UNIVERSITY OF PÉCS

Doctoral School of Biology and Sport Biology

Characteristics of The Immune and Molecular Microenvironment of The Intra-and Extra-Parenchymal Tumors of The Central Nervous System

PhD thesis

Amina Soltani

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Amina Soltani

PhD Supervisor: Prof. Dr. Judit E. Pongrácz

Co-supervisor: Dr. Luca Járomi

University of Pécs

Department of Pharmaceutical Biotechnology

Faculty of Pharmacy

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Abbreviations

ABC	ATP-binding cassette
AC	Adenocarcinoma
ACTB	Beta-actin
AKT1	V-akt murine thymoma viral oncogene homolog1
APC	Antigen-presenting cells
APC	Adenomatous polyposis coli
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BBTB	Blood-brain tumor barrier
BCRP	Breast cancer resistance protein
BSA	Bovine serum albumin
CaMKII	Calcium/calmodulin-dependent protein kinase II
CCL	C-C motif chemokine ligand
CD	Cluster of differentiation
CDC42	Cell division control protein 42 homolog
CK1	Casein kinase 1
CNS	Central nervous system
CRD	Cysteine-rich domain
CSF	Cerebrospinal fluid
CTLA-4	Cytotoxic T lymphocyte-associated Protein 4
CXCL	C-X-C motif chemokine ligand
CX3CR1	CX3C chemokine receptor 1
DC	Dendritic cell
DKK	Dickkopf
DNA	O6-methylguanine deoxyribonucleic acid
DVL	Dishevelled
EGFR	Epithelial growth factor receptor
EMR1	Epidermal growth factor like module containing mucin-like hormone receptor 1
EPC	Endothelial progenitor cells
ER	Estrogen receptors

FAT1	FAT atypical cadherin 1
FDA	Food and drug administration
FOXP3	Forkhead box P3
FOXM1	Forkhead box M1
FZD	Frizzleds
GABA	Gamma-aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBM	Glioblastoma multiform
GnRH	Gonadotropin-releasing hormone
GPCR	G protein-coupled receptors
GSK-3-beta	Glycogen synthase kinase 3-beta
GTP	Guanosine triphosphate
GUSB	Glucuronidase β
HGG	High grade gliomas
HPRT1	Hypoxanthine phosphoribosyl-transferase 1
KLF4	Krüppel-like factor 4
KRAS	Kirsten rat sarcoma viral oncogene homologue
KREMEN 2	Kringle containing transmembrane protein 2
ICAM-1	Intercellular adhesion molecule 1
ICBs	Immune checkpoint blockers
ICI	Immune checkpoint inhibitors
IDO-1	Indoleamine 2, 3-dioxygenase 1
IHC	Immunohistochemistry
IL-1	Interleukin
INF-γ	Interferon gamma
IDH	Isocitrate dehydrogenase
JNK	Jun N-terminal kinase
LC	Lung carcinoma
LDL	Low-density lipoprotein
LEF	Lymphoid enhancer factor
LGG	Low grade gliomas
LRP	Low-density lipoprotein receptor-related protein
MAGUNK	Membrane-associated guanylate kinase

MB	Medulloblastoma
MCP-1	Monocyte chemoattractant protein-1
MDR	Multidrug resistance
MDRP1	MDR protein 1
MET	Proto-oncogene, receptor tyrosine kinase
MGMT	O6-methylguanine-DNA methyltransferase
MHC II	Major histocompatibility II
MiR	Micro ribonucleic acid
MMR	Mismatch repair
MNG	Meningioma
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
m-TORC1	Rapamycin complex-1
MVP	Microvascular proliferation
NCAM	Neural cell adhesion molecule
NFAT	Nuclear factor of activated T-cells
NO	Nitric oxide
NSCLC	Non-small cell lung cancer
NF2	Neurofibromatosis type 2
NK	Natural killer
NKD	Naked cuticle1
PAX5	Paired box 5
PCP	Planar cell polarity
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death protein ligand 1
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PKB	PI3K/ protein kinase B
PLAGL2	Pleomorphic adenoma gene-like 2
PNS	Peripheral nervous system
PSD-95	Postsynaptic density protein-95
PTEN	Phosphatase and tensin homolog
qRT-PCR	Real-time quantitative polymerase chain reaction

RAC	Ras-related C3 botulinum toxin substrate
ROCK	Rho-associated coiled-coil-containing protein kinase
RHO	Ras homolog gene
RQ	Relative quantification
PR	Progesterone receptors
SAPK	Stress-activated protein kinases
SCC	Squamous cell carcinoma
SEM	Standard error means
SFRP1	Secreted frizzled-related protein 1
SV2	Synaptic vesicle protein 2
TAMs	Tumor-associated macrophages
TCF	T-cell factors
TERT	Telomerase reverse transcriptase
TGF-β	Transforming growth factor beta
Th1	T helper type 1
TMZ	Temozolomide
TNF-α	Tumor necrosis factor α
TNM	Tumor-node-metastasis
TRAF7	TNF receptor-associated factor 7
TRH	Thyrotropin-releasing hormone
Treg	Regulatory T cells
Trp	Tryptophan
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VEGF-A	Vascular endothelial growth factor-A
WBC	White blood cell
WHO	World health organization
WNT	Wingless-related integration site
WT	Wild type

1. INTRODUCTION

1.1 The complex structure of the central nervous system

1.1.1 Complex anatomical structure

The central nervous system (CNS) is a complex network of over 100 billion individual nerves which control our senses and movements. It has several particular anatomical characteristics. The brain and spinal cord are the main organs that form the CNS. The nervous tissue is protected by the skull, vertebrae, cerebrospinal fluids (CSF) and meninges. It is intensely delicate and even the smallest force can induce damage. Additionally, the blood-brain barrier (BBB) is another physical and enzymatic protection that prevents any harmful substance to cross from the blood circulation to the CNS. The brain is formed by cerebrum, cerebellum, and brainstem. The cerebrum is the largest part of the CNS and consists of left and right cerebral hemispheres. Even though each cerebral hemisphere tends to handle different roles, the hemispheres are not entirely separate systems [1]. They are constantly communicate with each other via 200 million myelinated nerve fibers that are called corpus callosum [2]. Each cerebral hemisphere consists of a complex outer layer of gray matter, named the cerebral cortex. Underneath the cerebral cortex is an inner core of white matter that is composed of myelinated nerve fibers. Each of these hemispheres divide into four lobes: frontal, parietal, occipital, and temporal [3]. Every lobe has a specific function in the brain [4]. The cerebellum is neuron-rich, as it has 80% of the brain's neurons prearranged in a dense cellular layer [5]. It is the wide part of the hindbrain, which is located in the posterior cranial fossa, behind the fourth ventricle, the medulla oblongata, and the pons. The cerebellum is separated from cerebrum by tentorium cerebelli, which is an extension of the dura mater. It is formed of two hemispheres linked by the vermis and is sub-divided into three lobes flocculonodular, anterior, and posterior. The anterior and posterior lobes are separated by the primary fissure, whereas the posterolateral fissure divides the posterior and flocculonodular lobes [6] (**Figure 1**).

