000). Li suggested p53-mutant fibroblasts are more sensitive to streptonigrin and paraquat when deleted for Ku80 as compared with Ku70. Thus, Ku80 may function outside the Ku heterodimer to influence DNA damage repair (Li H, 2009).

1.32 Ku80 and radiosensitivity

The integrity of a cell's DNA is of paramount importance for survival; therefore, all living cells have evolved mechanisms for repairing DNA lesions. DSBs are among the most critical lesions in chromosomal DNA are easy induced by ionizing radiation (Moll U, 1999). Cells mutated by the deletion of any of the previously mentioned genes are hypersensitive to ionizing radiation and defective in repairing DNA DSBs. The capability for DNA DSBs repair is crucial for inherent radiosensitivity of tumor and normal cells. The success of DSBs repair in tumor cells is the major cause for radiotherapy failure, leading to prolonged tumor cell survival. Thus, molecules that are involved in DSBs repair may be potential prognostic markers for the prediction of radiotherapy outcome, and hence, for optimization of treatment.

Although there are exceptions, it is supported by results from several clinical studies that Ku protein expression is correlated with radiation treatment outcome (Friesland KL, 2003; Harima S, 2003). Upregulation of the Ku80 protein following ionizing radiation exposure has been reported previously (Otomo H, 2004). Conversely, tumors with a low percentage of Ku80-positive cells tend to be radiosensitive.

1.33 Ku80 in CNS and glioma

Ku80, DNA-PKcs and Ku70 mRNAs were expressed in all normal tissues with relatively little variation in their levels. Many, but not all, cortical neurons in the gray matter and in the subcortical white matter stain strongly positive for DNA-PKcs and

Ku80. A significant subset of glial cells in the grey and white matter also stain strongly positive for DNA-PKcs and Ku80. Microglia were always negative for DNA-PKcs and Ku80. Parasympathetic ganglia, as well as myelin-producing Schwann cells of the autonomic nervous system have also been tested strongly positive for DNA-PKcs and Ku80 (Moll U, 1999).

Although most cancerous tissues were consistently positive for Ku80 (Moll U, 1999), radiotherapy is an important component of multimodal treatment for many malignant brain and spinal gliomas in clinic. But the research efforts on Ku80 with CNS glioma are rare. There are few studies performed with established cell lines about Ku80 expression in gliomas (Takashi O, 2004; Dhandapani KM, 2007; He F, 2007; Holcomb VB, 2006). Takashi O described 2 human glioblastoma cell lines, U87MG and A172 presenting a greater percentage of radioresistancy. The expression of DNA repair gene Ku80 in these cell lines could be found in a large percentage (Takashi O, 2004). Dhandapani KM studied glioma cell lines with the Ku80 inhibitor Curcumin, and investigated a potential therapeutic role in glioma. The results suggested that the expression of Ku80 might contribute to generally poor prognoses in human (T98G, U87MG, and T67) and rat (C6) glioma cell lines (Dhandapani KM, 2007). Besides Except studies performed with established glioblastoma cell lines, no other one is known about Ku80 expression in brain glioma, and so far, the expression and significance of Ku80 in spinal glioma has not been analyzed.

1.4 Ki67 introduction

1.41 Ki67 protein molecular structure and function

Ki67 is a large nuclear protein antigen and correlate with cell-cycle and proliferation. Its molecular weight is 345kd and 395kd. The Ki67 gene is located on chromosome 10q. The expression of Ki67 is mainly in the mid to late G1 phase, S phase and G2 phase of the cell-cycle, then the expression increases gradually and reaches the top in mitotic phase. It is an important structure for the maintenance of mitosis and it plays an important role in keeping the structure of DNA stable, it is a sensitive indicator of

cell proliferation (Sallinen P, 1994; Onda K, 1994).

The proliferation activity of tumours can be assessed by means of Ki-67 immunohistochemistry, which is now widely used in clinical pathology and neuropathological diagnostics. Burger and co-workers were the first to show the strong association between Ki-67 immunopositivity and glioma grade in freshly frozen samples of brain astrocytomas (Burger PC, 1986). Based on a large number of studies, the added prognostic information provided by Ki-67 immunostaining is now well established with regard to malignant tumors.

1.42 Ki67 and the proliferation

The latest WHO classification of central nervous system tumors (Louis DN, 2007) includes Ki-67 as an additional tool in histological typing and grading, although it cannot be regarded as being entirely prognostic in individual cases.

Haapasalo showed with immunostaining that Ki67 is useful as an adjunct of morphological diagnosis and grading of astrocytic tumors. Ki67 prognostic value is best expressed for optimal sensitivity and specificity with limit values of 7.5%, 10%, and 12.5 %, respectively. Using these limits a highly significant distinction was found in survival analysis (Haapasalo J, 2005).

More recently Ki67 have been widely used as markers to predict outcome in various malignancies. Ki67 is an established marker for proliferative index in cycling cells. Its presence in large proportion of cells suggests an aggressive neoplasm (Tsutsumi Y, 1995; Kammerer U, 2001; Sallinen P, 2000).

1.43 Ki67 in glioma

In a retrospective study on 70 ependymomas study treated between 1994 and 2001 Vaishali found that the expression of p53 and Ki 67 was correlated with the grade of tumor. The mean Ki -67 and p53 indices were significantly higher in grade III tumors as compared to grade I and II tumors. The difference in expression of Ki67 and p53 was highly significant between grade II and grade III tumors (*P value* < 0.0001). Four out of 11 cases of clear cell ependymomas showed higher Ki 67 indices as compared

to classical grade II ependymomas (Vaishali SS, 2004). Other studies looked at the expression of Ki67 in various grades of brain ependymomas and recurrent tumors. These authors confirmed that the Ki67 labelling index is a very important prognostic marker for brain ependymomas (Rushing EJ, 1981; Suzuki S, 2001; Versteegen MJ, 2002). Haapasalo et al studied Ki67 in different grades of astrocytic tumors and found that Ki67 revealed proliferating astrocytoma nuclei, which were scarce in low grade tumours and abundant in glioblastoma (Haapasalo A, 2005).

Despite many studies performed with established brain glioma showing the expression and significance of Ki67 in CNS tumor, there is, to our knowledge, no literature about Ki67 expression in spinal glioma so far. Maybe it is also due to the spinal glioma infrequency.

1.5 Questions and aims of the study

As mentioned above, spinal glioma, as one of the more uncommon CNS tumor, has not been given enough attention due its rare incidence. Until nowadays, there is no consensus regarding the management of spinal cord glioma. Surgery, radiation therapy, and chemotherapy have been used in various combinations; the treatment modalities of what extent of resection and how or when using of adjuvant chemotherapy or radiation therapy are not clearly associated with a survival advantage. The prognostic significance of some clinical factors as patient's age, tumor location, volume of resection, protocols of adjuvant therapy have been stated but other studies have yielded conflicting results. More precise molecular prognostic factors are important not only for division of patients with spinal glioma into specific risk groups for identification and assessment of appropriate therapies and to predict the survival, but also for comparing results of clinical trials. It is clear that the biological mechanisms involved in pathogenesis of spinal glioma are complex but deserve further study. Obviously, a better insight of its biology is crucial to choose the best treatment regimen and improve the prognosis. If it were possible to interfere with the chemosensitivity and radiosensitivity and reasonably block spinal tumors prone for

recurrence, an alternative therapeutic approach might be found.

Given that MGMT is one of the most important factors determining drug resistance, Ku80 determining radiosensitivity, Ki67 determining prone of relapse, the expression of MGMT, Ku80 and Ki67 in spinal glioma remains unclear. Herein, we postulate that the expression of the MGMT, Ku80 and Ki67 in spinal glioma is of interest for the therapy and its recurrence. It is for this reason that our study was designed to detect the expression of MGMT, Ku80 and Ki67 on spinal glioma by IHC staining to address the following questions: 1) to determine the expression level of Ku80 and MGMT for correlation with the clinical status of the patients. 2) Evaluate the relationship between MGMT and Ki67, Ku80 and Ki67 expression level and clinical outcomes. 3) Appraise the relationship between Ku80 expression level and radiotherapy, MGMT expression level and chemotherapy effects, Ki67 expression level and chemotherapy effects. 4) Judge the expression level of Ki67 for correlation with the histological grade or subgroups of patients. 5) Estimate the relationship between Ki67 expression level and recurrence of the tumor. Thus determine, if these immunophenotypes are prognostic factors in spinal glioma. They may be new markers for anticipating curative effects. There also might be a strategy for an optimal individualized treatment according their molecular prognostic factors.

2. Materials and methods

2.1 Patients and tissue samples

2.11 Patients and eligibility

We have collected spinal gliomas tissues and clinical data from 65 patients. Among them 39 patients were diagnosed and treated at the Department of Neurosurgery, University Medical Center of Schleswig-Holstein, Campus Kiel, Germany; 26 patients were diagnosed and treated in China, their tissues were kindly provided by Prof. Sien Zeng (Department of Pathology, affiliated hospital of Guilin Medical College, China), Xinwei Li (Department of Neurosurgery, 3th Hospital affiliated School of Medicine, Zhejiang University, China) and Yi Luo (Department of Neurosurgery, Liu Zhou hospital of Chinese Traditional Medicine, China).

Patients selected and excluded criterions for this study: (1) Histological characteristics of these cases fulfilled the criteria of the latest World Health Organization (WHO) classification of tumors of the nervous system (Louis DN, 2007). (2) Patients were required to have a life expectancy greater than 1 month, with possible detailed clinical data at diagnosis and therapy and during follow up. (3) Availability of adequate tissue specimens for histologic typing and immunohistochemistry. (4) Adequate hematologic, renal and hepatic function. (5) Patients who had a history of cerebral glioma then transfer to spinal were excluded from the study. (6) Patients previously treated by MGMT inhibitor (for example: *O*6-Benzyl-2'-deoxyguanosine (dBG)) or by chemotherapy with alkylating within 3 months prior to diagnosis were excluded. (7) Patients previously treated by Ki67 and Ku80 inhibitor (for example: Gamma-linolenic acid (GLA)) within 3 months or received radiotherapy within 1 year of diagnosis were excluded. Patients who died from postoperative complications were not included in the study.

2.12 Clinical data of patients

Patients treated from April 1992 to August 2009 were included in the study. Fifty-nine tumor tissues were newly diagnosed, and 6 were recurrent after prior

operation. All tissues were reviewed by neuropathologists affiliated with the Department of Neurosurgery, University Medical Center of Schleswig-Holstein, Campus Kiel, Germany. Tissues from China had additional H.E staining (stain nucleus with Hematoxylin and stain cytoplasm with Eosin) and dehydrate and were classified by a neuropathologist of the Department of Neurosurgery, UK S-H Campus Kiel according to the WHO histopathologic criteria. Clinical follow-up was obtained until October 27, 2009, or until death or loss of follow-up. 56 patients had undergone tumor total removed and 9 patients had undergone subtotal tumor resection. 18 patients have received alkylating chemotherapy, 16 patients received radiotherapy and 7 patients received chemotherapy in combination with radiotherapy. Except for the operation, the treatment of patients varied. There were no standard criteria for adjuvant treatment. This was mainly done depending on individual histological diagnosis, if the tumor was relapsing, the stage of the disease, the health condition and the individual hospital. All patients were followed up postoperatively by clinical examination and MRI scanning at least at 12 month intervals or when recurrence was suspected. Those patients with a complete remission or the stable disease were followed up by phone calls and were not always reexamined if being in good health.

2.13 Histological diagnosis and classification

Tumour diagnosis and grading were definitively established according to the WHO histopathologic criteria (Louis DN, 2007) and were systematically reviewed by an expert neuropathologist. These glial tumors encompass: (i) grade I (including Ependymoma I, Ganglioglioma I and Pilocytic astrocytoma I), (ii) grade II (including Ependymoma II, Ganglioglioma II and Astrocytoma II) ,(iii) grade III (including Ependymoma III and Astrocytoma III) and (iiii) grade IV (Glioblastoma IV). Distribution of the patients according to the WHO classification is presented in Table1. The spinal gliomas were located as follows: In 5 patients the tumors were located mainly in medulla oblongata, in 18 patients the tumors were located mainly in cervical myelon, in 22 patients the tumors were located mainly in thoracic myelon and in 20 patients located mainly in the lumbar myelon.

Tab 1. Demographic and pathological characteristics (N=65)

Histologic subtypes and grades	Patients number N=65	Patients (%)
Grade I	13	20.0
Grade II	35	53.5
Grade III	5	7.7
Grade IV	12	18.5

Grades I to IV: according to the World Health Organization

2.2 Immunohistochemistry (IHC)

2.21 Experimental instruments

Pressure cooker: Sicomatic-L, Germany

Electronic precision scale: A200S-*DI, SARTORIUS GMBH GOETTINGEN, Germany

Micropipettes and tips: EPPENDORF, Germany

Optical microscopy: OLYMPUS, BH-2, Japanese

2.22 Experimental reagents

(1) TBS (Tris-GerufferK Kodsaltzlg pH7.8)

Tri-sodium base	(Sigma T 1503)	0.9 g
Tris-HCL	(Sigma T 3253)	6.85g
NaCL	(Merck 1.06404)	8.78g
Distilled water		1000 ml

(2) EDTA-Puffer (TEC-Puffer pH7.8)

Tri-sodium base	(Sigma T 1503)	2.5 g
EDTA	(Merck 1.08418)	5.0 g
Tri-Sodium Citrate	(Merck 1.06448)	3.2g
Distilled water		1000 ml

(3) AEC Reagent: Sigma.

(4) Ku80,Ki67 Mouse Monoclonal Antibody: 1:200 LAB VISION CORP ORAT ION.

(5) MGMT Mouse Monoclonal Antibody: 1:60 THERMO SCIENTIFIC.

(6) Second antibody: Anti-Mouse and Rabbit Histofine: MEDAC, Germany.

- | | |
|-----------------------------|----------------|
| (7) Tris | Merck Germany. |
| (8) HCL | Merck Germany. |
| (9) NaCL | Merck Germany. |
| (10) Skimmed milk powder 5% | Roth Germany. |
| (11) Hematoxylin Solution | Merck Germany. |
| (12) 3% hydrogen peroxide | Merck Germany. |

2.23 Control samples

According to antibody data sheets, colon cancer tissue served as normal control samples for MGMT, normal tonsil tissues for Ku80, and Ki67. For positive and negative (omission of first antibody) control of the staining reaction, these sections were stained parallel to all cases in our study cohort. Each antigen has a preferred method of antigen retrieval, and each antibody was optimal diluted. Positive control experiments were performed to find optimal staining conditions before immunohistochemical stains could be proceeded.

First, we stained without antigen retrieval, and also used the heat-induced epitope retrieval with a pressure cooker. Antigen retrieval was tested in Tris/EDTA pH 6.0, 7.8 and 9.0 buffers. The antigen retrieval time was controlled 3-4 minutes for MGMT, Ku80 and Ki67 as soon as the cooker had reached full pressure. Antibody concentrations were diluted to 1:20, 1:40, 1:60, 1:80 for MGMT, to 1:100, 1:200 for Ku80, and 1:100, 1:200 for Ki67 as recommend on the data sheets. Finally a dilution of 1:60 was determined as the optimal dilution for MGMT, 1:200 for Ku80 and 1: 200 for Ki67.