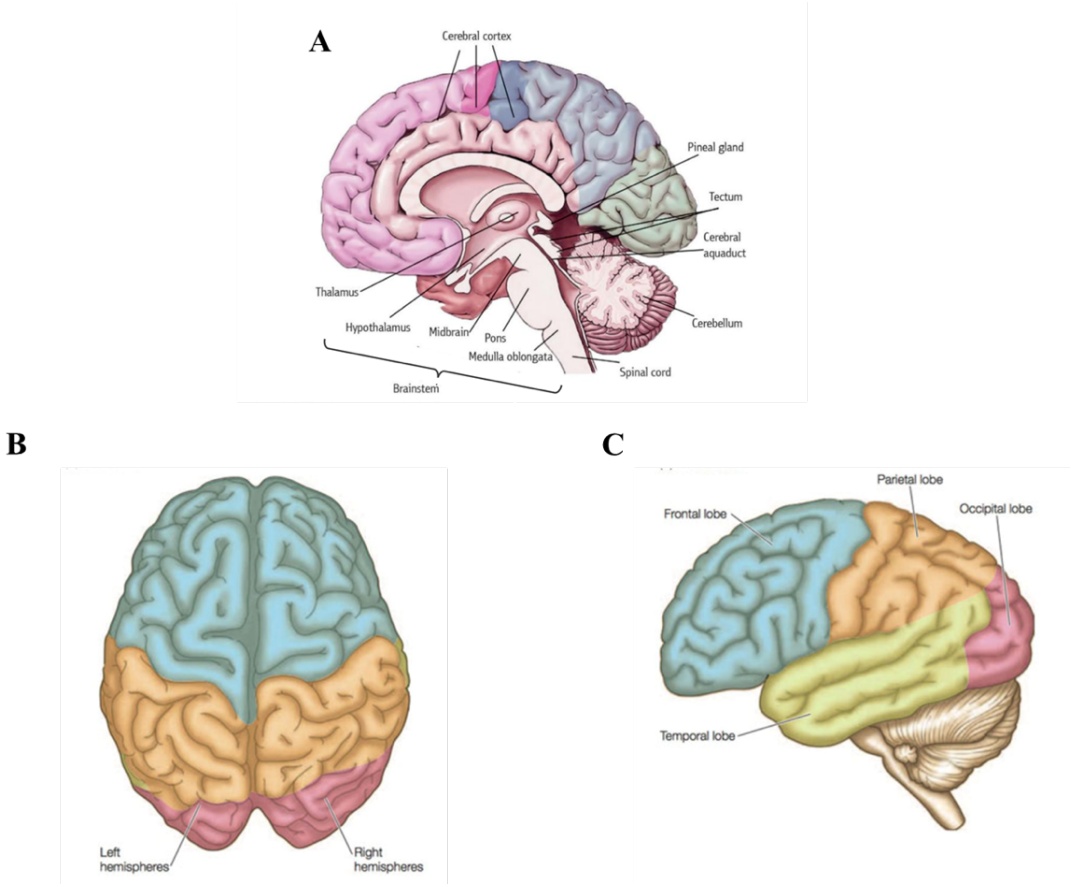

Figure 1. The main anatomical structure of central nervous system. **A**: CNS can be divided into two parts; the brain and the spinal cord. The brain is formed by three continuous sections: cerebrum, cerebellum, and brainstem. **B**: Lateral view of cerebral cortex lobes. It is divided into four lobes: frontal, parietal, occipital, and temporal lobe. **C**: Dorsal view of cerebral cortex. Note that the brain has distinctive shape of two halves; right and left hemispheres [7] [8].

The brainstem is the structure that connects the spinal cord at bottom and cerebellum. It contains several critical collections of grey and white matter. The brainstem is composed of four sections: the medulla oblongata, pons, midbrain, and diencephalon [9]. Diencephalon is a highly developed structure of the human CNS. It is the superior part of the brainstem that is located deep in the brain [10]. Diencephalon consist of four main components: the hypothalamus, subthalamus, thalamus, and epithalamus [11].

1.1.2. Designated function

The CNS is a central computer that controls all functions of the body. As described previously, the CNS is divided into two main parts, the brain and the spinal cord.

The brain. Each part of the brain coordinates particular functions:

The cerebrum is broken down into the right and left hemispheres, those hemispheres are responsible for different behaviors. The right hemisphere is more in control of creativity and spatial abilities. However, the left hemisphere is more dominant with logic, language, and math abilities [12]. Each one of these hemispheres has distinct fissures that divide the brain into four lobes, and every lobe has distinguished function:
Frontal lobe, is the biggest lobe of the brain, located in front of the central sulcus. It is divided into different significant areas. The dorsolateral frontal lobe is separated into three major areas, which include the primary motor cortex, the prefrontal cortex, and the premotor cortex. Any damage to these areas might lead to impaired execution and weakness of motor tasks of the contralateral areas [13].
Temporal lobe, the location of this lobe is on the auditory cortex, which is essential for interpreting the heard sounds and languages. Moreover, the temporal lobe is responsible for processing the information from the auditory sounds as a primary function, also from the senses of smell and taste. Furthermore, it helps in formatting long-term memories, as well as verbal and visual memories [14].
Parietal lobe is located in the center of the somatic sense. This lobe has several main functions; primary plays a significant role in the analysis of somatic sensation (position of the limbs, touch, temperature), analysis of space through all stimulus modalities, and determination of spatial relations for the motor system [15].
Occipital lobe occupies around 12% of the neocortex surface of the brain. It is mainly responsible for vision (movement and color recognition, and visual-spatial processing). Any damage to the occipital lobe may cause visual agnosia or partial or complete blindness, relating to the location and sternness of the damage [16].
Cerebellum plays a role in receiving somatosensory spinal cord and motor signals from the cerebral cortex, also inputs information about balance from vestibular organs inside the inner ear. This integration of the variant signals in the cerebellum organizes the timing, planning, and configurating of the contraction of skeletal muscle during movement activities [17].

Brainstem is the interconnection of the brain to the spinal cord, it is involved with sensory and motor function, also with the regulation of respiratory function, temperature, cardiac function, and consciousness [18]. Each section of the brainstem has a critical function:
Medulla is located at the bottom of the brainstem. It has roles in autonomic functions, some of those functions are critical for survival. The medulla is considered as a

monitor of the respiratory system, through chemoreceptors which are able to recognize any changes in the chemistry of the blood. Moreover, it is the vasomotor and cardiovascular center [12].

Pons is a part of the brainstem. It contains an extensive number of neurons that receive signals from the cerebral hemispheres to the cerebellum. Between, the pons and cerebellum there are many closely related motor functions [17].

Midbrain is the minuscule brainstem component. Many regions of the midbrain play direct role in eye movements whereas, others have an essential role in sensory and motor pathways [19].

Thalamus and hypothalamus are the main parts of the diencephalon. The thalamus has multiple roles in human physiology, it is composed of variant nuclei, where each of them serves a unique function [20]. The thalamus regulates and distributes most of the sensory and motor signals going to the cerebral cortex [17]. The hypothalamus regulates the secretion of hormones and the autonomic nervous system. It contains many types of neurons which release variant hormones such as the thyrotropin-releasing hormone, gonadotropin-releasing hormone, somatostatin, and dopamine. It has extensive efferent and afferent connections with the midbrain, the thalamus, and certain cortical regions that receive signals from the autonomic nervous system [17], [21].

The spinal cord is the most caudal part of the CNS, lodged in the vertebral column of the spinal column. The spinal cord receives sensory signals from the joints, skin, and muscles of the limbs and trunk that have in turn the motor neurons responsible for reflex and voluntary movements. Moreover, it receives sensory signals from the internal organs and controls several visceral functions through a cluster of neurons [17].

The spinal cord has 31 segments, eight cervical, twelve thoracics, five lumbar, five sacral, and one coccygeal [22]. These segmentations consist of 31 pairs of spinal nerves, each spinal nerve branches into two roots; dorsal and ventral [17]. The dorsal nerve roots are composed of sensory neurons, that transmit the signals from the peripheral nervous system (PNS) to the CNS [23]. The ventral roots carry the motor axons that innervate muscles, besides preganglionic, and parasympathetic axons [17].

1.2 Immunology of the central nervous system

1.2.1 Immune cells in the central nervous system

The structural integrity of the brain is highly protected by several layers mentioned above. Under physiological conditions, the CSF maintains the transport of hormones, neurotransmitters, and cytokines. The CSF is produced in the choroid plexus in the ventricles of the brain, and it occupies the subarachnoid space. It flows inside and around the brain via the ventricles and the spinal cord via the central canal. In spite of the CSF has reduced number of white blood cells, a few antigen-presenting cells (APCs) are present. T-cell surface glycoprotein and CD4+ T lymphocytes are also present [24]. The main population of T-cells that enter the CSF from the systemic circulation are activated memory T cells [25].