2.24 Experimental process

The immunohistochemistry protocol was as follows.

2.241. Fixing and embedding the tissue

The spinal glioma tissue was formalin-fixed and paraffin-embedded.

2.242. Cutting and mounting the section

After fixation and paraffin-embedding, the probes are cut in a microtome to the desired thickness of 3 microns and mounted onto the slide.

2.243 Deparaffinizing and rehydrating the sections

The sections were dewaxed in xylene and rehydrated by passage through a graded ethanol series to distilled water. The step in details is performing the following washes in proper order with sections placed in a rack.

- 1). Xylene: 2 x 5 minutes
- 2). Xylene 1:1 with 100% ethanol: 2 x 5 minutes
- 3). 100% ethanol: 2 x 2 minutes
- 4). 96% ethanol: 2 x 2 minutes
- 5). 70 % ethanol: 2 minutes
- 6). Distilled water to rinse

2.244 Antigen retrieval

The appropriate antigen retrieval buffer is added into the pressure cooker and the slides transferred from the distilled water to the pressure cooker. When the cooker has reached full pressure, the slides remain there up to 3 minutes for Ku80 and Ki67 antigen retrieval and 10minutes for MGMT. Once de-pressurized, the slides are rinsed immediately with distilled water.

2.245 Immunohistochemical staining process

- 1). Slides are kept in distilled water for 1 minute.
- 2). Slides are kept in 70% ethanol for 1 minute.
- 3). 96% ethanol for 1 minute.
- 4). Endogenous peroxidases were blocked with 3% hydrogen peroxide in Tris-buffered saline (TBS) for 10 minutes.
- 5). 96% ethanol for 1 minute.
- 6). 70% ethanol for 1 minute.
- 7). Distilled water for 1 minute.
- 8). Blocking of nonspecific binding was accomplished in 5% skimmed milk for 10 minutes. (This step is only used for MGMT).
- 9). Slides were washed in distilled water for 1 minute. (This step is only used for MGMT).
- 10). Sections were washed 3 times in TBS for 2 minutes.
- 11). Slides were drained for a few seconds.

- 12). Primary antibody was diluted (1:60 for MGMT, 1:200 for Ku80 and 1:200 for Ki67) according to the manufacture protocol. 100 μ L of primary antibody fine-tuned was added.
- 13). Anti-MGMT mouse monoclonal antibody remained in room temperature overnight (18 hours), anti-Ku80 in room temperature for 30 minutes, and anti-Ki67 at 37°C for 40 minutes.
- 14). Slides were washed in distilled water for 2 minutes after incubation.
- 15). Sections were washed 3 times in TBS for 2 minutes.
- 16). Sections were incubated at room temperature for 30 minutes with 100 μ L of anti-mouse and rabbit histofine (Secondary antibody).
- 17). Sections were washed 3 times in distilled water for 5 minutes.
- 18). Slides were than washed 2 minutes in TBS 3 times.
- 19). Slides were incubated for 20 minutes at room temperature with 100 μ L AEC complex (2ml AEC +1 μ L 30% hydrogen peroxide), and then washed in distilled water for 1 minute
- 20). Sections were counterstained with hematoxylin for 5 minutes.
- 21). Slides were washed in running tap water for 5 minutes and then washed in distilled water for several seconds, mounted using one drop of aqua tex (Merck, Germany).

2.25 Viewing the staining under the microscope and results evaluation

Two independent experienced scientists (one a experienced neuropathologist) without prior knowledge of the patients' clinical outcomes investigated all histologic specimens. Each tumor was evaluated for these gene proteins and given a score for the percentage of immunoreactive positive cells density of all tumor cells. One thousand neoplastic cells per specimen were evaluated at x400 magnification and the ratio (%) of MGMT, Ku80, and Ki67 immunoreactive neoplastic cells were recorded. The final result is the sum of the percentage score. On the basis of the percentages of positive cells in the tumors, these tumors were defined as low MGMT expression, low

Ku80 expression when there are no or fewer than 50% positive cells, and high expression when positive rate was more than 50%. For Ki67, low expression when there are no or fewer than 10% positive cells, and high expression when positive rate was more than 10%.

2.3 Statistical analysis

Tumor complete resection was defined as the disappearance of all contrast enhancements in MRI or according the operation record. Tumor partial resection was defined as the tumor residual at contrast enhancements in MRI or according to the operation record. Tumor relapse also was defined as the enhance MRI in tumor size compared with the baseline or according patient's history. End points of the study were overall survival (OS) and progression-free survival (PFS). PFS was evaluated from the first day of treatment to relapse, progression or death, or to the last date of follow-up, and OS was calculated from the first day of treatment of the tumor to death for any reason or to the last date of follow-up. Patients who did not experience the event of interest with respect to OS or PFS were considered as censored observations with time from first diagnosis to last follow-up visit as the censoring time.

A descriptive study of all the variables included in the study was carried out. The absolute expression level of MGMT, Ku80 and Ki67 in all groups classified according to variables was analyzed by One-way ANOVA method. The quantitative variables were expressed in terms of their centralisation and dispersion measurements, and in some cases were categorized in accordance with their median value. Chi-square test was applied to estimate the relation between the expression of MGMT, and Ki67, Ku80 and Ki67, recurrence and patient characteristics, metastasis and patient characteristics. Correlation analysis was performed between the death/survival, chemotherapy, radiotherapy, recurrence, group, subgroup and the expression of MGMT, Ku80, Ki67. Kaplan–Meier methodology was applied in order to determine the effect of the different variables on survival and on PFS. Parameters possibly correlated with disease progression and survival were age, gender, tumor localization,

surgical type, and use of alkylating agents, radiotherapy, expressions of MGMT, Ku80 and Ki67 protein. These variables were estimated with their mean and 95% confidence interval. The end-point variable of interest was OS and PFS. The log-rank test was applied in order to verify the probabilities of accumulated survival and PFS in accordance with different strata of variables. A $p < 0.05$ value was considered to be of statistical significance. Analyses were performed with the use of SPSS 17.0.

3. Results

3.1 Patient characteristics and treatment

There are 65 patients with spinal glioma in this study. All data of these patients are lined in Table 2a and Table 2b.

Tab 2a. Primary clinical data of the patient characteristics

No	Se	Ag	Co	Diagn (grad)	Re	Met	Tu:Site	Op. Type	CT (Al)	RT	MGMT (%)	Ku80 (%)	Ki67 (%)	KPS	PFS (Mon)	OS (Mon)	S/D
1	M	64	G	GBMIV	no	no	MO- C2	TR	yes	yes	41.6	69.2	20	80	13	13	S
2	F	42	G	E II	no	no	L2	TR	no	no	88.2	66	7	80	135	135	S
3	M	42	G	E II	no	unk	C4-T4	SR	yes	no	3	55.8	7	unk	unk	unk	unk
4	F	58	G	E II	yes	no	C4-T2	TR	no	yes	18.4	77.2	2	50	1)10, 2)131	141	S
5	F	16	G	E II	yes	no	C2-5	TR	unk	unk	4.2	84	5	unk	1)9, 2)29	38	unk
6	M	38	G	E II	yes	no	L2	TR	no	yes	90.6	72	5	100	1)23, 2)31	54	S
7	M	9	G	E II	no	no	L1-3	TR	no	yes	6.4	74	10	90	54	54	S
8	F	42	G	E II	no	no	L1-2	TR	no	no	95.2	51	2	90	51	51	S
9	F	21	G	E I	yes	no	L2-3	TR	unk	unk	89	21	2	90	1)48,2)12,3)36	96	unk
10	F	38	G	E II	no	no	C3-5	TR	no	no	86.4	68.8	2	80	42	42	S
11	M	35	G	E II	no	no	C6	TR	no	no	94	71	1	70	9	9	S
12	F	46	G	E II	no	no	L2	TR	no	no	81	89.2	1	60	24	24	S
13	F	33	G	E II	no	no	T8-12	TR	unk	unk	94.4	78	1	70	9	9	unk
14	F	50	G	E II	no	no	L2	TR	no	no	18.2	2	2	80	10	10	S
15	F	48	G	E II	no	no	L1	TR	no	no	79	74	5	unk	3	3	unk
16	F	53	G	E I	no	no	C7-T1	TR	no	no	78.2	83.2	1	70	78	78	S
17	M	47	G	E I	no	no	L2	TR	no	no	16	82.8	1	90	73	73	S
18	F	62	G	E I	no	no	T4-L1	TR	no	no	51	68	2	100	72	72	S
19	M	49	G	E I	no	no	L1-2	TR	no	no	16	73	1	80	65	65	S
20	F	22	G	E I	yes	no	T12-L4	TR	unk	unk	24.4	81	1	unk	1)2,2)48,3)96	146	unk
21	M	22	G	E II	unk	no	L3	TR	no	no	51.2	73.6	5	unk	40	40	unk
22	F	30	G	E II	yes	unk	C4-6	TR	unk	unk	3	76	1	unk	1)47,2)9,3)20	76	unk
23	F	43	G	E I	no	no	L1	TR	no	no	98.6	48	1	unk	22	22	unk
24	F	57	G	E II	no	no	C6-7	TR	no	no	9	85.5	1	50	107	107	S
25	F	44	G	E I	no	no	T12-L12	TR	no	yes	94.2	89.2	1	100	100	100	S

26	M	57	G	E I	no	no	L1-2	TR	no	yes	2	67.6	1	70	92	92	S
27	F	27	G	E II	no	no	T6-9	TR	no	no	3	62.8	2	80	175	175	S
28	F	64	G	E II	no	no	C5-7	TR	no	no	28.4	32	0	90	175	175	S
29	F	53	G	E II	unk	unk	T12-L1	TR	no	no	35	84	3	0	174	174	D
30	F	36	G	E II	no	no	T12-L2	TR	unk	unk	90.6	30	1	unk	88	88	unk
31	F	34	G	E II	yes	no	L1-2	1)SR,2)TR	unk	unk	32.8	22.4	2	unk	unk	unk	unk
32	M	37	G	E II	no	no	T3-5	TR	unk	unk	22.2	34.8	1	unk	35	35	unk
33	M	61	G	E II	no	unk	T12-L1	TR	no	no	7	66	2	50	162	162	S
34	M	61	G	E II	no	no	C5-6	TR	unk	unk	8.8	41.6	1	unk	10	10	unk
35	F	62	G	E II	no	no	L1-2	SR	yes	no	90.4	93.2	1	60	10	10	S
36	F	69	G	A II	yes	no	T2	SR	no	no	38	38	1	80	1)4, 2)22	26	S
37	M	11	G	Gli II	no	no	C1-2	SR	yes	yes	75.2	40	5	60	31	31	S
38	F	30	G	GBMIV	yes	no	T7	TR	unk	unk	89.2	44	80	unk	1	1	unk
39	F	41	G	A III	no	no	MO- C1	TR	yes	yes	8	74	20	90	1	1	S
40	F	46	C	E II	no	no	C4-T1	TR	yes	no	76	42.4	1	50	102	102	S
41	F	43	C	E III	yes	no	T1-T6	TR	yes	yes	16	0	10	70	41	41	S
42	M	34	C	E I	no	no	T2-T7	TR	yes	no	30	48	1	100	62	62	S
43	F	21	C	A III	yes	yes	L1-2	TR	no	no	58	37.2	15.2	0	1)14, 2)26	40	D
44	F	36	C	E II	no	no	T11-T12	TR	yes	yes	52	84.4	0	60	94	94	S
45	F	27	C	E II	no	no	C5-T1	TR	yes	no	80	91.6	10	80	13	13	S
46	M	56	C	E II	no	no	C5-C7	TR	unk	unk	68	94.8	1	90	6	6	S
47	F	25	C	E II	no	no	MO-C4	TR	yes	no	24	86	2	50	17	17	S
48	M	5	C	A I	no	no	T7-L1	TR	no	no	33.2	84	6.4	50	15	15	S
49	M	45	C	E I	no	no	L1	TR	no	no	52	22	0	80	40	40	S
50	F	12	C	GBMIV	yes	no	C3-C7	TR	no	no	82	58	20.8	0	1)8, 2)5	13	D
51	M	40	C	GBMIV	yes	no	T1-T3	TR	yes	yes	0	94	17.2	0	1)45, 2)3	48	D
52	F	16	C	GBMIV	yes	yes	T11-T12	TR	no	no	0	70.4	6.8	0	4	5	D
53	F	27	C	E III	no	no	L1-L2	TR	yes	yes	72	72.2	9.8	100	22	22	S
54	F	54	C	E I	no	no	T12-L1	TR	no	no	12	97	12.2	70	27	27	S
55	F	42	C	E II	no	no	C4-6	TR	no	no	16	0	11	90	45	45	S
56	M	24	C	GBMIV	yes	no	C4-7	SR	no	no	62	0	2.4	0	7	20	D
57	F	52	C	GBMIV	yes	yes	C2-3	TR	yes	no	0	0	29	0	33	36	D
58	F	18	C	GBMIV	yes	yes	C3-C6	TR	no	no	0	0	29.2	0	8	14	D
59	M	35	C	GBMIV	yes	unk	T10-L2	SR	yes	yes	0	0	11.2	0	27	38	D
60	F	32	C	E I	no	no	T5-6	TR	unk	unk	68	6.4	1.6	unk	40	40	unk
61	F	38	C	MBMIV	yes	yes	MO-C4	SR	yes	yes	42	82.4	19.6	0	11	21	D
62	F	35	C	E II	yes	no	C4-8	TR	no	no	26.6	94.6	1	70	14	14	S
63	M	41	C	A III	no	no	T1-3	TR	yes	yes	36	73	22	100	95	95	S
64	M	43	C	GBMIV	yes	no	T5-L1	TR	1)y,2)n	1)y,2)n	66	74	8.2	0	1)108, 2)16	124	D
65	F	37	C	GBMIV	yes	unk	MO-C2	SR	no	no	63	80.4	9	0	4	8	D

Abbreviations: Se, Sex; Ag, Age; Co, country; Diagn, diagnosis; Re, relapse; Met, metastasis; Tu, tumor; Op, operation; TR, Total resection; SR, Subtotal resection; OS, overall survival; PFS, progress free survival; M, male; F, female; S, survival; D, death; G, Germany; C, China; CT,

chemotherapy; Al, Alkylating; RT, radiotherapy; RC, radiochemotherapy; NT, no treatment; MO, medulla oblongata; C, cervical myelon; T, thoracal myelon; L, lumbar spine; Grad, grade; E, ependymoma; A, astrocytoma; GBM, glioblastoma; Gli, Glioma; Mon, month; Unk, unknown; Special note: for example 1)48,2)12,3)36 in PFS: 2)12 mean the number of operation is the second operation and the month of progress free survival of this operation is 12 month.

Tab 2b. The constitute of patients from Germany and China

Country	Tumor types	Number(%)
Germany	E I	9 (23.1%)
	E II	25 (64.1%)
	E III	0
	A I	0
	A II	1 (2.6%)
	A III	1 (2.6%)
	G II	1 (2.6%)
	GBMIV	2 (5.1%)
Total		39(100%)
China	E I	4 (15.4%)
	E II	7 (26.9%)
	E III	2 (7.7%)
	A I	1 (3.9%)
	A II	0
	A III	2 (7.7%)
	G II	0
	GBMIV	10 (38.5%)
Total		26(100%)

There are 65 patients in our study, 39 patients recruited in Germany and 26 come from China. The group comprised 22 men and 43 women with a male/female ratio of 0.51; age ranged from 5 to 69 years (mean 39 years). The spinal gliomas were located as follows: In 5 patients the tumors were located mainly in medulla oblongata, in 18 patients the tumors were located mainly in cervical myelon, in 22 patients tumors were located mainly in thoracic myelon and 20 tumors were located mainly in lumbar/lumbosacral myelon.

All patients were diagnosed as spinal gliomas by a neuropathologist according to the World Health Organization (WHO) criteria. 9 patients had undergone subtotal resection at the time of initial diagnosis and 55 tumors were totally resected, 1 patient was subtotally resected at first operation and totally resected in a second operation. 18 patients were scheduled for alkylating chemotherapy, 16 patients were treated with radiotherapy and 11 patients underwent both radiotherapy and alkylating chemotherapy. No adjuvant therapy was applied in 30 patients (Tab 3).

Tab 3. Treatment protocols in 65 patients

Treatment	Radiotherapy	No radiotherapy	total
Chemotherapy	11 (20.8%)	7 (13.2%)	18
No chemotherapy	5 (9.4%)	30 (56.6%)	35
Total	16	37	53

For 12 patients a possible adjuvant therapy is unknown

One patient was treated by polychemotherapy with a CCNU and methotrexate (MTX) protocol: MTX was given by intrathecal application, a TMZ protocol was used in 3 patients; a MeCCNU protocol was accepted in 2 patients; 8 patients were chosen for CCNU chemotherapy and in 4 patients the correct chemotherapy regimen could not be identified. In total 18 patients received chemotherapy including alkylating agents.

16 patients underwent fractionated spinal radiotherapy with 30-54 Gy (1.8-2 Gy/day). Among them, 2 recurrent patients received radiotherapy after the second operation. 37 patients had no radiotherapy. 53 patients were followed up in detail; there are still 12 patients which were lost in follow up. The time of follow up varied from 1 month to 175 months, mean 54.6 months. It was terminated until to death for any reason or to the last date of follow-up. The overall survival (OS) from 1 month to 175 months, mean 54.6 months, and PFS varied from 1 month to 145 months, mean 46.1 months. There was only one serious complication during the radiotherapy and chemotherapy with one female patient, who suffered radiation myelitis followed by paraplegia rapidly 1.5 months after radiotherapy.

Tab 4. Expressions of MGMT, Ku80, and Ki67 analyzed by One-way ANOVA method

Variables	MGMT (mean±SD)	MGMT 95% CI	pvalue	KU80(mean±SD)	KU8095%	pvalue	ki67 (mean±SD)	Ki67 95% CI	pvalue
Overall	44.6±33.6	36.3-56.9		59.0±29.3	51.8-66.3		7.2±11.7	4.3-10.0	
Sex(n)									
M(n=23)	34.7±28.8	22.3-47.2		60.5±26.7	49.0-72.1		6.2±6.7	3.3-9.1	
F (n=42)	49.9±35.0	39.0-60.7	0.065	58.2±31.0	48.6-67.8	0.761	7.7±13.7	3.4-11.9	0.632
Age									
< 50(n=48)	48.8±34.4	38.9-58.7		57.9±29.0	49.6-66.2		7.9±12.6	4.3-11.5	
≥ 50(n=17)	31.6±28.0	16.7-46.5	0.05	62.5±31.1	9-79.0	0.594	4.9±8.3	0.5-9.3	0.390
Tumor site									
MO(n=5)	35.7±20.8	9.9-61.5		78.4±6.7	70.3-86.7		14.4±11.4	3.9-24.4	
C(n=18)	40.6±37.5	21.9-59.2		53.5±34.1	36.6-70.5		11.4±19.6	1.6-21.1	
T(n=22)	39.7±32.3	25.5-54.0		58.1±30.3	44.6-71.5		5.3 ±6.0	2.6-7.9	
L(n=20)	55.7±33.0	40.2-71.2	0.358	60.2±26.5	47.8-72.6	0.420	3.7±4.0	1.8-5.6	0.089
Surg. Type									
TR(n=56)	44.5±34.2	35.3-53.6		61.2±28.1	53.6-68.7		7.3±12.4	3.9-10.6	
SR (n=9)	45.2±30.8	21.5-68.8	0.955	45.8±34.9	19.0-72.6	0.146	6.7±6.1	1.8-11.2	0.85
Diagnosis									
Grade:									
Gral(n=13)	42.2±33.8	28.8-69.7		60.8 ±29.3	43.1-78.5		2.4±3.3	3.9-4.4 (1,3)	0.000
Grall(n=35)	46.3±34.4	34.5-58.1		63.4 ±25.8	54.5-72.2		2.9±3.0	1.9-4.0 (1,4)	0.009
Gralll(n=5)	38.1±27.1	4.3-71.7		15.4±5.6	8.4-22.3		15.4±5.6	8.5-22.4(2,3)	0.007
Grallv(n=12)	37.2±35.4	14.7-59.6	0.779	47.7±37.3	24.0-71.4	0.407	21.1±20.4	8.2-34.1(2,4)	0.000
							Total sig:		0.000
Group:									
Ependymoma and astrocytoma (glioblastoma):									
E (n=47)	46.9±34.5	36.7-57.0		62.1±27.8	54.0-70.2		3.0±3.3	2.0-4.0	

A (n=18)	36.8±31.2	23.1-54.1	0.377	51.0±32.6	34.8-67.2	0.176	17.9±17.7	9.2-26.7	0.000
Subgroup:									
E(I,II,n=46)	46.8±34.4	36.6-57.0		62.7±26.7	54.8-70.6		2.7±3.0	1.8-3.6 (1,2)	0.002
E(III,IV,n=2)	44.0±40.0	-311.7-399.7		36.1±51.1	-422.6-49.48		9.9±0.1	8.6-11.2 (1,3)	0.168
A(I,II,n=2)	54.2±29.7	-212.6-52.9		62.0±31.1	-217.5-341.5		5.7±1.0	3.2-14.6 (1,4)	0.002
A(III,IV,n=15)	36.5 ±32.8	36.3-52.9	0.745	50.4±34.5	51.8-66.3	0.361	20.7±4.7	10.6-30.7 (2,3)	0.099
								(2,4)	0.426
								(3,4)	0.274
							Total sig:		0.000
Chemotherapy(A)									
Yes(n=18)	39.6±31.3	24.0-55.2		60.0±32.1	44.1-76.0		10.8±8.7	6.5-15.1	
No(n=35)	43.0±33.7	30.9-55.2	0.452	60.1±27.9	50.5-69.7	0.994	7.1±14.2	2.3-12.0	0.235
Radiotherapy									
Yes(n=16)	39.4±31.7	22.5±56.5		58.2±31.7	41.3-75.1		15.7±18.4	5.9-25.5	
No(n=37)	46.7±34.5	35.2±58.2	0.471	60.9±28.3	51.4-73.3	0.758	5.2±7.4	2.8-7.7	0.004
Both CT and RT									
Yes(n=11)	37.2±28.0	18.4±56.0		60.3±32.7	38.8-82.2		13.0±7.2	8.2-17.8	
No(n=30)	47.5±34.3	34.6-60.3	0.379	61.2±27.6	50.9-71.5	0.931	4.7±6.7	2.2-7.2	0.001
Metastases									
Yes(n=5)	20.0±28.0	-14.7-54.7		38.0±38.4	-9.7-85.9		20.0±9.5	8.1-31.8	
No(n=54)	49.3±33.3	40.2-58.4	0.063	61.3±27.8	53.7-68.9	0.088	6.3±11.8	3.0±9.5	0.015
Unk(n=6)									
Relapse									
Yes(n=23)	35.1±33.1	20.8-49.5		50.5±34.0	35.8-65.2		12.4±17.1	5.0-19.8	
No(n=40)	50.8±33.5	40.1-61.5	0.078	63.9±25.7	55.7-72.1	0.111	4.4±5.8	2.5-6.2	0.038
Unk(n=2)									
KPS									
KPS ≥80(n=23)	48.9±32.5	34.8-62.9		58.5±27.2	46.8-70.3		5.8±6.9	2.8-8.8	
KPS <80(n=29)	41.3±33.7	28.5-54.1	0.417	62.8±32.9	50.3-75.3	0.620	7.6±8.5	4.4-10.8	0.410
Unk(n=13)									
PFS									
PFS ≥60Mon(n=18)	43.3±32.2	27.3-59.3		67.3±18.2	58.3-76.4	(1,2) 0.08	3.1±5.2	0.5-5.7	
PFS <60Mon(n=17)	42.3±35.9	23.9-60.8		40.5 ±33.7	23.2-57.8	(1,3) 0.900	7.3±7.8	3.3-11.3	
PFS <24Mon(n=28)	48.7±34.1	35.5-61.9	0.792	66.4±28.1	55.5-77.3	(2,3) 0.013	9.8±15.8	3.7-16.0	0.166
Unk(n=2)							Total sig:		0.005
D/S									
S(n=38)	45.5±32.3	34.9-56.2		65.4±26.5	56.7-74.1		4.7±5.9	2.8-6.6	
D(n=12)	34.0±32.2	13.6-54.4	0.286	48.3±38.4	23.9-72.8	0.089	14.3±9.1	8.5-20.1	0.004
Unk(n=15)									
MGMT expression									
Low(n=35)	16.6±13.6	11.9-21.3		57.5±32.0	46.5-68.5		7.5±8.6	4.6-10.4	
High(n=30)	77.2±15.0	71.6-82.8	0.000	60.8±26.3	51.0-70.6	0.658	6.7±14.6	1.3-12.2	0.793
Ku80 expression									
Low(n=22)	44.0±32.9	29.5-58.6		23.2±18.6	14.9-31.4		9.4±17.9	1.5-17.4	

Hight(n=43)	44.8±34.3	34.3-55.4	0.930	77.4±10.9	74.0-80.7	0.000	6.0±6.5	4.0-8.0	0.390
Ki67 expression									
Low(n=49)	49.2±33.1	39.7-58.7		62.1±25.6	54.7-69.4		2.6±2.5	1.9-3.3	
Hight(n=16)	30.5 ±31.8	13.5±47.4	0.05	49.7±38.1	29.4-70.0	0.237	21.1±17.0	12.1-30.1	0.001

Abbreviations: M, male; F, female; MO, medulla oblongata; C, cervical myelon; T, thoracic myelon; L, lumbar myelon; D, died; S, survived ; Grad, grade; E,ependymoma;A, astrocytoma, CT, chemotherapy; RT, radiotherapy; RC, radio-chemotherapy; Mon, month; Unk, unknown; CI, confidence interval; SD, standard deviation; Surg, surgery; PFS, progression free survival;KPS, Karnofsky

3.2 MGMT expression in spinal glioma

The level of MGMT expression was determined by MGMT immunohistochemistry in 65 spinal glioma tissues. The absolute expression levels of MGMT in all groups classified according to variables were analyzed by One-way ANOVA method and listed in Table 4. There is a considerable variability in MGMT expression level ranging between 0% and 98.6% in spinal glioma (mean 44.56%).

We found that there was not statistically significant difference of the expression of MGMT in spinal gliomas (table4), that included sex, tumor site, surgical type, tumor grade and treatment protocol. But, for the variable age, we could find a significant difference of the expression of MGMT between patients of age < 50 years subgroup and the age ≥ 50 years subgroup (P=0.05). Some similar results were obtained from variables of metastases group (P=0.063), relapse group (P=0.078).

35 tumor samples (54%) demonstrated a low MGMT staining (positive rate < 50%, mean 16.6%) (Fig 1-4), and 30 (46%) tumor samples demonstrated intermediate and high MGMT expression (positive rate ≥ 50%, mean 77.2%)(Fig 5,6). The MGMT expression level in spinal glioma (N = 65) was neither correlated with age of the patient nor with diagnosis. (There was not significant difference in MGMT expression between different gender (P=0.179) and histological diagnosis (P=0.475). The differences in MGMT expression between patients less than 50 years and older than 50 years reached statistical significance (P=0.044). This revealed that the MGMT level in age ≥ 50 years patients is usually lower than in patients whose age is less than 50 years.

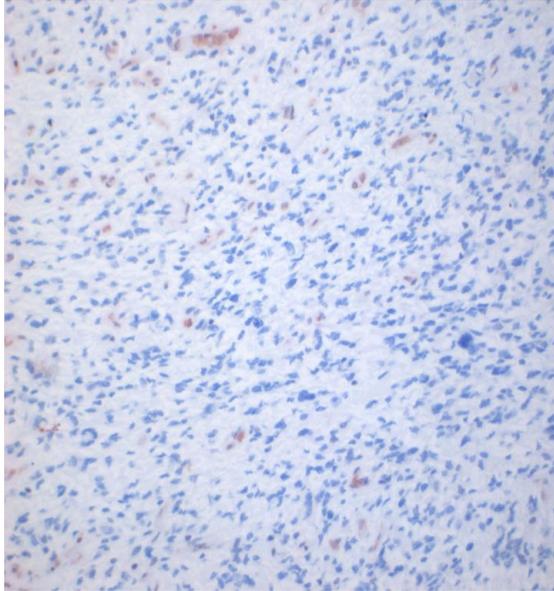


Fig 1 .MGMT staining $\times 200$ (0%)

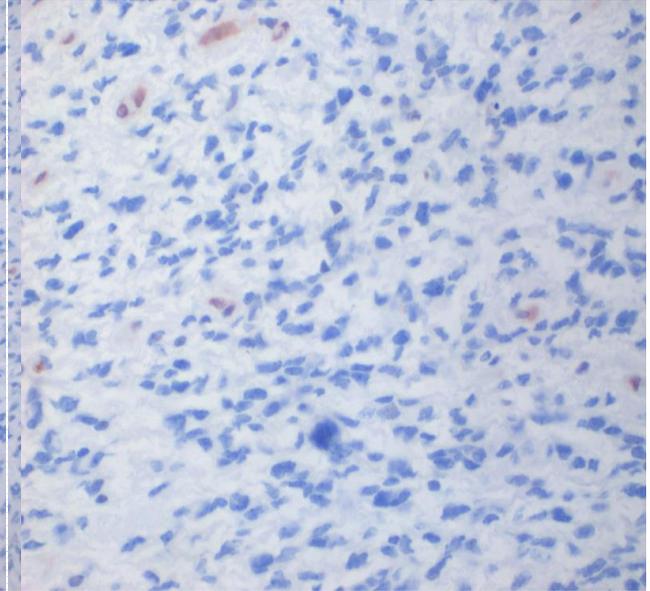


Fig 2 . MGMT staining $\times 400$ (0%)