Dendritic cells (DCs) are specialized in uptake of the antigen, processing, and presentation to T- cells thus induce adaptive immunity. DCs are key cells which can be found in circumventricular organs and meninges within the steady-state CNS [26]. Migration of DCs is coordinated by a certain set of chemokine receptors, which allows response to the expressed chemokines. In peripheral tissues, under homeostatic state, inducible chemokines are expressed at low levels. Otherwise, the expression of this chemokines increases drastically, to facilitate the influx of DCs [27]. Macrophages are myeloid cells, that have distinct phenotypes and perform different and opposing functions. They survey the local and immediate environment by intake and degradation of dead cells and potentially hazardous agents. Macrophages are present in most tissues and circulate throughout the body to maintain tissue homeostasis [28]. Several myeloid cells are present in the CNS, such as choroid plexus and meningeal macrophages. Those macrophages are likely to originate from yolk sac and/or the fetal liver [29]. It was reported that these macrophage populations share many myeloid and macrophage-specific markers (CX3C chemokine receptor 1 (CX_3CR1) and human epidermal growth factor (EGF)-like module containing mucin-like hormone receptor 1 (EMR1)); but have varied functions.

Strategically placed at the CNS barriers such meningeal, perivascular, and choroid plexus, macrophages modulate the phenotype of immune cells and the entry during inflammation [24]. In a homeostatic state, peripheral macrophages tend to be absent in the CNS. However, during inflammation and damage of the CNS damage,

peripheral macrophages can infiltrate the CNS parenchyma by breaking down the BBB [30] (**Figure 2**).

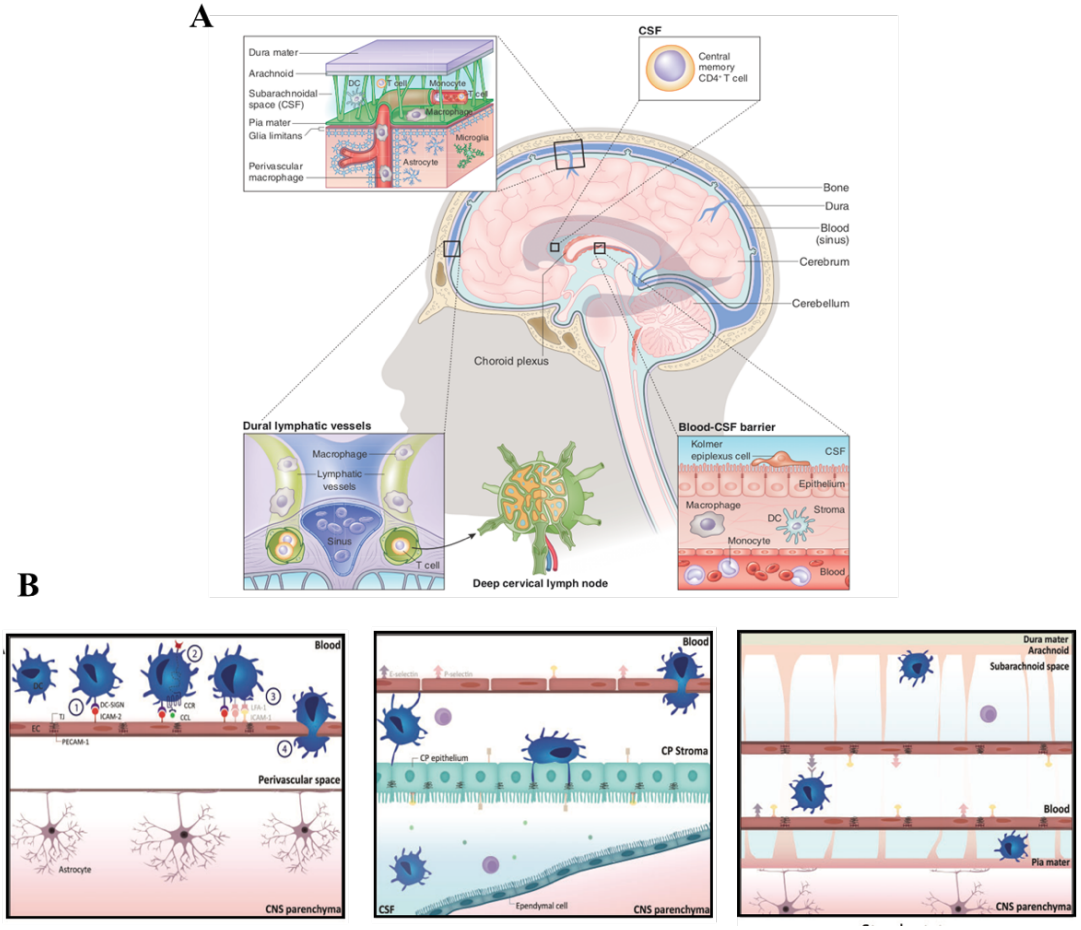

Figure 2. A schematic illustration of the neuroimmune system during homeostasis. **A**: Cells involved in ensuring CNS integrity in steady-state [24]. **B**: Transmigration paths of DCs into CNS via choroid plexus and meningeal vessels in steady-conditions [27].

1.2.2 Unique immune environment of the brain

In recent decades, research the focus on physiological interactions of the immune system and the CNS has intensified. The physiology of the immune system in the CNS- has unique features. To begin with, the entire region of the brain parenchyma is excluded from the peripheral immune system. Moreover, the baseline parenchymal immunity is replaced by the tissue-resident macrophages of the brain and microglia [31]. Microglia are tissue resident macrophages, responsible for immune surveillance and innate immunity within the CNS. Microglia represent around 10% of the total glial cells. They are more enriched in the gray than the white matter. Resting

ramified microglia are activated through the identification of amyloid beta, interferon gamma (INF-γ), and lipopolysaccharides. Afterwards, they can initiate the innate immune response via upregulation of immunomodulatory surface proteins, chemokine and cytokine production, such as monocyte chemoattractant protein-1 (MCP-1), nitric oxide (NO), tumor necrosis factor α (TNF-α), interleukin 1 (IL-1), and interleukin 6 (IL-6). The above activity results in the breakdown of the BBB and allows systemic immune cells to enter. The expression of major histocompatibility II (MHC II), CD80, CD86, and CD40 on activated microglia interacts with T-cells and induce their activation. They can also induce T-cell apoptosis through the Fas pathway [32]. Astrocytes are an abundant cell type in the CNS. The main task of these cells is to preserve the physiological homeostasis of neurons [33]. In normal and inflamed CNS, astrocytes play a role similar to immune cells in both innate and acquired immune responses.

During inflammation, astrocytes contribute to the penetration of lymphocytes to the CNS via effecting BBB permeability through expression of adhesion molecules vascular cell adhesion protein 1 also known as vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), and also by secretion of chemokines such as C-X-C motif chemokine ligand (CXCL), CXCL10, CXCL8, C-C motif chemokine ligand (CCL) CCL2, and CCL5. Astrocytes can also express MHC I and II molecules, CD80, CD86 to activate $CD4^+$ and $CD8^+$ T-cells. However, astrocytes can suppress the activity of T-cells by inducing regulatory T-cell differentiation. Additionally, they upregulate the expression of cytotoxic T lymphocyte antigen (CTLA-4) on activated T-cells [32]. Recent studies have described the mechanisms used to regulate T-cell activation by neurons. Neurons have the ability to release soluble factors such as; cytokines, neuropeptides, and soluble Fas ligand, therefore they can decrease the activation of microglia and T-cells [32].

1.3. Tumors of the central nervous system

Primary brain tumors are a various group of neoplasms arising from different cells of the CNS [34]. They are either benign or malignant. Benign brain tumors grow relatively slowly and do not tend to invade other parts of the brain tissues. Malignant brain tumors are fast growing tumors and tend to spread to other areas of the brain and are considered to be the most aggressive tumors [35]. More than 30% of patients with

primary cancers in other parts of the body also develop brain metastases. Of all cancer types, lung cancer has the highest metastatic rate to the brain (about of 20%) [36].

1.3.1 Brain tumor types

Due to the biological and histological heterogeneity, brain tumors are divided into two main groups; glioma and non-glioma [37] (**Figure 3**).

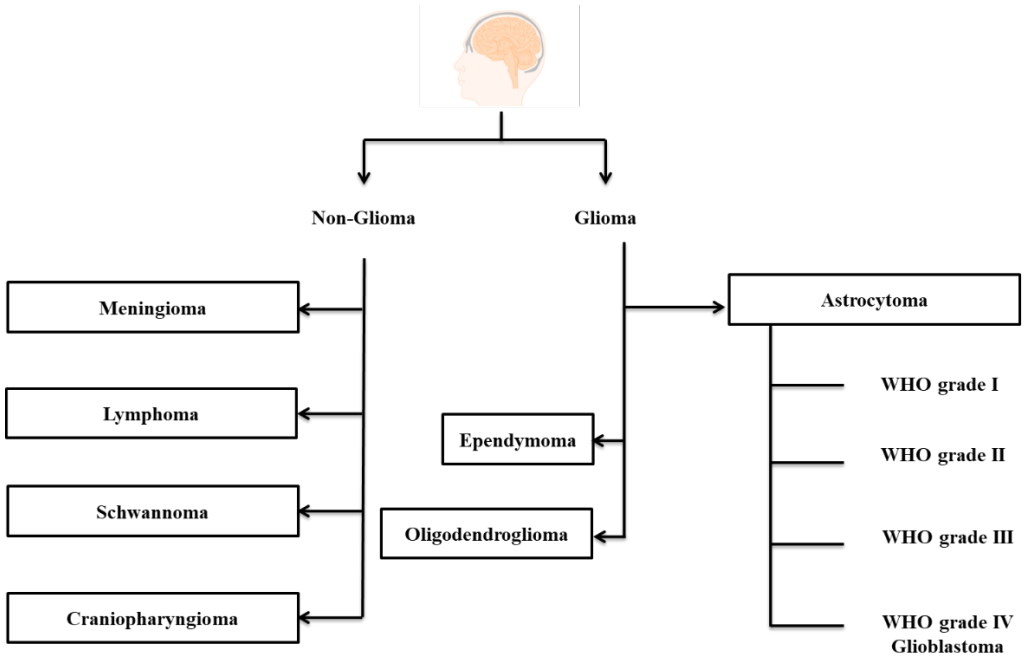

Figure 3. Different types of brain tumors, figure adapted from [37].

1.3.1.1 Glioma

Glioma is a malignant primary brain tumor, originating from transformed progenitor glial or neural stem cells [38]. The World Health Organization (WHO) has characterized glioma into two sub-groups; low-grade glioma (LGG) grade I and II and high-grade glioma (HGG) grade III and IV, depending on the histological and the genetic features [39]. According to the phenotype, glioma can be classified into astrocytic, ependymal, and oligodendrocyte subtypes [40]. Dysfunction of the BBB could occur under any pathological condition, brain tumors. In LGG, the function and the vascularization of blood-brain tumor barrier (BBTB) stay intact and resemble the normal conditions of BBB. Nevertheless, HGG is characterized by critical alterations to normal vascular function [41].

1.3.1.1.1 Astrocytoma

Astrocytoma is a category of glioma originating from a particular type of glial cells called astrocytes. It accounts for the vast majority of gliomas (75%) [42]. The WHO classified astrocytic tumors into different degrees based on their level of aggressiveness including pilocytic astrocytoma (grade I), diffuse astrocytoma (grade II), anaplastic astrocytoma (grade III) and glioblastoma (grade IV) [43].

1.3.1.1.2 Glioblastoma

Glioblastoma (GBM) is considered as the most aggressive malignant form of astrocytic lineage [44]. It displays extensive molecular and cellular heterogeneity [45]. GBM represents 16% of all primary brain tumors and more than 54% of all glioma types [46]. Based on several characteristics, glioblastoma can be subdivided into primary and secondary types [47]. Primary glioblastoma is the most frequent subtype which occurs widely in patients over the age of 50. From the beginning of the tumor, it is considered grade IV glioblastoma. Generally, the secondary glioblastoma arises in young patients and/or from the progression of LGG anaplastic astrocytoma or diffuse astrocytoma [41].

GBM remains an irremediable disease with a median overall survival rate of 15 months, even though the use of a combination of therapies [48]. The poor survival outcome in GBM patients were correlated to the molecular aberrations due to mutations in distinct genes. Around 40% of GBM tumors are distinguished by overexpression and amplification of epithelial growth factor receptor (EGFR) [37]. Genetically, primary GBM is characterized by gene alteration of EGFR, telomerase reverse transcriptase (TERT) promoter mutation, phosphatase and tensin homolog (PTEN) deletion or mutation. One of the most critical gene alterations in secondary GBM and also in LLG is a mutation in isocitrate dehydrogenase (IDH) [49]. O^6-methylguanine deoxyribonucleic acid (DNA) methyltransferase (MGMT) is another marker which is ordinarily tested as part of the routine clinical examination in GBM patients [50]. MGMT is a DNA repair enzyme that effectively protects cells against methylating agents such as temozolomide (TMZ) [51]. MGMT promoter methylation is mainly abundant in secondary GBM with 75% versus 36% in primary GBM. Consequently, methylation of MGMT promoter in patients allow alkylating agents to be more effective [50].

The high metabolic rate of GBM creates hypoxic areas that increase the expression of angiogenesis and vascular endothelial growth factor (VEGF). Hence, this leads to the formation of abnormal vessels, which dysfunctions the BBTB [52]. Beyond that, GBM is known to activate the efflux of molecules and induce non-uniform permeability of the BBB [53], [54].

1.3.1.2 Non-glioma

Non-glioma is a group of tumors that originate from other type of brain cells except glial cells. These tumors include medulloblastomas, pituitary adenomas, meningiomas and central nervous system lymphomas.

1.3.1.2.1 Medulloblastoma

Medulloblastoma (MB) is a highly aggressive malignant tumor which represents approximately 10% of pediatric brain tumors. It is noted that the incidence of MB is highest in children among 3-4 and 8-10 years of age. It is reported that up to 30% of cases are adults [55]. MB begins in the cerebellar vermis and spreads to form a variable size of tumors along the ventricles [56]. Understanding the molecular mechanisms of MB allows understanding of its pathophysiological mechanisms. MB is divided into four subgroups according to the affected signaling pathways [57].

1.3.1.2.2 Pituitary adenoma

Pituitary adenoma is a slow-growing tumor that causes no metastasis. Generally, pituitary adenomas are divided into two groups; functioning and non-functioning tumors. Functioning tumors are endocrine-active tumors, representing around 70% of pituitary adenomas, and are further classified based on their secretory products [58]. Non-functioning tumors include almost 30% of all pituitary adenoma. They are endocrine-inactive tumors which don’t secret pituitary hormones [59].

1.3.1.2.3 Meningioma

Meningioma (MNG) arises from the meninges consequently is not a brain tumor but it constitutes 20-30% of all primary intracranial tumors [60]. Meningioma arises from the arachnoidal cap cells of the leptomeninges [61]. According to the WHO, 70-80% meningioma is generally benign (grade I). However, 5-20% have the potential to become atypical (grade II) and 1-3% malignant tumors (grade III) [62].