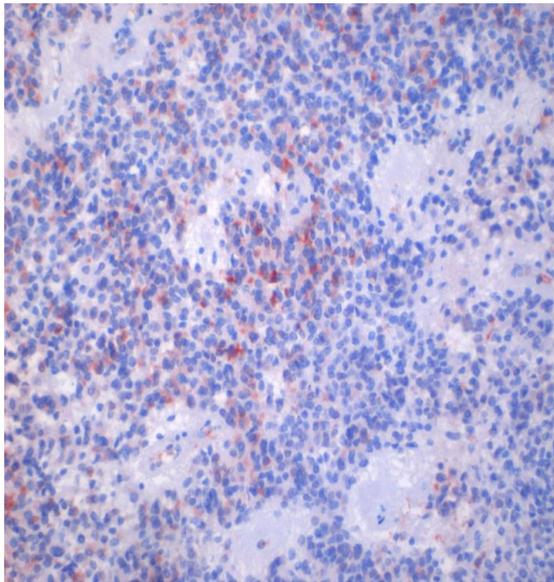


Fig 3 . MGMT staining $\times 200$ (3.0%)

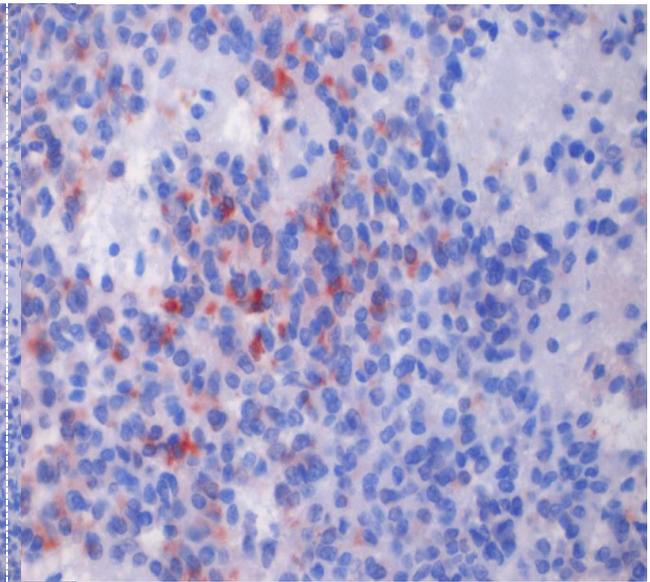


Fig 4 . MGMT staining $\times 400$ (3.0%)

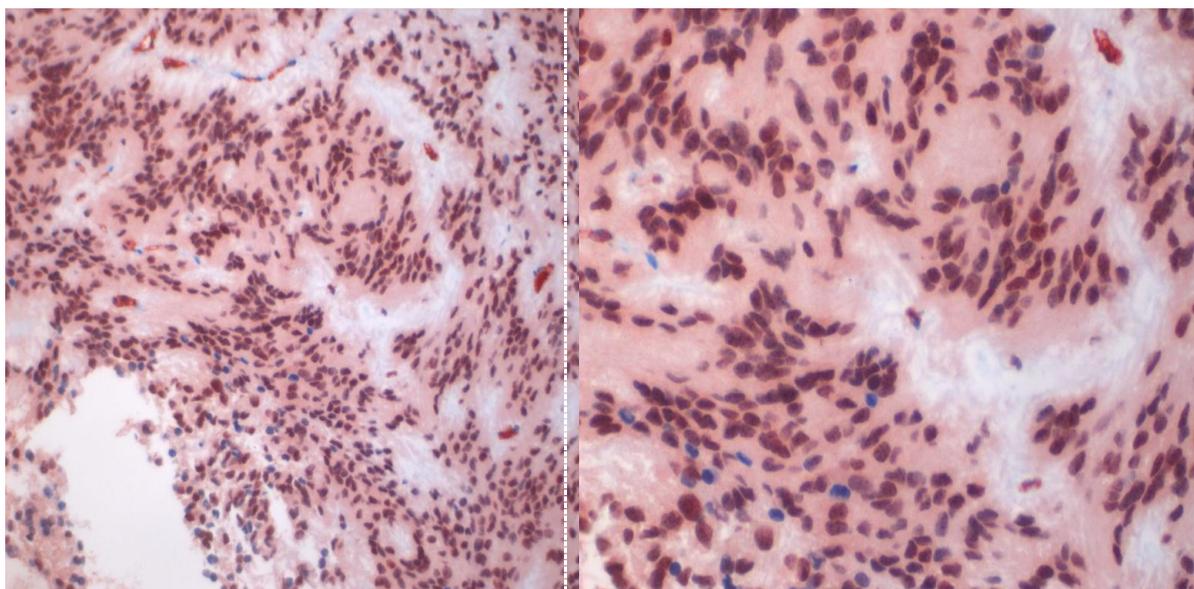


Fig 5. MGMT staining ×200 (90.6%)

Fig 6. MGMT staining ×400 (90.6%)

Fig. 1~6 Representative photomicrographs showing immunostaining for MGMT in spinal glioma samples. Fig 1, 2 (glioblastoma IV) Immunohistochemical staining demonstrates the negative expression of MGMT in the spinal glioma tissue, and low cytoplasmic staining (3.0%) can be observed in Fig 3,4 (ependymoma II), and high cytoplasmic staining (90.6%) can be observed in Fig 5, 6 (ependymoma II).

Tab 5. Baseline patient characteristics and MGMT expression

Study, n (%)	MGMT (weak)	MGMT (strong)	p value
Sex			
Male(n=23)	15 (23.1%)	8 (12.3%)	0.179
Female (n=42)	20 (30.8%)	22 (33.9%)	
Age			
< 50 years(n=48)	23 (35.4%)	25 (38.5%)	0.044
≥ 50 years(n=17)	12 (18.5%)	5 (7.75%)	
Diagnosis			
Ependymoma(n=47)	24 (36.9%)	23 (35.4%)	0.475
Astrocytoma(GBM) (n=18)	11 (16.9%)	7 (10.8%)	

3.3 Ku80 expression in spinal glioma

The level of Ku80 expression was determined by Ku80 immunohistochemistry in 65 spinal glioma tissues. The absolute expression levels of Ku80 in all groups classified according to variables were analyzed by One-way ANOVA method and listed in Table 4. There is a considerable variability in expression level ranging between 0% and 97.0% in spinal glioma (mean 59.2%). There are no statistical significant differences of the Ku80 expression in spinal gliomas among most of variables analyzed including sex, age, tumor site, surgical type, tumor grade and treatment protocol. For the PFS group, we could find a statistical significant difference of the expression of Ku80 between patients in $PFS < 60$ months subgroup and in $PFS \geq 60$ months subgroup ($P=0.005$). Otherwise, there is significant difference in the expression of Ku80 between patients that suffered metastases and those who were free of metastases ($P=0.088$), although it did not reach statistical significance. Similar results were found in the death/survival group ($P=0.089$).

The results of the Ku80 expression for the age, gender, diagnosis subgroups are presented in Table 6. 22 tumor samples (33.9%) demonstrated low Ku80 staining (positive rate $< 50\%$, mean $23.2\% \pm 18.6\%$) (Fig 7-10), and 43 (69.1%) tumor samples demonstrated high Ku80 expression (positive rate $\geq 50\%$, mean $77.4\% \pm 10.9\%$) (Fig 11-14).

The Ku80 expression level in spinal gliomas ($N = 65$) could not be correlated with gender, age and diagnosis of the patient. A significant difference in Ku80 expression could not be found between gender ($P=0.673$), age ($P=0.804$) and histological subgroups ($P=0.271$)(Tab 6).

Tab 6. Baseline patient characteristics and Ku80 expression

Study, n (%)	Ku80 (low)	Ku80 (high)	p value
Sex			
Male(n=23)	7 (10.8%)	16 (24.6%)	0.673
Female (n=42)	15 (23.1%)	27 (41.5%)	
Age			
< 50 years(n=48)	17 (26.2%)	31 (47.7%)	0.804
≥ 50 years(n=17)	5 (7.7%)	12 (18.5%)	
Diagnosis			
Ependymoma(n=47)	8 (12.3%)	39 (60.0%)	0.271
Astrocytoma(GBM) (n=18)	14 (21.5%)	4 (6.23%)	

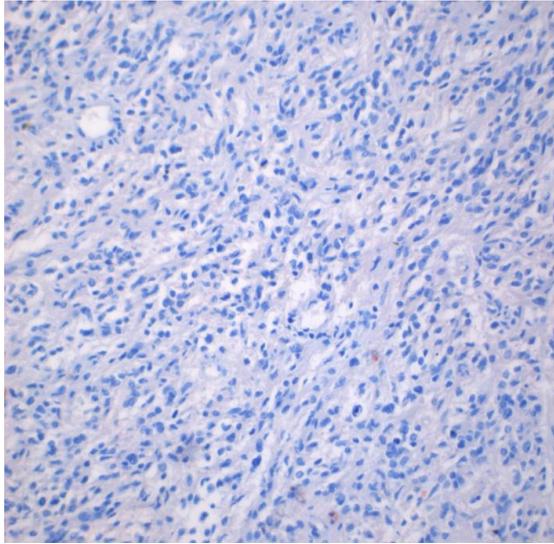


Fig 7. Ku80 staining ×200 (0%)

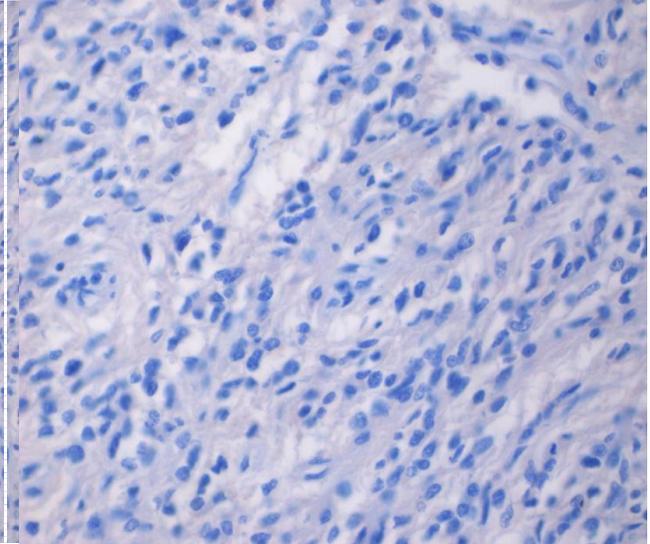


Fig 8. Ku80 staining ×400 (0%)

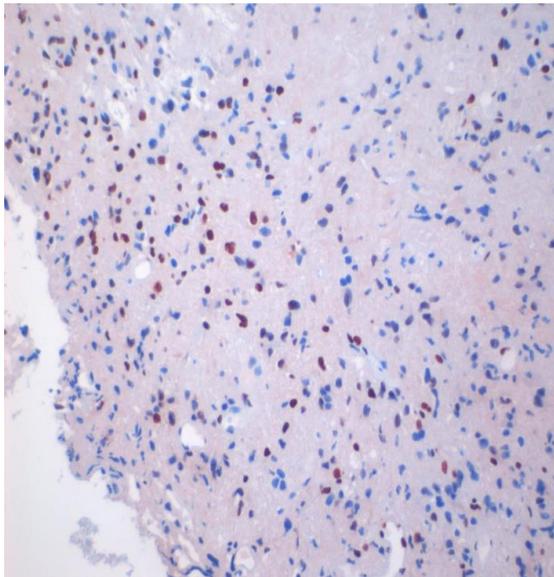


Fig 9. Ku80 staining ×200 (37.2%)

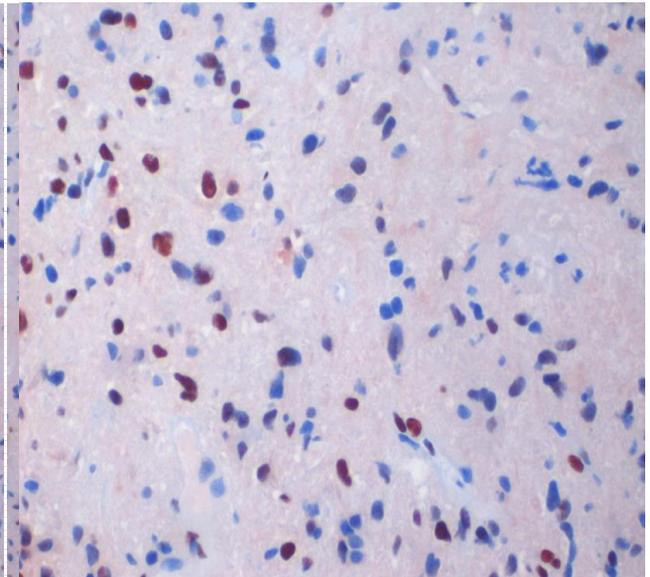


Fig 10. Ku80 staining ×400 (37.2%)

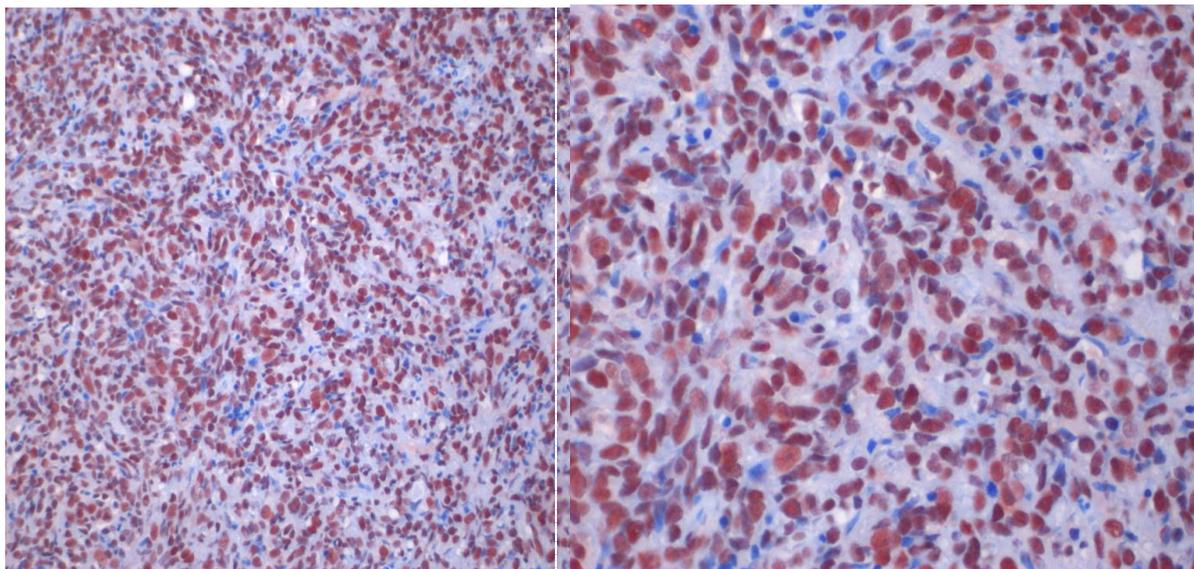


Fig 11. Ku80 staining ×200 (73.0%)

Fi12. Ku80 staining ×400 (73.0%)