Most meningiomas are caused by an inactivating mutation in the neurofibromin 2, or NF2 gene [63], but recently further mutations have also been

discovered including TNF receptor-associated factor 7 (TRAF7), Krüppel-like factor 4 (KLF4), v-akt murine thymoma viral oncogene homolog1 (AKT1), and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) were also reported in some meningiomas [64]. Additionally, down-regulation of micro ribonucleic acid (miR)-200a has been identified as an inducer of cell proliferation in meningiomas, through increasing the expression of β-catenin and cyclin D1 (Wingless-related integration site (WNT)/β-catenin signaling pathways) [2]. Although meningiomas are mostly not malignant, their continuous growth and intracranial location often leads to serious or even lethal consequences [65].

Elderly women have the highest incidence of this type of tumor [66]. It has been proposed that female sex hormones affect the development and growth of meningiomas [67]. The first cytological analysis of meningioma revealed that 10% of meningioma contains estrogen receptors (ER) and two-thirds of meningioma contains progesterone receptors (PR) [68]. It was found that in women there is a strong association between breast carcinoma and meningioma [69]. More recent evidence demonstrated that each anatomic location of meningioma have different immune landscape [70].

1.4 Incidence of brain tumors

Although brain tumors are rare, they are among the highest causes of mortality [71]. Globally, there were 330,000 incidents of CNS cancers and about 227,000 deaths in 2016. East Asia is considered as the region with the highest incidence (108,000 cases) for both genders, followed by Western Europe (49,000 cases) and South Asia (31,000 cases) [72]. Between 1990 and 2016, the United States had the highest incidence of brain tumors after China [73]. Worldwide, around 240,000 cases of primary CNS and malignant brain tumors are reported per year. In 2019, approximately 86,970 patients were diagnosed in the United States with primary brain tumors, among which 26,170 patients were diagnosed with primary malignancy and 60,800 with non-malignant tumor [74]. CNS tumors in young adults and children account for 20% and 30% of cancer deaths, respectively [71]. According to estimates from the WHO in Hungary, 765 brain and CNS cancer cases and almost 661 cancer deaths occurred in 2020 [75].

1.5 Survival of CNS tumors

The prognosis for brain tumor is extremely dependent on their grade (**Figure 4**). Generally, brain tumors are organized according to the grading systems that order them from least grade (grade I) to the most aggressive grade (grade IV), as previously described (**section 1.3.1**) [36]. Only, 40% of people live longer than one year after being diagnosed with a malignant brain tumor, and even less (around 20%) live for 5 years [76].

Regardless of the age, the gender of the patient is considered as the most important factor that affects the incidence of brain tumors [36]. In the United States, it was reported for the same period, only 36% of benign brain tumors occurred in men compared to 64% in women. However, around 55% of malignant brain tumors ensued in men compared to 45% in women [77].

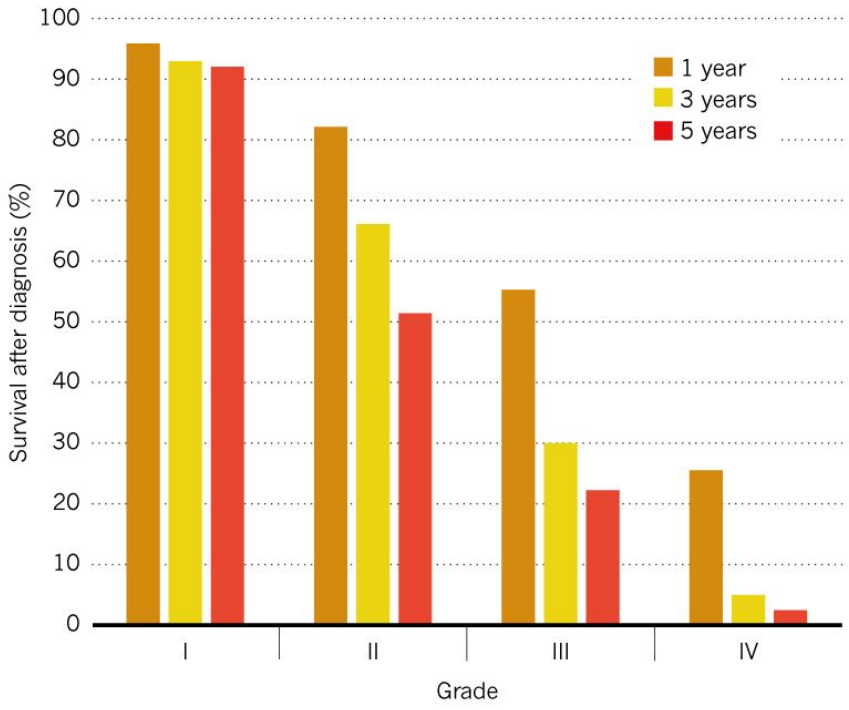

Figure 4. The relation between the survival rate and the grade of the brain tumor[36].

1.6 Current therapeutic approaches for tumors in the CNS

Environmental and genetic factors are both play a role in the pathogenesis of brain tumors [78]. The treatment is highly dependent on the stage of the tumor. The therapeutic regimen is chosen very carefully based on several factors such as the type

and stage of the tumor, age and overall health of the patient, and rate of progression [79]. Despite recent advances in medical knowledge, there is still no specific and highly effective drug targets for brain tumors.

1.6.1 Surgery

The first and the most common treatment modality for patients diagnosed with brain tumors is surgery. It helps to reduce the pressure on the CNS and enhances the efficacy of chemo- and radiation therapy. Patients with low grade primary tumors can be effectively cured with surgery if the tumor is at an operable location. However, high grade tumors are often treated with radio- and/or chemotherapy before and also after surgery [80]. Combination therapy is routinely applied in higher grade gliomas (grade II and VI) [81].

Beside the grade of the tumor, its location also has an effect on the survival of the patient. After surgery, the survival rate of patients with glioblastoma located in temporal and parietal lobes are shorter than those with frontal lobe glioblastomas [79].

In spite of the acceptance of surgery as treatment of brain tumors, surgery has evident limitations. These include reduced or lost body functions caused by damage to the brain tissue surrounding the tumor [82]. Although new surgical techniques and tools have been developed to help the surgeons locating the tumor more precisely (e.g. magnetic resonance imaging (MRI)-guided laser ablation and channel-based resections), it is improbable that all brain tumors could become surgically removable [83].

1.6.2 Radiotherapy

Radiotherapy can cause DNA damages to both normal and tumor tissues [84]. The three main forms of radiation therapy are proton therapy, high energy X-ray, and gamma knife radiosurgery [79]. In the clinical setting, an external beam of radiation is the most commonly used approach.

Biologically, the radiation can affect cellular DNA directly or indirectly through free radicals derived from excitation and ionization of the water element of the cells [85]. Neuro-oncologists face a huge controversy about the role of radiotherapy in treating LLG patients [86]. In inoperable and non-completely resected LGG cases, radiation is the treatment of choice [87]. The majority of HGG cases are treated with combination therapy, including postoperative adjuvants; radio and/or chemotherapy [88]. Unfortunately, not all tumor cells are sensitive to the radiation.

Hypoxic tumors are considered more radioresistant, because their DNA is less likely to be damaged [84]. Additionally, meningiomas (grade II and III) are also treated with radiotherapy after surgical resection. However, frequent recurrence of meningiomas require re-treatment (re-resection and re-irradiation) [89].

1.6.3 Chemotherapy

Through different mechanisms chemotherapy is used to manage tumors, including blocking a distinct signaling pathway, and depleting nutrients critical for cell growth. It is a major challenge to treat brain tumors with chemotherapy due to the presence of the BBB [79].

There are different strategies to deliver chemotherapeutic drugs to the brain tumors:

Localized chemotherapy

In the United States, the only chemotherapeutic agents approved as local treatment for brain tumors is carmustine wafer also called BCNU wafer (Gliadel® wafer) [79]. It is an alkylating drug, able to bind the DNA via O^6 position of guanine causing cell damage by preventing DNA replication. Polifeprosan 20 is the matrix where the active ingredient is embedded (3.85% of BCNU). The wafers implanted into the surgical cavity, which after a while degrades to release the active component [90]. Although BCNU brakes down the BBB, it has several limitations, among them is the short half-life of the drug (half-life of 15 min) and its low bioavailability. Moreover, many studies have reported serious intracranial infections and leakage of CSF in the group treated with Gliadel (commercial name of carmustine) [91]. It has also been reported that carmustine wafer extends the survival of HGG patients with just a few months (2-4 months) [92], [93]. Due to all of the adverse reactions, brain tumor patients tend to seek other medical strategies [79].

Systemic chemotherapy

The effectiveness of systemic chemotherapy in the brain is limited due to the existence of the BBB [94].

The chemotherapeutic drug most frequently used to treat tumors of the brain is temozolomide (TMZ) (commercially called Temodar). TMZ is an orally applied alkylating agent and it induces cell cycle arrest [95], [96]. In 2005, Temodar was approved by the United States Food and Drug Administration (FDA) as a promising

treatment for anaplastic astrocytoma [97]. It has also been licensed in Europe as a therapy for refractory HGG [46]. TMZ is most effective in GBM patients who lack the expression of MGMT [98]. The anti-tumor effect of TMZ is due to its ability to methylate DNA at O^6-guanine (5%), N^7-guanine (>70%) and N^3 adenine (>9%) [99] (**Figure 5**).

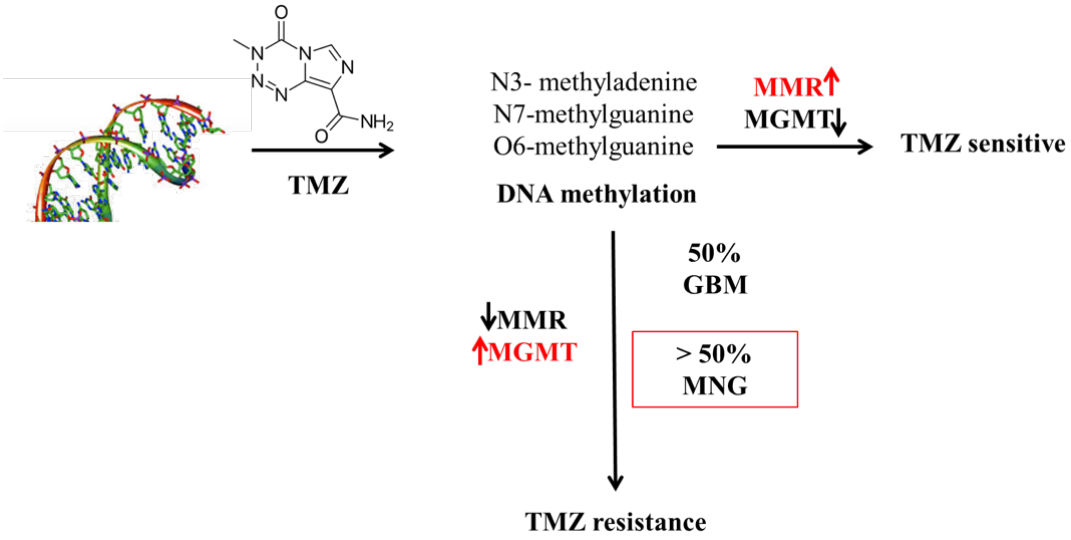

Figure 5. Temozolomide and temozolomide resistance mechanism**.** MGMT overexpression limit temozolomide efficacy. 50% of glioblastoma patients have up-regulated MGMT gene. Image adapted with modification from [100].

1.6.4 Hormone therapy

Several studies revealed that meningioma tumors express female sex hormone receptors. Less than 31% express estrogen receptors, while around 70% express progesterone receptors [101]. Therefore, hormonal therapies became a therapeutic approach [102].

Mifepristone (RU486), a progesterone receptor inhibitor showed some beneficial effect in certain inoperable meningioma patients. However, there is no clear evidence to recommend mifepristone for all recurrent or inoperable cases [103][104]. Similarly, inhibition of estrogen receptors using tamoxifen showed little benefits [105]. Thus, hormonal therapies have a limited role and have shown to be mostly ineffective [106].

1.6.5 Immunotherapy

Life quality of a brain tumor patient is significantly reduced, due to long-term toxicities and the adverse effects of the standard treatments. There are a limited number of immunotherapy clinical trials for brain tumors and currently immune checkpoint inhibitors (ICI) are still under investigation [107].

In preclinical trails, ICI-s present promising results for GBM treatment [108]. In 2011, the FDA approved ipilimumab, an anti–cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) antibody which can effectively activate the immune system against melanoma [109]. It is an immunoglobulin G1 (IgG1) human monoclonal antibody directed against CTLA-4 that leads to enhance T-cell activation [110]. In 2014 the FDA approved the first anti-programmed death-1 (PD1) antibody, namely, nivolumab for treating patients with advanced stages of non-small cell lung cancer (NSCLC) [111]. It is a fully human monoclonal antibody able to inhibit immunoregulatory cell surface receptor protein PD-1. It is expressed highly on the surface of $CD4^+$ and $CD8^+$ T-cells that are chronically exposed to antigens [112]. Programmed death ligand-1 (PD-L1) is the ligand of PD-1, its' interaction maintains peripheral tolerance. PD-L1 is expressed by tumor cells to evade immune elimination [113]. The binding of PD-1 to PD-L1 recruits tyrosine phosphatases which generates an inhibitory signal and blocks the downstream effects of phosphoinositide 3-kinases (PI3K)/ protein kinase B (PKB) [114]. In the last few years, the use of anti-PD-1 has highly increased, contrary to the rarely used anti-CTLA-4 for GBM in clinical trials. The combination of ICI-s (anti-PD-1 and anti-CTLA-4) showed a higher efficacy in melanoma compared to the monotherapy. Recently, trials are on ongoing in pediatric and adult populations, one of these trials aims to assess the effect of ipilimumab in pediatric GBM. Notably, none of these trials have considered the PD-L1 expression in the selected patients [107], [115].

1.6.6 Inhibition of vascularization

In 2009, the FDA has approved recombinant humanized monoclonal antibody called Avastin (bevacizumab) as a treatment for recurrent GBMs. It blocks angiogenesis through inhibition of VEGF-A. The overall survival of patients who received bevacizumab was 9 months compared to those who didn't receive the drug with 6.1 months. With this monoclonal antibody the treatment regime could increase the survival rate to 41% instead of 18% [79].

1.7 Chemotherapy resistance

Chemoresistance is the ability of cancer cells to avoid response to chemotherapy and the tumor cells carry on proliferating. Statistical data revealed that over 90% mortality of tumor patients is attributed to drug resistance. Multidrug resistance (MDR) of cancer cells can be associated with a variety of mechanisms including increased activation of drug transporters [116], [117].

1.7.1 Drug transporters

One of the largest drug transporter families is the adenosine triphosphate (ATP)-binding cassette (ABC) transporter family. ABC transporters are transmembrane proteins that transport a range of substrates across extra/intra-cellular membranes [118]. In humans, 49 different ABC transporters were identified [119]. They are divided into 7 subfamilies, from A to G, based on their domain organization and sequence similarities [120], [121]. Many studies focus on the influx and efflux mechanisms to evaluate the potency of drugs in tumor therapy. Current researches focus on ABCB1 and ABCG2, members of ABC transporter family which are highly involved in drug resistance [122], [123]. ABCB1, also called multi-drug resistant (MDR) protein 1 (MDRP1) and is abundantly expressed in cell membranes of the kidneys, liver and blood-barriers [124]. In lymphocytes and also in other immunological components MDRP1 have a putative role in trafficking cytokines [125]. ABCG2 gene encodes the breast cancer resistance protein (BCRP), and it is an ABC efflux transporter [126]. ABCG2 protein is expressed on the apical cell membranes of the brain, placenta and many other organs [127]. ABCG2 is widely expressed in a population of stem cells derived from the bone marrow, where it acts as a protective barrier against oxidative stress that may cause genetic damages [128].