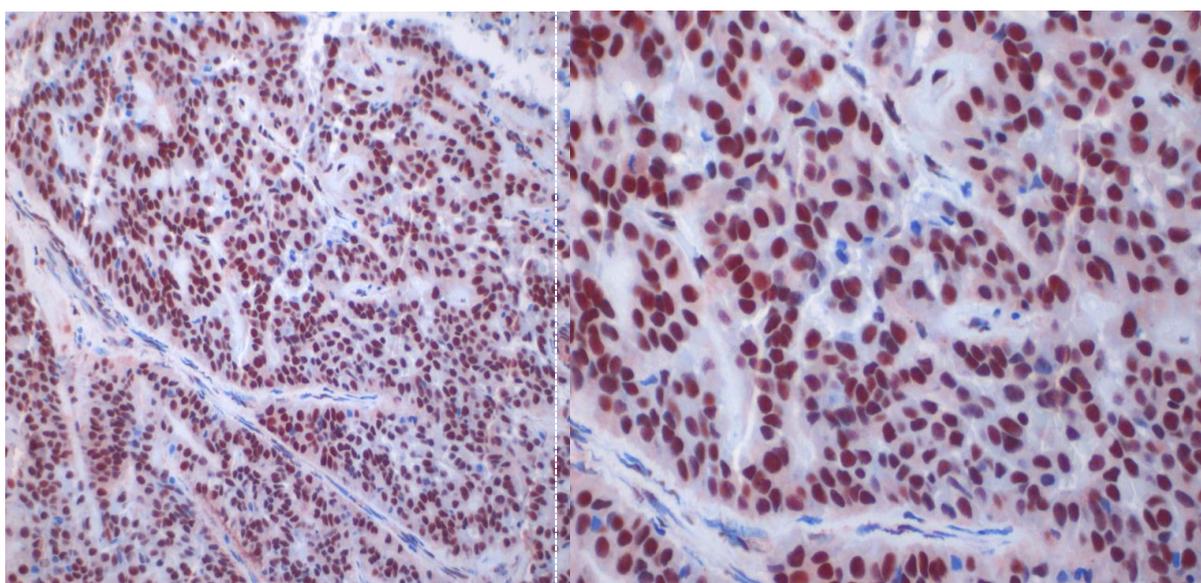


Fig 13. Ku80 staining ×200 (91.6%)

Fig.14. Ku80 staining ×400 (91.6%)

Fig. 7~14 Representative photomicrographs showing immunostaining for Ku80 in spinal glioma samples. Fig 7, 8 (ependymoma III) Immunohistochemical staining demonstrating the negative expression of Ku80 in the spinal glioma tissue. Low nuclear staining (37.2%) could be observed in Fig 9, 10 (astrocytoma III). Strong nuclear staining (73.0%) could be observed in Fig 11,12 (astrocytoma III). Very strong nuclear staining (91.6%) could be observed in Fig 13,14 (ependymoma II).

3.4 Ki67 expression in spinal glioma

The Ki67 expression was determined by Ki67 immunohistochemistry in 65 spinal glioma tissues. A considerable variability in Ki67 expression level was found ranging between 0% and 80.0% in spinal gliomas (mean 7.15%). The absolute expression level of Ki67 in all groups classified according to variables were analyzed by One-way ANOVA method and listed in Table 4. There is statistical significant difference of the expression of Ki67 between patients in death/survival (D/S) group ($P=0.004$). Similar results were found for the relapse group ($P=0.038$), the metastases group ($P=0.015$), the group receive both or not radiotherapy and chemotherapy ($P=0.001$), radiotherapy group ($P=0.004$), histological diagnosis (including grades, ependymoma and astrocytoma) groups ($P=0.000$).

There are not statistically significant differences for the expression of Ki67 in spinal gliomas, for the sex, age, tumor site, surgical type (Table 4).

The results about Ki67 expression of age, gender, diagnosis are presented in Table 7. 49 tumor samples (75%) demonstrated low Ki67 staining (positive rate $< 10\%$, mean $2.6\% \pm 2.5\%$) (Fig 15, 16), and 16 (25%) tumor samples demonstrated high Ki67 expression (positive rate $\geq 10\%$, mean $21.1\% \pm 17.0\%$) (Fig 17-20). The Ki67 expression level in spinal glioma ($N = 65$) could not be correlated with gender and age of patients. A significant difference in Ki67 expression could not be found between gender ($P=0.842$) and age ($P=0.538$). But the differences in Ki67 expression between the histological diagnoses (ependymoma and astrocytoma (including GBM,)) reached statistical significance ($P=0.001$): The Ki67 level in patients with ependymoma is usually lower than in patients who suffered astrocytoma (including GBM).

Tab 7. Baseline patient characteristics and Ki67 expression

Study, n (%)	Ki67 (low)	Ki67 (high)	p value
Sex			
Male(n=23)	17 (26.2%)	6 (9.2%)	0.842
Female (n=42)	32 (49.2%)	10 (15.4%)	
Age			
< 50 years(n=48)	36 (55.4%)	12 (18.5%)	0.538
≥ 50 years(n=17)	13 (20.0%)	4 (6.2%)	
Diagnosis			
Ependymoma(n=47)	42 (64.6%)	5 (7.7%)	0.001
Astrocytoma(GBM) (n=18)	7 (21.5%)	11 (16.9%)	

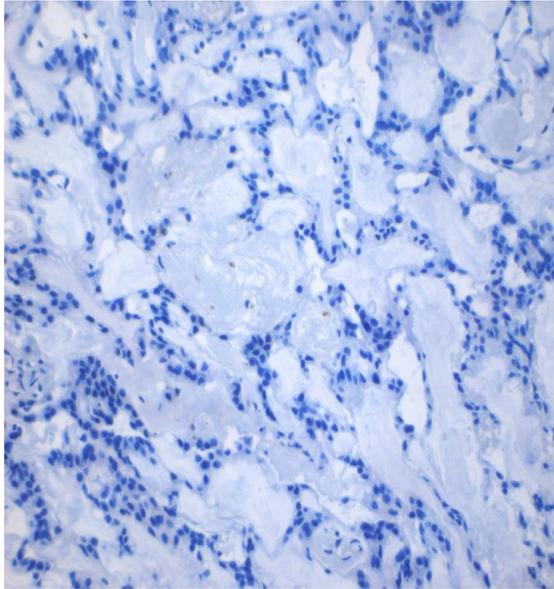


Fig 15. Ki67 staining $\times 200$ (0%)

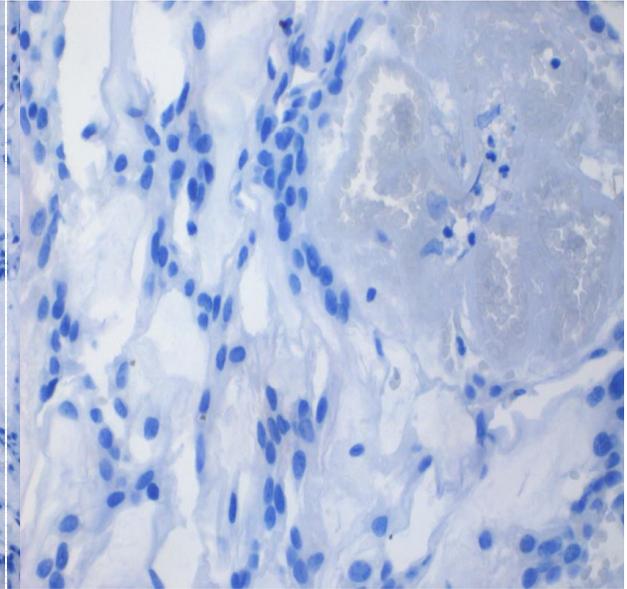


Fig 16. Ki67 staining $\times 400$ (0%)

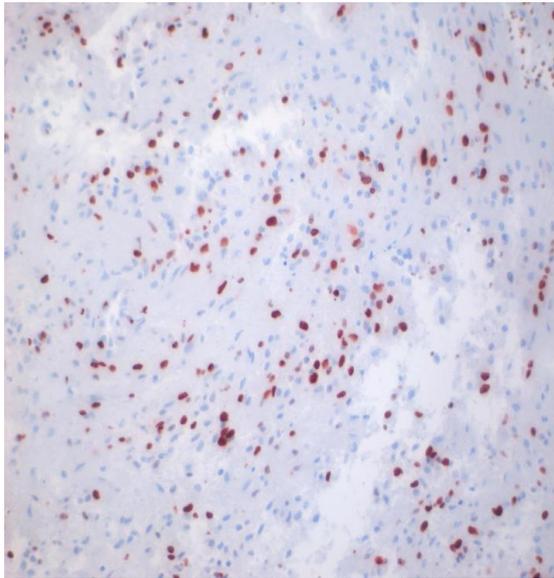


Fig 17. Ki67 staining $\times 200$ (29.0%)

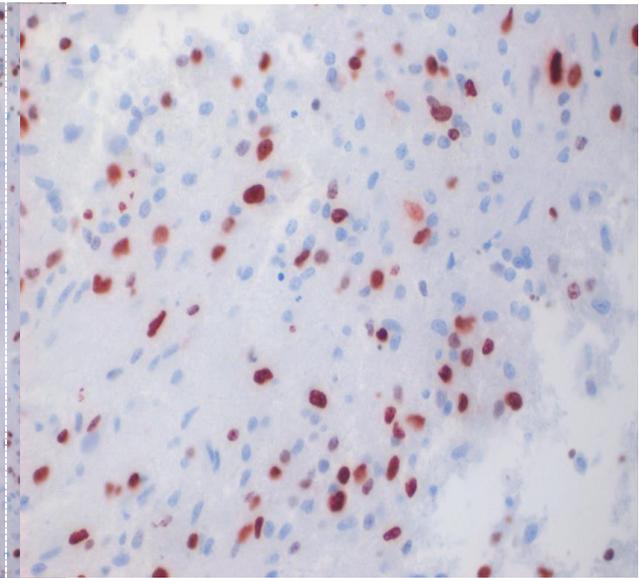


Fig 18. Ki67 staining $\times 400$ (29.0%)

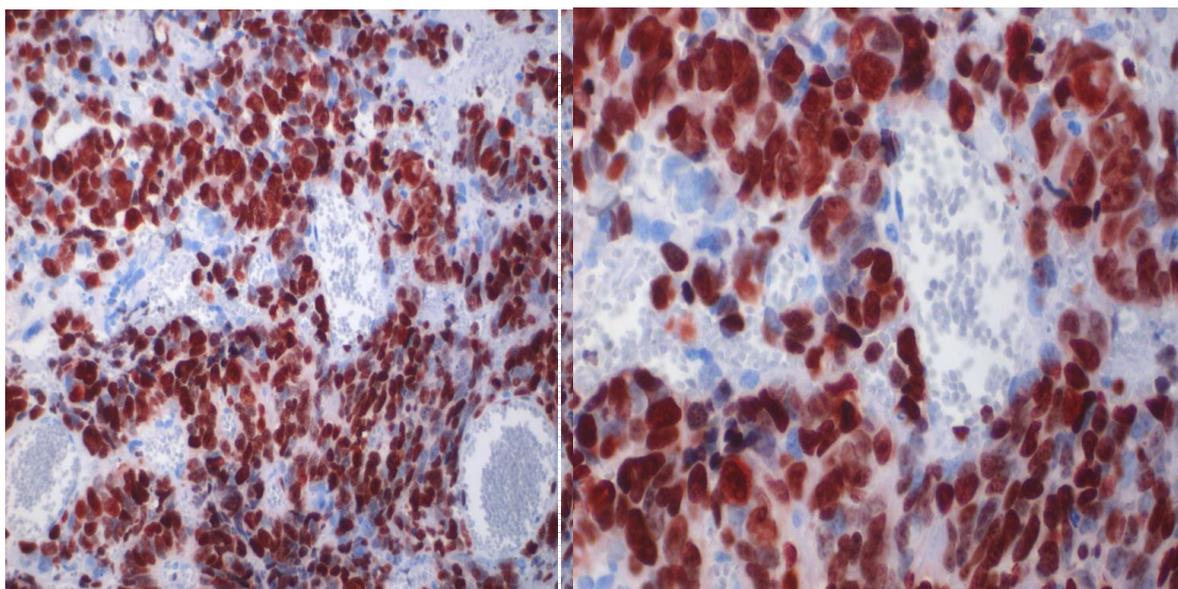


Fig 19. Ki67 staining ×200 (80.0%)

Fig 20. Ki67 staining ×400 (80.0%)

Fig. 15~20 Representative photomicrographs showing immunostaining for Ki67 in spinal glioma samples. Fig 15, 16 (ependymoma I) Immunohistochemical staining demonstrating the negative expression of Ki67 in the spinal glioma tissue. High nuclear staining (29.0%) could be observed in Fig 17, 18 (glioblastoma IV). A very high nuclear staining (80.0%) could be observed in Fig 19, 20 (gliblastoma IV).

3.5 Relevance analysis between MGMT expression and Ki67 expression

Strong positivity of MGMT staining was observed in 30 (46%) of 65 spinal glioma cases and strong Ki67 expression was found in 16 (25%) patients. Among 65 cases that were interpretable for high expression each proteins, only 4 cases (6%) showed high expression for both MGMT and Ki67, 26 cases (40.0%) showed high expression only for MGMT, 12 cases (19%) showed high expression only for Ki67, and 23 cases (35%) showed low expression for both proteins (Tab 8).

Tab. 8 Correlation between MGMT expression and Ki67 expression

Protein	Ki67 (low)	Ki67 (high)	Total
MGMT (low)	23 (35.4%)	12 (18.5%)	35
MGMT (high)	26 (40.0%)	4 (6.2%)	30
Total	49	16	65

Sig: 0.853

Ki67 high expression could be statistically significantly correlated with tumor recurrence ($P=0.038$), tumor grade ($P=0.000$), D/S ($P=0.004$), metastases ($P=0.015$) (Tab 4). We could find that patients have a strong trend toward that poor prognosis in MGMT low expression but Ki67 high expression, as compared with MGMT high expression and Ki67 low expression cases (Tab 4, Tab 8). Ki67 expression was statistical significant correlated with OS ($r=-0.283$, $P=0.024$), tumor recurrence ($r=-0.242$, $P=0.038$), tumor grade ($r=-0.415$, $P=0.001$) and tumor subgroups ($r=0.569$, $P=0.000$) (Tab 9). There is also a statistically significant difference observed between levels in MGMT expression and Ki67 expression ($P=0.05$, Tab 4; $r=-0.24$, $P=0.05$, Tab9). There were only 5 patients with a negative MGMT expression and a very high Ki67 expression in our study group (MGMT: 0%). All of them were spinal glioblastomas (gradeIV). We could find that MGMT and Ki67 have significant negative correlation. Litter co-expression of MGMT and Ki67 exists in patients with spinal glioma (6.2%, Tab 8).

Tab. 9 The correlations among variables

		PFS	OS	D/S	CT	RT	Ki67	MGMT	Ku80	RE	G	SG
PFS	P.C(2-tailed)	1	.857**	-.255*	0.036	0.041	-0.237	-0.096	0.091	.403**	-.304*	-.279*
	Sig(2-tailed)		0	0.044	0.779	0.75	0.061	0.455	0.477	0.001	0.015	0.027
	N	63	63	63	63	63	63	63	63	63	63	63
OS	P.C(2-tailed)	.857**	1	-0.133	0.162	0.085	-.283*	-0.166	0.122	0.155	-.317*	-.304*
	Sig(2-tailed)	0		0.299	0.206	0.506	0.024	0.195	0.341	0.225	0.011	0.015
	N	63	63	63	63	63	63	63	63	63	63	63
D/S	P.C(2-tailed)	-.255*	-0.133	1	.481**	.498**	-0.058	0.06	-.266*	-.295*	0.057	0.086
	Sig(2-tailed)	0.044	0.299		0	0	0.649	0.635	0.033	0.017	0.654	0.495
	N	63	63	65	65	65	65	65	65	65	65	65
CT	P.C(2-tailed)	0.036	0.162	.481**	1	.770**	-.364**	0.014	-0.083	0.104	-.343**	-.363**
	Sig(2-tailed)	0.779	0.206	0		0	0.003	0.91	0.511	0.408	0.005	0.003
	N	63	63	65	65	65	65	65	65	65	65	65
RT	P.C(2-tailed)	0.041	0.085	.498**	.770**	1	-.493**	0.04	-0.117	0.165	-.415**	-.437**
	Sig(2-tailed)	0.75	0.506	0	0		0	0.752	0.353	0.189	0.001	0
	N	63	63	65	65	65	65	65	65	65	65	65
Ki67	P.C(2-tailed)	-0.237	-.283*	-0.058	-.364**	-.493**	1	-0.242	-0.12	-.258*	.524**	.569**
	Sig(2-tailed)	0.061	0.024	0.649	0.003	0		0.052	0.343	0.038	0	0
	N	63	63	65	65	65	65	65	65	65	65	65
MGMT	P.C(2-tailed)	-0.096	-0.166	0.06	0.014	0.04	-0.242	1	0.01	0.099	-0.09	-0.06
	Sig(2-tailed)	0.455	0.195	0.635	0.91	0.752	0.052		0.937	0.434	0.475	0.634
	N	63	63	65	65	65	65	65	65	65	65	65
Ku80	P.C(2-tailed)	0.091	0.122	-.266*	-0.083	-0.117	-0.12	0.01	1	0.116	-0.139	-0.093
	Sig(2-tailed)	0.477	0.341	0.033	0.511	0.353	0.343	0.937		0.357	0.271	0.459
	N	63	63	65	65	65	65	65	65	65	65	65
RE	P.C(2-tailed)	.403**	0.155	-.295*	0.104	0.165	-.258*	0.099	0.116	1	-.467**	-.442**
	Sig(2-tailed)	0.001	0.225	0.017	0.408	0.189	0.038	0.434	0.357		0	0
	N	63	63	65	65	65	65	65	65	65	65	65
G	P.C(2-tailed)	-.304*	-.317*	0.057	-.343**	-.415**	.524**	-0.09	-0.139	-.467**	1	.960**
	Sig(2-tailed)	0.015	0.011	0.654	0.005	0.001	0	0.475	0.271	0		0
	N	63	63	65	65	65	65	65	65	65	65	65
SG	P.C(2-tailed)	-.279*	-.304*	0.086	-.363**	-.437**	.569**	-0.06	-0.093	-.442**	.960**	1
	Sig(2-tailed)	0.027	0.015	0.495	0.003	0	0	0.634	0.459	0	0	
	N	63	63	65	65	65	65	65	65	65	65	65

● Abbreviations : PFS, progress free survival; OS, overall survival; S, survival; D, death; CT, chemotherapy;

RT, radiotherapy; RE, recurrence; G, Group; SG, subgroup; PC, Pearson Correlation; N, Number.

. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed)

3.6 Relevance analysis between Ku80 expression and Ki67 expression

Strong positivity of Ku80 staining was observed in 43 (66.2%) of 65 spinal glioma cases and strong Ki67 expression was found in 16 (24.7%) patients. Among 65 cases that were interpretable for both proteins, there are 9 cases (13.9%) with a high expression for both Ku80 and Ki67, 34 cases (52.3%) with a high expression only for Ku80, 7 cases (10.8%) with a high expression only for Ki67, and 15 cases (23.1%) with a low expression for both proteins (Tab. 10). There is no significant difference observed between levels in Ku80 expression and Ki67 expression ($P=0.237$, Tab. 4), and it seems, there is not significant correlation was found between levels in Ku80 expression and Ki67 expression too.

However, we also found a phenomenon with only 6 patients showing a negative Ku80 expression (0%) in our study group. 5 of them are spinal glioblastomas (grade IV) and the other one is ependymoma (grade III). As documented previously, there are only 11 glioblastomas and 2 ependymomas (Grade III) in our study group altogether (Tab. 1) All of them showed a high Ki67 expression. Moreover there were only 3 patients with both a negative MGMT and Ku80 staining (0%) in our study group. They were all glioblastomas.

Otherwise, we found that there was significant difference in Ku80 expression between $PFS \geq 60\text{Mon}$ and $PFS < 60\text{Mon}$ ($P=0.08$). These differences revealed that the low expression level of Ku80 is usually accompanied high expression of Ki67 and with poor PFS.

Tab 10. Correlation between Ku80 expression and Ki67 expression

Location Protein	Ki67 (low)	Ki67 (high)	Total
Ku80 (low)	15 (23%)	7 (11%)	22
Ku80 (high)	34 (52%)	9 (14%)	43
Total	49	16	65

Sig: 0.262

3.7 Relevance analysis among others variables

We used the Bivariate Correlations method to calculate the relevance among variables in this study. The significance of the correlations in variables and Pearson correlation was calculated. The relevance of patients were analyzed for progress free survival (PFS), overall survival (OS), death/survival (D/S), chemotherapy (CT), radiotherapy (RT), MGMT, Ki67, recurrence (RE), tumor group (G), and subgroup (SG). Through the bivariate correlations analysis among variables (Tab 9) we could find that OS (P=0.000), D/S (P=0.044), RE (P=0.001), G (P=0.015), SG (P=0.027) were significant related with PFS in spinal glioma; Ki67 (P=0.024), G (P=0.011), SG (P=0.015) were significantly related with OS in spinal glioma; CT (P=0.000), RT (P=0.000), Ki67 (P=0.033) and RE (P=0.017) were significant related with OS (Tab 9). Additionally, we could find, that PFS (P=0.001), OS (P=0.017), Ki67 (P=0.038), G (P=0.000) and SG (0.000) were statistical significant related with tumor relapse (RE) in spinal glioma. Radiotherapy (RT) (r=0.165, P=0.189) could not be shown to be correlated. But in our study group only 16 patients received radiotherapy. 14 patients received radiotherapy after initial diagnosis and the first operation. 2 patients received radiotherapy after tumor recurrence and second operation. Of these 16 patients, 11 patients are still in remission. This group included 2 grade I tumors, 4 grade II, 4 grade III and 1 grade IV. 2 recurrent tumors are still progression free (113 months and 31 month). As high grade tumor usually received radiotherapy and are prone to relapse, there was not statistical significant correlation between tumor recurrence and radiotherapy in our study group compared to the low grade tumor group.

Similar reasons maybe also adapt for the correlation of chemotherapy and tumor relapse. Otherwise, there are 11 patients received adjuvant both chemotherapy and radiotherapy. So the correlation between tumor recurrence and chemotherapy (alkylating agents) also was more complicated for the clinical result in our study group. We found both chemotherapy and radiotherapy have definitely effects not only for initial diagnosis spinal glioma but also for recurrent spinal glioma.

3.8 Some variables regarding recurrence of spinal glioma

We used the Chi-square test for different variables regarding recurrence of spinal tumors in this study. The significance of the Chi-square test in variables was calculated. The variables of patients were analyzed including surgical type, chemotherapy, radiotherapy. In table 11, we show that there is statistical significant difference observed for tumor recurrence with tumor total removed and tumor subtotal removed (P=0.016). Similar results were obtained for patients for spinal glioma grade (I+II) receiving radiotherapy or not (P =0.013), and receiving chemotherapy or not in the same group (P =0.002). However, there was no statistical significant difference obtained for tumor relapsed in spinal glioma grade (III+IV) receiving radiotherapy or not (P =0.056), and received chemotherapy or not (P =0.056). Nonetheless, there is a near statistically significant difference. We could show that surgical type (P=0.016), radiotherapy for spinal glioma grade (I+II) (P=0.013) and chemotherapy for grade (I+II) could be significantly correlated with tumor recurrence and prognosis in spinal glioma (Tab 11).

Tab 11. Chi-square test for different variables regarding recurrence of spinal tumors

Variables	Recurrence	No Recurrence	p value
Surgical type			
Total remove	16	38	
Subtotal remove	7	2	0.016
Radiotherapy			
grade (I+II)			
Yes	0	6	
No	5	27	0.013
grade (III+IV)			
Yes	6	4	
No	7	0	0.056
chemotherapy			
grade (I+II)			
Yes	1	7	
No	4	24	0.002
grade (III+IV)			
Yes	6	4	
No	7	0	0.056

3.9 Some variables regarding metastasis of spinal glioma

We used the Chi-square test for different variables and their influence on metastasis formation of spinal tumors in our study. The significance of the Chi-square test in variables also was calculated. The different variables of patients were analyzed including diagnosis and its subgroups, the low and high expression of MGMT, Ku80 and Ki67. We could find a statistical significant difference observed among spinal glioma grade I, II, III, IV (P=0.002, Tab 12). Similar results were obtained for ependymomas and astrocytomas (P=0.000, Tab 12) and the low and high expression of Ki67 (P=0.005, Tab 12). However, there was no statistical significant difference obtained for the influence on metastasis formation of spinal tumors between the low and high expression of MGMT (P=0.212, Tab 12) and Ku80 (P=0.155, Tab12).

Tab12. Chi-square test for variables and their influence on metastasis formation

Variables	Metastasis	No metastasis	p value
Diagnosis			
Grade I	0	12	
Grade II	0	31	
Grade III	1	4	
Grade IV	4	8	0.002
Ependymoma	0	42	
Astrocytoma	5	13	0.000
MGMT			
Low	4	28	
High	1	27	0.212
Ku80			
Low	3	16	
High	2	39	0.155
Ki67			
Low	1	43	
High	4	12	0.005

3.10 Survival analysis in spinal glioma

We used the Kaplan-Meier method to calculate the survival rates for this study. The significance of the difference in the survival curves was calculated with the log-rank tests. Kaplan-Meier survival curves of patients were analyzed according to sex, age, tumor location, surgical approach, treatment protocol, alkylating agents, and the expression of MGMT, Ku80 and Ki67. Tumor locations divided into four groups stated as above: the tumors located mainly in medulla oblongata, the tumors located mainly in cervical myelon, tumors located mainly in thoracic myelon and located mainly in lumbar myelon. Treatment protocol was categorized into four groups described as above: chemotherapy, radiotherapy, radiochemotherapy and no adjuvant therapy. These patients also classified into alkylating agents group and non-alkylating agents group, radiotherapy group and non-radiotherapy group and both alkylating agents and radiotherapy group and non-both alkylating agents and radiotherapy group. The surgical type was categorized into tumor total removed and tumor subtotal removed. The expression of MGMT, Ku80 and Ki67 was evaluated by immunohistochemistry staining, those patients were divided into low expression group (positive rate is less than 50% in MGMT, Ku80 and positive rate is less than 10% in Ki67) and high expression group (positive rate is not less than 50% in MGMT, Ku80 and positive rate is not less than 10% in Ki67).

Progression-free survival and overall survival were estimated by the Kaplan–Meier method and compared with the use of the log-rank test. Kaplan-Meier analysis between variables with OS revealed that patients with tumors located mainly in cervical myelon, thoracic myelon and lumbar myelon had statistically significantly longer median survival time (MST) than patients with tumors located mainly in medulla oblongata (MST: 124.4 months, 140.5 months, 127.1 months vs. 17.8 months; $P = 0.004$; log-rank test). Similar results were obtained between tumor total remove and subtotal remove group (MST: 147.5 months vs. 27.7 months; $P = 0.000$, log-rank test), patients who received radiochemotherapy and no radiochemotherapy treatment protocol (only for grade III and grade IV patients) (MST: 77.2 vs. months, 16.7; $P = 0.002$; log-rank test; Tab 13). This result supports the conclusions that alkylating agents and radiotherapy play an important role in treatment of patients with grade III and grade IV spinal glioma patients. Also

surgical resection, tumor location are related with OS prognosis in spinal glioma. Significantly statistically different results were obtained for patients who had high vs. low Ki67 expression (MST: 52.4 vs.157.2 months P =0.000, log-rank test). Different results were obtained for patients who had high and low MGMT expression (MST: 110.4 months vs. 138.7months; P =0.895, log-rank test) and high and low Ku80 expression (MST: 144.9 months vs. 127.0 months; P =0.424, log-rank test), these differences did not reach statistical significance. Similar results were obtained for male and female patients (P =0.406, log-rank test), patients of the < 50 years and \geq 50 years group (P =0.275, log-rank test). For the radiotherapy group and alkylating group, overall survival of patients who received these therapies was shorter than who not received these protocols. This is due to the poorer WHO tumor grade in this group (grade III and grade IV glioma). Results obtained for patients who grade I and II receiving both alkylating agents and radiotherapy or not group showed no positive effect for low grade tumors (P =0.457). Kaplan-Meier analysis between variables with PFS revealed the almost same statistical results as OS (Tab 14). We could show that surgical type (P=0.000), radiochemotherapeutic treatment protocol (P=0.000) and Ki67 (P=0.010) were significantly related with PFS prognosis in spinal glioma (Tab 14).

Tab13. Kaplan–Meier analyses for the correlation between variables and OS

Variables	OS Mean	95% CI	p value
Sex			
Male	126.3	90.2-162.4	
Female	146.2	125.0-167.3	0.406
Age			
< 50 years	127.5	101.7-153.2	
≥ 50 years	159.1	130.7-187.6	0.275
Tumor location			
MO	17.8	9.9-25.6	
C	124.4	83.5-165.2	
T	140.5	111.8-169.2	
L+S	127.1	112. 2-141.9	0.004
Surgical type			
Total remove	147.5	128.9-165.9	
Subtotal remove	27.7	17.6-37.9	0.000
Treatment protocol(grade III +IV)			
Radiochemotherapy	77.2	34.1-120.3	
No Radiochemotherapy	16.7	6.6-26.7	0.002
Treatment protocol (grade I+II)			
Chemotherapy	40.8	5.5-76.1	
Radiotherapy	88.2	56.4-120.1	
Radiochemotherapy	69.8	1.9-128.3	
No treatment	74.4	49.4-99.4	0.457
Alkylating agents			
yes	88.7	58.3-119.0	
No	144.0	121.9-166.2	0.220
Radiotherapy			
Yes	108.6	83.4-133.8	
No	136.9	112.3-161.6	0.908
Both alkylating agents and radiotherapy			
yes	85.7	47.3-124.2	
no	142.7	121.5-164.0	0.184
MGMT expression			
Low	138.7	113.3-164.1	
High	110.4	92.19-128.6	0.895
Ku80 expression			
Low	127.0	92.2-161.8	
High	144.9	123.6-166.3	0.424
Ki67 expression			
Low	157.2	140.9-173.5	
High	52.4	32.8-71.9	0.000

Tab14. Kaplan–Meier analyses for the correlation between variables and PFS

Variables	PFS Mean	95% CI	p value
Sex			
Male	113.9	76.3-151.5	
Female	102.9	74.9-131.0	0.298
Age			
< 50 years	92.4	63.3-121.6	
≥ 50 years	137.0	99.9-175.7	0.167
Tumor location			
MO	12.3	7.0-17.5	
C	81.8	37.3-126.4	
T	114.8	79.9-149.8	
L+S	102.9	76.2-129.8	0.097
Surgical type			
Total remove	114.9	90.6-139.3	
Subtotal remove	15.3	6.6-29.0	0.000
Treatment protocol (grade III+IV)			
Radiochemotherapy	59.0	25.9-92.1	
No Radiochemotherapy	7.5	4.6-10.4	0.000
Treatment protocol (grade I+II)			
Chemotherapy	34.0	2.3-65.7	
Radiotherapy	55.8	20.6-91.0	
Radiochemotherapy	62.5	0.8-124.2	
No treatment	65.1	41.7-88.5	0.511
MGMT expression			
Low	102.6	73.2-132.0	
High	90.2	66.4-114.0	0.488
Ku80 expression			
Low	90.4	50.5-130.0	
High	114.7	86.5-142.8	0.267
Ki67 expression			
Low	122.3	96.6-147.9	
High	43.8	24.4-63.2	0.010

In the same time, we also analyzed the influences of MGMT, Ku80 on PFS and OS on using alkylating agents and radiotherapy between different subgroups (Tab 15). There we could show that patients with high expression of MGMT had a statistical significantly longer median PFS than patients with low expression MGMT (108.0 months vs. 46.4 months; $P = 0.038$; log-rank test) in the alkylating agent group.