1.7.2 Expression of drug transporters in tumors

Over time, tumor cells have the ability to develop resistance against structurally and mechanistically unrelated drugs [129]. ABCB1 and ABCG2 are often related with the development of MDR in human cancers where it could result in relapse [130], [131]. They are highly expressed in solid tumors including lung and breast cancers [132] as well as in blood tumors such as chronic lymphocytic leukemia, acute lymphocytic leukemia, and multiple myeloma [133]–[135]. Higher expression of ABCB1 and ABCG2 has been correlated with reduced chemosensitivity.

1.7.2.1 Expression of drug transporters in lung tumors

In lung carcinoma (LC), resistance to chemotherapy is a major obstacle to achieve a successful treatment. This tumor is the common cause of cancer related deaths worldwide. Adenocarcinoma (AC) of the lungs is the most common subtype of non-small cell lung cancer (NSCLC), which represents around 40% of all LC cases [136].

Although platinum-based drugs can increase the survival rate of LC, MDR remains a major issue [137], [138]. The treatment protocol is mainly based on the combination of cisplatin, as a main drug, with paclitaxel, gemcitabine, vinorelbine, or docetaxel to increase the effectiveness [139]. However, it has been revealed that cisplatin via the induction of canonical WNT signaling pathway can affect the expression of ABCB1 and ABCG2. Overexpression of both of these transporters modulate drug response [123]. Interestingly, the expression pattern of drug transporters might be influenced by the background mutation and molecular microenvironment of the tumor. The expression as well as the activity of ABCB1 and ABCG2 drug transporters alteration are determined by the mutation background (EGFR or kirsten rat sarcoma viral oncogene homologue (KRAS)).

1.7.2.2 Expression of drug transporters in brain tumors

One of the complications associated with CNS tumors is their weak response to anti-neoplastic drugs [140]. The effectiveness of chemo and immunotherapy is impaired by the BBB/BBTB [141], [142]. Interestingly, the expression of ABC transporters in BBB/BBTB has directly been related to chemoresistance versus several of their anticancer drug substrates [143].

Although actively investigated [140], [144] there is still limited knowledge about the expression and the activity of ABC transporters in CNS tumors. Certainly, ABCB1, ABCG2, ABCC1, ABCC4, and ABCC5 up-regulation has been reported in glioma cells in recent research [53], [140], [145]. Thus, brain tumors can be regarded as being located behind a multibarrier system, which defends the cancer cells from chemotherapeutics agents [146].

1.7.2.3 Regulation of ABC drug transporter expression

It has recently been discovered that expression of the ABC drug transporter family is regulated by the WNT signaling pathway [147]. The canonical WNT pathway seems to play the most important role in the process [148]. It has been

detected that the expression of ABCB1 and ABCG2 transporters can be upregulated via modulation of the canonical WNT signaling pathway in NSCLC [123]. Additionally, WNT/β-catenin signaling regulates the expression of ABCC3 which affects the sensitivity of colon cancer cells to anticancer agents [149].

1.8 Molecular regulation of WNT signaling in brain tumors

Recently, the WNT pathway has been recognized as a significant regulator of many biological processes such as: embryonic development, maintenance and differentiation of stem cells, and self-renewal [150]. Any abnormalities in this signaling pathway leads to pathological conditions including carcinogenesis [151].

The WNT family in human beings contains nineteen secreted glycol-lipo-protein ligands. WNT ligands interact with several receptors. Their primary receptors are the ten Frizzled (FZDs) transmembrane receptor proteins. They consist of approximately 500 to 700 amino acids. The extracellular chain of a FZD protein has a cysteine-rich domain (CRD). The CRD is the binding site of the WNT molecules. FZDs are related to the atypical G protein-coupled receptors (GPCR) superfamily. They contain conserved seven hydrophobic domains (**Figure 6A**) [152]. Low-density lipoprotein receptor-related protein (LRP) is an essential co-receptor in WNT signaling. Structurally, LRP is similar to other members of the low-density lipoprotein (LDL) receptors. LRP recognizes around thirty different biological ligands. The ligand binding site in LRP occurs in four distinctive clusters (**Figure 6B**). Most of the LRP ligands bind in cluster II and IV [153]. WNT signaling is divided into β-catenin dependent (canonical) and β-catenin independent (non-canonical) WNT signaling pathways [154].

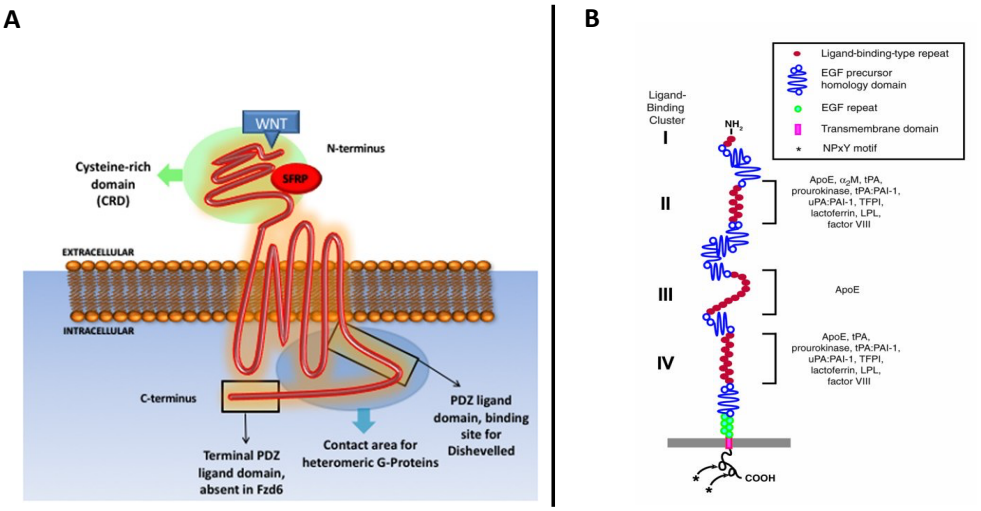

Figure 6. FZD and LRP receptor structures. **A**) FZD protein domains and ligands interaction areas [155]. **B**) Binding of LRP ligands in different clusters [153].

1.8.1 Beta-catenin dependent WNT signaling

β-catenin was known as cadherin-binding protein. It plays an important role in the regulation of cell-cell adhesion. Moreover, it is a main mediator of WNT signaling [156]. Generally, in the absence of WNT ligands, β-catenin destruction complex is formed. It mainly consists of adenomatous polyposis coli (APC), axis inhibition protein (axin), casein kinase 1 (CK1), and glycogen synthase kinase 3-beta (GSK-3-beta). β-catenin is phosphorylated by this complex at the serin and threonine sites leading to its ubiquitination and degradation in the proteasome (**Figure 7A**) [157]. In the presence of the WNT molecules, the induction of dimerization of FZD and LRP5/6 is caused by ligand-receptor-coreceptor interaction. This leads to disheveled (DVL) activation and recruitment of the destruction complex to the cell membrane (**Figure 7B**). Consequently, activated DVL directly inhibits GSK-3β-APC-axin complex by assisting the interaction between axin and LR5/6 [158]. Cytoplasmic accumulation of β-catenin enters the nucleus, where it interacts with transcription factor family of T-cell factors (TCF1, TCF3, TCF4) and lymphoid enhancing factor (LEF) [159].