Significantly different results were obtained for patients in the no-alkylating agents subgroup (132.48 months vs. 87.68 months; P =0.082, log-rank test), radiotherapy subgroup for Ku80 (68.8 months vs. 31.4 months; P =0.510, log-rank test) and the no radiotherapy subgroup for Ku80 (140.4 months vs. 90.6 months; P =0.153, log-rank test). These differences did not reach statistical significance. Although the expression of MGMT and Ku80 varies in spinal glioma, high expression of MGMT and Ku80 maybe have correlation with longer PFS in the spinal glioma.

Tab 15. Influence of MGMT, Ku80 on PFS and OS between different subgroups

Subgroup	Variables	OS Mean	p value	PFS Mean	p value
Alkylating agents (n=18)	MGMT Low (n=10)	65.9		46.4	
	MGMT High (n=8)	114.1	0.448	108.0	0.038
No alkylating agents (n=35)	MGMT Low (n=10)	120.3		87.68	
	MGMT High (n=25)	141.7	0.651	132.48	0.082
Radiotherapy (n=16)	Ku80 Low (n=5)	56.0		31.4	
	Ku80 High (n=11)	138.7	0.196	68.8	0.510
No Radiotherapy (n=37)	Ku80 Low (n=11)	106.3		94.6	
	Ku80 High (n=26)	156.8	0.571	140.4	0.153

We could show that patients with high expression Ki67 had a statistical significantly shorter median PFS than patients who with low expression Ki67 (43.8 months vs 122.3 months; P =0.010; log-rank test). Similar results were also obtained for the MST (48.4months vs. 85.6months; P =0.115; log-rank test), although this difference did not reach statistical significance. Ki67 (P=0.010) was also related with PFS prognosis in spinal glioma.

Tab 16. The influences of the expression Ki67 on PFS and OS

Group	Variables	OS Mean (Mon)	p value	PFS Mean (Mon)	p value
1	Ki67<10%(n=47)	157.2		122.3	
2	Ki67≥10%(n=16)	52.4	0.000	43.8	0.010

4. Discussion

As primary spinal cord gliomas are rare, there has not been paid sufficient attention especially for its unsatisfactory therapy. There is no consensus regarding the management of spinal glioma. Nowadays spinal glioma remains incurable in a party of patients. Surgery, radiation therapy, and chemotherapy have been used in various combinations; however, the extent of resection and the use of adjuvant chemotherapy, radiation therapy and the prevention of tumor recurrence and the treatment for tumor relapse are not clearly associated with a survival advantage. (Jallo GI, 2003). Which group or subgroup of spinal gliomas tends to relapse and needs radiotherapy, chemotherapy or both radiochemotherapy? Which chemotherapeutic agents are most effective in the treatment of spinal glioma? A better insight into its biology may be crucial to answer these questions and to improve the prognosis of spinal glioma. Special attention has been paid in recent years to identifying prognostic factors related to the molecular biological characteristics of the tumor, in an attempt to improve the effective treatment and the prognosis (Richard AR, 2007). This study was aimed at detecting the expression of MGMT, Ku80 and Ki67 on spinal glioma by immunohistochemistry (IHC) staining to evaluate the relationship between MGMT, Ku80 and Ki67 expression level to clinical characteristics and outcomes, thus determine whether these immunophenotypes were prognostic markers in spinal glioma and could be a guide to chose an optimal and reasonable individualized treatment.

4.1 MGMT expression level in spinal glioma

Many studies concerning MGMT expression, including IHC studies, have been performed in cerebral gliomas and other tumors. Most association analyses between MGMT and glioma were performed in brain glioma, especially for the subgroup of glioblastomas. The MGMT status in spinal glioma is still unclear. Only one case report in the literature correlating MGMT expression with the clinical response of a high-grade spinal cord astrocytoma treated using TMZ, suggesting a clinical value of

MGMT evaluation in treatment benefit from alkylating agents in spinal glioma patient. The expression of MGMT protein tested by IHC is also not reported for spinal glioma in the literature.

O6-methylguanine- DNA-methyl-transferase (MGMT) is an enzyme in the DNA repair process that specifically removes cytotoxic O6-alkylguanine adducts, thus mediating resistance to alkylating agents. This DNA repair enzyme plays a role in maintaining the integrity of the DNA in normal cells but also protects tumor cells against alkylating and methylating chemotherapeutic agents, resulting in drug resistance. It is therefore assumed that the good prognosis of brain glioma with low MGMT expression was caused by a better response to alkylating agents such as fotemustine, which a cytotoxic agent belonging to the nitrosurea family. It has been observed that MGMT gene expression seems to be related to the methylation of the MGMT promoter, MGMT enzyme activity, protein expression and cell resistance to anti-tumor alkylating agents could predict a possible chemosensitivity (Kaina B, 2007). Therefore the study of MGMT status could be of therapeutic and prognostic interest.

In our study, the level of MGMT expression was detected by IHC method in 65 spinal glioma tissues. 30 (46.2%) tumor samples demonstrated intermediate and high MGMT expression respectively. There are no significant differences in MGMT expression level with patients' gender, locations of tumors, group and sub group of diagnosis, PFS, D/S. But for the age group, we could find a significant difference of the expression of MGMT between patients in age < 50 years subgroup and in age \geq 50 years subgroup reaching statistical significance (P=0.044). This revealed, that the MGMT level in age \geq 50 years patients is usually lower than in patients with a age less than 50 years.

For the metastases group, we could find a difference of the expression of MGMT between metastases group and no metastases group (P=0.063), although it did not reach a statistical significant difference. Some similar results were obtained from variables of relapse group (P=0.078), and Ki67 expression group (0.05). For the Ki67 high expression statistical significant correlation with malignant and benign of spinal

gliomas, we found that there is a strong trend that poor prognosis in MGMT low expression but Ki67 high expression patients, as compared with MGMT high expression but Ki67 low expression cases. This trend is also discovered for the Ki67 expression with a statistical significant correlation with OS, tumor recurrence, tumor grade, and tumor subgroup of patients. Although there is no statistical significant difference observed between levels in MGMT expression and Ki67 expression ($P=0.051$, Tab 4; $r=-0.24$, $P=0.052$). Otherwise, there are only 5 patients with a negative MGMT expression. Otherwisw Ki67 expression was very high in our study group (MGMT: 0%) with all of them being spinal glioblastomas (grade IV) except one anaplastic ependymoma. We could show that MGMT and Ki67 have significant negative correlation.

No significant difference on overall survival between patients who had high MGMT expression and patients with low MGMT expression was observed by Kaplan-Meier method ($P=0.895$, log-rank test). Similar results were observed for PFS ($P=0.488$). So, in this study, MGMT protein expression can not be considered as a definite predictor to chemotherapeutic (alkylating) response and prognosis in patients with spinal glioma.

We used the Bivariate Correlations method to calculate the relevance among variables in this study, the significance of the correlations in variables and Pearson correlation was calculated. Surprisingly,we could show that patients who underwent chemotherapy (alkylating agents) had significantly longer MST than patients who did not underwent chemotherapy (alkylating agents) ($P=0.000$). This supported the conclusion that alkylating agents play an important role in chemotherapy outcomes in patients with spinal glioma. There are three possible explanations to this surprising results: a, this phenomenon is caused by the error of analysis techniques, it may be one way to choose adequate method or combine with other methods such as methylation specific PCR for preferable assessing the MGMT status; b, sample size is not enough, further analysis with larger number of cases is necessary to clarify the utility of MGMT immunohistochemistry as a predictor of therapy of spinal glioma; c,

There are 11 patients received adjuvant both chemotherapy and radiotherapy in our study group, given alkylating agents prescribed as part of radiochemotherapy regimens, so that the correlation between tumor recurrence and chemotherapy (alkylating agents) also become more complicated. Both the alkylating agents and radiotherapy maybe play a role on tumor cells.

4.2 Ku80 expression level in spinal glioma and outcome.

As a DNA repair protein, Ku80 forms a heterodimer with Ku70, called Ku that binds to DNA ends, nicks, gaps, and hairpins. In vitro, Ku forms a complex called DNA-dependent protein kinase (DNA-PK) by associating with a 450-kDa catalytic subunit, DNA-PKcs (Ute Moll et al, 1999). Cells mutated by the deletion of any of these genes are hypersensitive to ionizing radiation. An absence of Ku80 results in an increased sensitivity to ionizing radiation. Although most cancerous tissues were consistently positive for Ku80 (Moll U, 1999), and radiotherapy is also an important component of multimodal treatment for many malignant brain and spinal gliomas in clinic, the research efforts on Ku80 with CNS glioma are rare. It is reported that hypersensitivity of Ku80-deficient cell lines to DNA damage is correlated with the effects of ionizing radiation on growth and development of cerebral glioma cell lines (Takashi O, 2004; Dhandapani KM, 2007; He F, 2007; Holcomb VB, 2006). Other than these studies performed with established glioblastoma cell lines, no other is known about Ku80 expression in brain glioma. So far, the expression and significance of Ku80 in spinal glioma has not been analyzed.

The level of Ku80 expression was determined by Ku80 immunohistochemistry in 65 spinal glioma tissues. There is variability in expression level ranging between 0% and 97.0% in our study group. There are no statistical significant differences of the expression of Ku80 in spinal gliomas between sex, age, tumor site, surgical type, tumor grade and treatment protocol. But, for the PSF group, we found a statistical significant difference for the expression of Ku80 in PFS < 60 months subgroup and in PFS \geq 60 months subgroup (P=0.005). Otherwise, there is a difference of the

expression of Ku80 for patients that suffered metastases and metastases free subgroup (P=0.088), although it did not reach a statistical significant difference. Similar result was found in death/survival group (P=0.089). FKU80 expression has usually the highest levels in normal neurons and glial cells of the central nervous system (Moll U, 1999). Low expression of Ku80 maybe has a correlation with the degree of malignance of spinal glioma.

No significant difference on overall survival between patients who had high Ku80 expression and patients with low Ku80 expression was observed by Kaplan-Meier method (P =0.424, log-rank test) with similar results observed for PFS (P=0.267). So it is difficult to considere Ku80 protein expression as a definite predictor for radiotherapy response and prognosis in patients with spinal glioma.

Although it is supported that Ku protein expression is correlated with radiation treatment outcome (Friesland SL, 2003; Harima YS, 2003;), there are exceptions. Some stated that Ku80 expression is not correlated with radiation sensitivity (Kasten-Pisula US, 2008). In our group, the Bivariate Correlations showed that patients who underwent radiotherapy, had significantly longer MST than patients with no radiotherapy (P=0.000). 2 recurrent tumors even did not progress so far (113 months and 31 month). Among 16 patients with radiotherapy in our group, 4 patients with recurrent spinal glioblastomas recurrent died and 1 patient was lost to follow up. The other 11 patients are still progression free. This also supports the conclusion that radiotherapy plays an important role on OS in patients with spinal glioma, although their Ku80 protein expression has not definite correlated with radiation sensitivity.

4.3 Ki67 expression level in spinal glioma and outcome, Ki67 maybe a prognostic marker of spinal glioma.

Several studies show the Ki67 index in intracranial glioma as a reproducible and robust prognostic factor (Burger PC, 1986; Haapasalo A, 2005; Vaishali SS, 2004; Rushing EJ, 1981; Suzuki S, 2001; Verstegen MJ, 2002). Verstegen showed for intracranial ependymoma a significant correlation of a low Ki67 index with a

favorable patient outcome. The Ki67 tumor cell proliferation index is an independent prognostic factor in ependymoma patients (Verstegen MJ, 2002); Haapasalo looked at Ki67 in different grades of astrocytic and found that Ki67 is expressed in proliferating astrocytoma nuclei, which are scarce in low grade tumours and abundant in glioblastoma (Haapasalo A, 2005). Although many studies looked at brain gliomas and showed the expression and significance of Ki67, there is few literature about Ki67 expression in spinal glioma so far.

The Ki67 expression in 65 spinal glioma tissues is found to be of a considerable variability ranging between 0% and 80.0% (mean 7.15%). 49 tumor samples (75.4%) had a low Ki67 staining (positive rate $< 10\%$, mean $2.6\% \pm 2.5\%$), and 16 (24.6%) tumor samples showed a high Ki67 expression (positive rate $\geq 10\%$, mean $21.1\% \pm 17.0\%$). The absolute expression level of Ki67 in all groups classified according to variables analyzed by One-way ANOVA method showed that no statistical significant differences of the expression of Ki67 in spinal gliomas in sex, age, tumor site and for the surgical therapy groups. But there are statistical significant differences of the expression of Ki67 for patients concerning the death/survival(D/S) groups ($P=0.004$), relapse groups ($P=0.038$), metastases groups ($P=0.015$), both or no radiotherapy and chemotherapy groups ($P=0.001$), the radiotherapy group ($P=0.004$) and the diagnosis groups ($P=0.000$, $P=0.002$). There are also statistical significant difference on overall survival between patients who had high Ki67 expression and patients with low Ki67 expression observed by Kaplan-Meier method ($P=0.000$, log-rank test) and for PFS ($P=0.010$). We could show that most spinal gliomas (75.4%) with low expression of Ki67 are low grade gliomas; the tumors with high expression of Ki67 are significantly correlated to malignant tumors. This relates with poor prognosis including poor D/S, OS and PFS. They tend to relapse earlier and are prone form metastases. The prognosis of astrocytoma is poorer than that of ependymoma. We could identify Ki67 as a prognostic marker of spinal glioma.

The proliferation activity of tumors can be assessed by means of Ki67 immunohistochemistry, which is now widely used in clinical pathology and

neuropathology diagnostics. Consistent with our results, Burger and co-workers could show the tight association between Ki-67 immunopositivity and glioma grade in freshly frozen samples of cranial astrocytomas (Burger PC, 1986). Others also have described the prognostic value of immunostaining with MIB-1 (Ki67) (Onda K, 1994; Cunningham JM, 1997). Based on a large number of studies, the added prognostic information provided by Ki-67 immunostaining is now well established with regard to astrocytic tumors of the brain. The latest WHO classification of central nervous system tumors (WHO Classification of Tumors of the Central Nervous System, 3rd Edition) includes Ki67 as an additional tool in histological typing and grading, although it cannot be regarded as being entirely prognostic in individual cases. Haapasalo also used ultrarapid Ki67 immunostaining in frozen section interpretation of gliomas for intraoperative guidance of the resection (Haapasalo, 2005). With this work we can confirm this correlation of Ki67 expression for spinal gliomas.

4.4 Relevance analysis between MGMT expression and Ki67 expression

In our study patients with a high Ki67 expression were associated with a low MGMT expression and reduced survival in Kaplan-Meier analysis. A strong trend was found that toward poor prognosis in patients with high Ki67 expression but low MGMT expression, as compared with low Ki67 expression but high MGMT expression cases. There is significant difference observed between the levels in MGMT expression and Ki67 expression ($P=0.05$). Furthermore, we found that the expressions of MGMT and Ki67 statistically correlated ($r=-0.24$, $P=0.05$). All 5 patients with negative MGMT expression showed very high Ki67 expression in our study group (MGMT: 0%), and all of them were spinal glioblastomas (grade IV). It indicates that MGMT and Ki67 have significant opposite correlation.

MGMT is DNA repair gene, which plays an important role of protecting tumor cells against DNA injuries caused by chemotherapy (alkylating agents). High expression of MGMT can lead to a failure of alkylating chemotherapy due to a tumor cell resistance to DNA damage impacted by these drugs. In most case, it has been shown that those tumors with abundance of MGMT protein were resistant to alkylating

chemotherapeutics, while those with low MGMT activity were sensitive to alkylating agents. MGMT behaves as a potential predictor of response to chemotherapy and also maybe a prognostic biomarker of spinal glioma just the same as Ki67. Given MGMT is one of the most important factors determining drug resistance to alkylation, some strategies have been developed to inhibit MGMT expression in tumors with the aid of MGMT inhibitors, and further enhance the anti-neoplastic efficiency of alkylating agents. (Krishnan M et al, 2007)

4.5 Relevance analysis between Ku80 expression and Ki67 expression

No significant difference was observed between the levels of Ku80 and Ki67 expression analyzed by One-way ANOVA in our study ($P=0.237$). A correlation was also not found for these two proteins ($r=-0.12$, $P=0.343$). However, we found a phenomenon of 6 patients with negative Ku80 expression (0%) and 5 of them being spinal glioblastomas (grade IV) and the other one an ependymoma (grade III). Overall there were only 11 glioblastomas and 2 ependymomas (grade III) in our study group - all of them with a high Ki67. Moreover there were 3 patients with both MGMT and Ku80 negative (0%) in our study group, all of them being glioblastomas. It could indicate that near half of the spinal glioblastoma with MGMT and/or Ku80 expression could be radiosensitive and/or chemosensitive (alkylating agents). We found there is not a statistical significant difference in Ku80 expression between $PFS \geq 60$ Mon and $PFS < 60$ Mon ($P=0.08$). These differences revealed that the low expression level of Ku80 is usually accompanied by a high expression of Ki67 resulting in a poor PFS. As an important repair gene of DSBs Ku80 potential prognostic marker for the prediction of radiotherapy outcome and also a prognostic biomarker for spinal glioma just like Ki67.

4.6 Relevance analysis between treatment protocols and tumor recurrence. Operation type, radiotherapy and chemotherapy may be prognostic markers for relapse in spinal glioma.

As spinal gliomas are rare tumors, there is no consensus regarding the management of

malignant spinal cord glioma. Surgery, radiation therapy, and chemotherapy have been used in various combinations; however, the extent of resection and use of adjuvant chemotherapy or radiation therapy are not clearly associated with a survival advantage (Jallo GI, 2003).

We used the Chi-square test to analyze the treatment protocols regarding recurrence of spinal tumors in this study. The variables of patients were analyzed including surgical type, chemotherapy and radiotherapy. We could demonstrate statistical significant difference regarding tumor recurrence with total versus subtotal removal of the tumor ($P=0.016$). Similar results were obtained for patients with benign spinal glioma (grade I+II) that received radiotherapy versus not ($P=0.013$), and received chemotherapy or not in the same group ($P=0.002$). Although without statistical significance we found a difference in tumor recurrence for patients with malignant spinal glioma (grade III+IV) that received radiotherapy versus not ($P=0.056$), and that received chemotherapy versus not ($P=0.056$).

Thus we could show, that radical tumor resection ($P=0.016$), radiotherapy for benign spinal glioma (grade I+II) ($P=0.013$) and chemotherapy for benign tumors (grade I+II) improved the rate of PFS statistically significantly.

With the advent of microsurgery and MRI, especially, using of MRI had dramatically improved the anatomical understanding of the surgical pathology, and the use of intraoperative neurophysiological monitoring has tremendously increased the understanding of the functional integrity of the spinal cord pathways during surgery and in the process of neurological recovery thereafter. Nevertheless, differences of opinion still exist about patients suffering from an intramedullary tumor and their optimal individualized treatment. In spite of the advances in surgical treatment, the old concept of 'biopsy and radiation' is apparently not extinct (O'Sullivan C, 1994; Houten JK, 2000), especially in some developing areas of the world. It has been stated, that a total or near-total resection is the best treatment for spinal gliomas (Kothbauer K, 2007). Our result can confirm this statement for spinal glioma with statistical significant results.

Despite lack of consensus, surgery followed by radiation therapy, especially for

incompletely resected lesions and recurrent tumors, is a common treatment paradigm. Several studies indicate that the use of postoperative radiation therapy modestly improves both local control and survival in spinal cord ependymomas and astrocytomas. Modern treatment planning and imaging allow more accurate target definition respecting the related normal tissue tolerances (Isaacson SR, 2000; Minehan KJ, 1995; Houten JK, 2000). Furthermore we found that radiotherapy has significant effects on PFS not only for initially treated but also recurrent spinal glioma. Several centers have treated the malignant spinal gliomas with multimodal treatment including chemotherapy (Allen JC, 1998). Concerning benign spinal gliomas, only recurrent patients have been treated with chemotherapy (TMZ) demonstrating only modest efficacy with acceptable toxicity in 22 patients (Chamberlain MC, 2008). Our study indicate that with the guide of molecular prognostic factors (e.g. MGMT, Ki67) there might be a base for adjuvant chemotherapy (Taylor MD, 2005; Richard A R, 2007).

4.7 Relevance variables regarding metastasis of spinal glioma

5 patients (8%) suffered tumor metastases in our study group. Analyzed by One-way ANOVA, there is statistical significant difference for the occurrence of metastases for high versus low Ki67 expression ($P=0.015$); although not statistical significant the metastases formation with high versus low MGMT expression ($P=0.065$), Ku80 expression ($P=0.088$) was obvious different. We also used the Chi-square test for different variables and their influence on metastasis formation of spinal tumors in our study. It showed a statistical significant difference observed for the different WHO grades ($P=0.002$). Similar results were obtained for ependymomas versus astrocytomas ($P=0.000$) and for low versus high expression of Ki67 ($P=0.005$).

Thus, we could show that WHO grades ($P=0.002$), histology ($P=0.000$) and the expression of Ki67 ($P=0.005$) significantly influenced the formation of metastases; the expression of MGMT ($P=0.065$) and Ku80 ($P=0.088$) probably influences the formation of metastasis.

4.8 Prognostic factors in spinal glioma

There is no consensus regarding treatment with most part of glioma (Richard AR, 2007). Prognostic factors are important not only for the discrimination of patients with spinal glioma into specific risk groups and for the identification and assessment of appropriate therapies on an individually base, but also for comparing results of clinical trials and planing those. However, it is difficult to identify prognostic factors in series from single institutions because of the limited number of cases. Rudà could show for intracranial and spinal ependymomas that a complete resection should be achieved whenever possible at first surgery or at reoperation. Involved field radiotherapy is recommended for anaplastic or incompletely resected grade II tumors (Rudà R, 2008). Craniospinal irradiation is reserved for metastatic disease (Rudà R, 2008). Chemotherapy is not advocated as primary treatment, but it is best utilized as salvage treatment for patients failing surgery and radiotherapy (Rudà R, 2008).

In our survival analysis, the expression of Ki67, extent of resection, radiotherapy, and chemotherapy were considered important predictors, whereas MGMT and Ku80 expression were not statistically significant. Nonetheless, we found, that MGMT and Ku80 also are potential prognostic markers in tumor recurrence, metastasis and for choosing adjuvant therapy.

Additonally , when the tumor involved regions like the medulla oblongata and cervical myelon ,that was associated with a poorer prognosis than that tumors of the thoracal myelon or the lumbar region.

5. Conclusion

The expression of MGMT, Ku80 and Ki67 was observed in the majority of spinal glioma. It is the first time to detect the expression of MGMT, Ku80 and Ki67 proteins in spinal glioma with immunohistochemistry method. Ki67 was predictive for survival in this study. MGMT and Ku80 are potential prognostic factor for patients with spinal glioma. In addition to extent of tumor resection, radiotherapy, chemotherapy (alkylating agents) and tumor location correlated significantly with PFS and OS. The role of these variables and the clinical relevance deserves to be

assessed in further studies.

6. Summary

Compared to cranial gliomas, the spinal cord glioma is a rare tumor of the central nervous system. Due to its infrequent incidence, there is no consensus regarding the management of spinal cord glioma until nowadays. Radical surgery, radiation therapy, and chemotherapy have been used in various combinations around the world; so far the extent of resection and the use of adjuvant chemotherapy or radiotherapy have not been clearly shown to be associated with a survival advantage. MGMT is one of the most important genetic factors determining drug resistance while Ku80 determining radiosensitivity, and Ki67 determining tumor proliferation. The expression of MGMT, Ku80 and Ki67 in spinal glioma remains unclear. If they interfere with the chemosensitivity, radiosensitivity and with the prognosis of spinal glioma, an optimized therapeutic protocol might be found based on these molecular factors. The aim of our study was to detect the expression of MGMT, Ku80 and Ki67 on spinal glioma by immunohistochemistry staining and to assess the relationship between MGMT and Ki67, and Ku80 and Ki67 expression level and clinical outcomes, thus determine whether these immunophenotypes were prognostic factors in spinal glioma. At the same time we evaluated the efficacy of surgery, radiotherapy and chemotherapy efficacy in a series of spinal gliomas through the retrospective analysis.

65 patients with spinal glioma from Germany and China were included in this retrospective study. All tissues were reviewed and diagnosed with H.E staining by a neuropathologist. The expression of MGMT, Ku80 and Ki67 in tumor samples was determined by immunohistochemistry using the MGMT, Ku80 and Ki67 monoclonal mouse antihuman antibody. One thousand neoplastic cells per specimen were counted. On the basis of the percentages of positive cells in the tumors, these tumors were defined as low MGMT expression or low Ku80 expression when there were fewer than 50% positive cells, and high expression when positive rate was more than 50%. A low Ki67 expression was defined as less than 10% positive cells and a high

expression when the positive rate was more than 10%. Analyses were performed with the use of SPSS 17.0.

The mean expression level of MGMT, Ku80 and Ki67 in 65 spinal gliomas was $44.6\% \pm 33.6\%$, $59.0\% \pm 29.3\%$ and $7.2\% \pm 11.7\%$. resp. A correlation was found between MGMT and Ki67 proteins expressions ($r = -0.242$, $P = 0.052$). A significant difference in MGMT expression could be found between age < 50 years old group and age ≥ 50 years old group ($P = 0.05$). Differences in Ku80 and MGMT expression between primary and recurrent spinal gliomas did not reach significance ($P > 0.05$). Kaplan-Meier analysis revealed that patients with tumors showing a high Ki67 expression had significantly shorter progress free survival (PFS) than patients who had low Ki67 expression (122.3 months vs. 43.8 months; $P = 0.010$). Expression of Ki67, extent of resection, radiotherapy, chemotherapy were related with prognosis in spinal glioma ($P < 0.05$), whereas MGMT and Ku80 expression were not statistically significant, but showed to be potentially related to tumor recurrence and metastases formation (MGMT ($P = 0.065$) and Ku80 ($P = 0.088$)).

This study showed that the expression of MGMT, Ku80 and Ki67 could be observed in the majority of spinal gliomas. It is the first time to detect the expression of MGMT, Ku80 and Ki67 proteins in spinal glioma with immunohistochemistry staining method, Ki67 even predicting survival in this study. In addition to, Ki67 and other clinical variables including tumor location, extent of resection, radiotherapy, chemotherapy are correlated significantly with OS and PFS.

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8. List of abbreviations

CI	Confidence interval
CT	Chemotherapy
CNS	Central nervous system
DNA	Deoxyribonucleic acid
DNA-PKcs	DNA-PK catalytic subunit
DNA-PK	DNA-dependent protein kinase
DSBs	DNA double-strand breaks
EGFR	Epidermal Growth Factor Receptor
GBM	Glioblastoma multiforme
GLA	Gamma-linolenic acid
IHC	Immunohistochemistry
IDH1	Isocitrate dehydrogenase
KPS	Karnofsky performance score
MGMT	O6-MethylguanineDNAmethyltransferase
MRI	Magnetic resonance imagine
MST	Median survival time
NaCl	Sodium Chloride
NHEJ	Nonhomologous end joining
OS	Overall survival
PBS	Phosphate buffered saline
PFS	Progression-free survival
PTEN	Phosphatase and Tensin homolog
RE	Recurrence
RC	Radio-chemotherapy
RT	Radiotherapy
TBS	Tris-buffered saline
TMZ	Temozolomide
WHO	World Health Organization

9. Acknowledgements

In particular, I want to express my sincere gratitude to my mentor Prof. Dr. med. Hubertus Maximilian Mehdorn, head and chairman of the department of neurosurgery, University of Kiel, for his kindly invitation to Kiel and great support of my study, and valuable support throughout the research. I really appreciate to his excellent ability of various neurosurgery operation skills, highly efficient organization in scientific research and his enthusiasm for work.

I appreciated deeply to Dr. med. Lutz Doerner, my supervisor of this project, for his providing me the idea to perform this important study, for his numerous efforts in helping me to achieve this work and for his scientific guidance, valuable statistical advice and careful correction of the thesis. He has always set time to discuss the course of this research patiently. I learned a lot of valuable experience of research from him.

I am deeply grateful to Dr. med. Heinz-Hermann Hugo and Dr. Tanja, Dr. Xinwei Li, Dr. Yi Luo, Prof. Dr. Sien Zeng, Dr. Yunqian Li, and Dr. Thomas Kriesen, for their great technical supports, for their great supports on collection clinical and pathological materials of patients during this study, for their collections of clinical data of patients. They gave me a great number of help in my experimental work.

Many thanks I give to Priv.-Doz. Dr. Arya Nabavi, Dr. med. Harald Barth, Priv.-Doz. Dr. med. Andreas Stark, Dr. med. Martin Hefti, Dr. med. Mehran Mahvash and all colleagues in the department of neurosurgery in Kiel. They have always given me a lot of helping during the total period in Kiel. I have really cherished the happy time working and living with them.

Finally, I would also like to thank my distinguished parents and parents-in-law, beloved wife and daughter, it is their strong supports and understanding that gives me the motivation to accomplish this thesis.

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