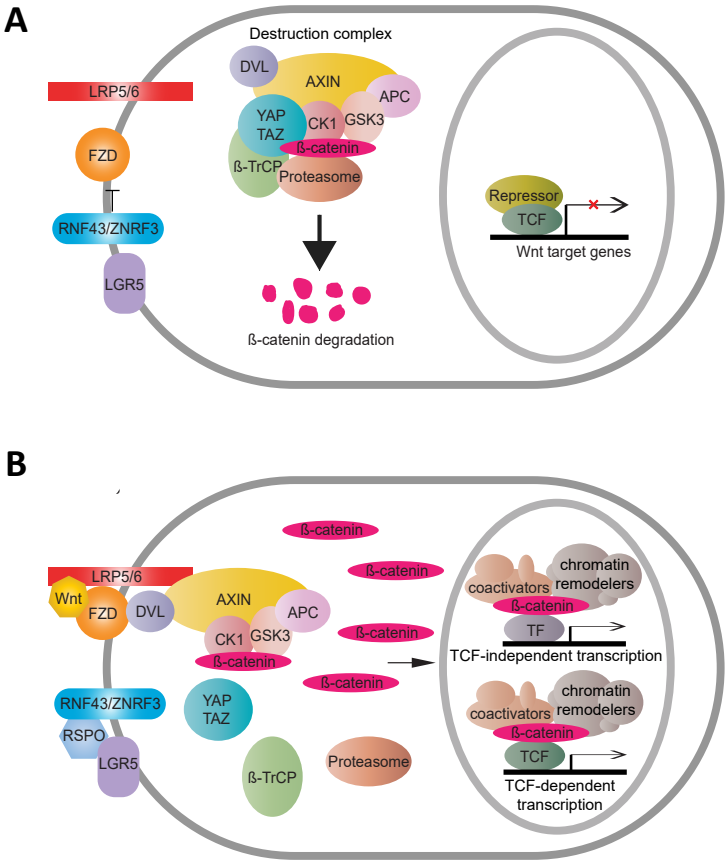

Figure 7. WNT canonical pathway. **A**) in the absence of WNTs, β-catenin is degraded by the destruction complex. **B**) in the presence of WNTs, the dissociation of β-catenin from the destruction complex [158].

1.8.2 Beta-catenin independent WNT signaling

Mostly, β-catenin independent WNT signaling includes two pathways, the WNT/Ca^{2+} and the planar cell polarity (PCP) pathways. Both of these pathways are activated by various WNT ligands including WNT4, WNT5A, WNT7B, WNT11, and WNT16 [157].

WNT/Ca^{2+} pathway is initiated via increasing the intracellular concentration of Ca^{2+} and activating protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (CaMKII) [156]. This pathway regulates transcription factors including nuclear factor of activated T-cells (NFAT) [160]. WNT5A is a prominent member in WNT/Ca^{2+} pathway. It initiates the signal transduction through DVL3. WNT5a activates CaMKII and PKC via induction of an intercellular release of Ca^{2+}, and regulates different cell functions such as cell migration [156].

In the PCP pathway, binding of WNT ligands to the receptor complex leads to the activation of DVL. Activated DVL is able to transduce the signals via activation of small guanosine triphosphate (GTP)-ase of the Ras homolog gene (RHO) subfamily (Rho, Ras-related C3 botulinum toxin substrate (RAC), Cell division control protein 42 homolog (CDC42)). PCP pathway is divided into different branches, which activates different downstream targets [161]. Exemplified, WNT11 induces the activation of RHOA and RAC1. In turn, RHOA and RAC1 activate stress-activated protein kinases (SAPK), including Rho-associated coiled-coil-containing protein kinase (ROCK) and Jun N-terminal kinase (JNK). In order, this initiates reconstruction of the cytoskeleton leading to the modification of cell motility and adhesion [157].

1.8.3 WNT pathway in the central nervous system

WNT signaling has an important role in different aspects of the CNS. WNT molecules are secreted morphogens that control embryonic patterning. WNT1, WNT3A, WNT8C ligands, inhibitors including secreted frizzled-related protein 1 (SFRP) and dickkopf WNT signaling pathway inhibitor 1 (DKK1) are the important WNT signaling components in the tissue patterning process [162]. The WNT pathway plays a critical role in various stages of brain development (e.g. the hippocampus) [163]. At the level of the synapse, WNT pathways remain essential in mature brains. Recent research using mouse brains has revealed that even in the postnatal life the cortex-hippocampal circuit and the synaptic functions are closely regulated by WNT signaling [164]. In the cerebellum, WNT7A is known to increase the clustering of presynaptic proteins such as synaptic vesicle protein 2 (SV2), synaptotagmin, and synaptopysin, but has no effect on postsynaptic clustering [165], [166]. Furthermore, WNT7A and WNT3A induce exocytosis and recycling of synaptic vesicles to increase the synaptic transmission in hippocampal neurons [167]. Beyond that, WNT5A increases postsynaptic density protein-95 (PSD-95) clustering through WNT/JNK signaling pathway [168]. PSD-95 is a member of the membrane-associated guanylate kinase (MAGUNK). It is a multiprotein complex which interacts with molecules that participate in the regulation of glutamate receptors [169]. In the hippocampus, WNT5A regulates the inhibitory synapses, expression of gamma-aminobutyric acid (GABA) receptors and stimulates the recycling of activated $GABA_A$ receptors via

triggering CaMKII [163], [170]. In conclusion, it is well accepted that WNT signaling plays a vital role in the development and maintenance of the CNS.

1.8.4 WNT pathway in meningioma

Only a few studies focus on the involvement of WNT signaling pathway in meningioma [171]–[173]. It has recently been reported that some FZD receptor levels had a 3.7 fold increase in meningioma compared to leptomeningeal cells [174]. Based on microarray expression data [175], the expression of β-catenin is higher in anaplastic meningioma than meningioma grade I. Additionally, messenger RNA (mRNA) expression level of secreted FZD-related protein 1 (SFRP1) is significantly lower in recurrent and advanced meningioma compared to the primary meningiomas [176], [177]. Genomic and transcriptome analysis demonstrated that SFRP1, SFRP3, FZD7, and TCF3 are involved in malignant transformation of meningiomas [178], [179]. Forkhead box M1 (FOXM1) is a pro-mitotic transcription factor that is necessary for cell proliferation during development. It was discovered that the FOXM1/WNT signaling axis plays an important role in aggressive meningioma [180].

1.8.5 WNT pathway in glioblastoma

Recent reports have found that 13% of GBM cases have APC mutations [181]. FAT atypical cadherin 1 (FAT1) is negative regulator of WNT pathway. Mutations in FAT1 influence WNT signaling in GBM [182]. Another study has identified the epigenetic alteration that affects WNT pathways. Comparison of the micro-RNA expression profile in GBM versus normal brain revealed that the expression of miR-770-5p and miR-138-2-3p are significantly different [183]. The reported changes in miRNA expressions in GBM modulate β-catenin in many other tumor types such as hepatocellular carcinoma. *In vitro*, the expression of miR-34a in GBM stem cells induces the degradation of β-catenin [184]. In contrast, MIR22HG (long non-coding RNA) produces miR-22-3p and miR-22-5p which are responsible for WNT signaling activation. GBM stem cells have shown overexpression of MIR22HG [185]. Pleomorphic adenoma gene-like 2 (PLAGL2) is known as a protooncogene in malignant glioma. It induces the activation of β-catenin/WNT pathway through WNT6, FZD2, and FZD9 to contribute to stemness in GBM [186].

2. Aims of the study

The main goal of the current study is the comparison of the two main types of brain tumors, namely meningioma and glioma at the levels of the immune and the WNT signaling microenvironment.

To achieve that, we focused on the following well defined targets:

1. Identification of the phenotype of infiltrating immune cells into grade I meningioma and grade IV glioma.

- Based on the type of the markers presented on the immune cell surfaces, the expression of $CD3^+$ T, $CD56^+$ NK cell and $CD19^+$ B cell were tested.
- Moreover, immunosuppressive regulatory cell markers were to be investigated.

2. Evaluation of the expression levels of immunosuppressive proteins (cytokines, metabolic enzymes, and cell surface proteins) that are known to inhibit the immune system.

3. Study of the immune checkpoint targets in meningioma (grade I) and also in malignant glioma (glioblastoma).

4. Comparison of the expression levels of ABCB1 and ABCG2 drug transporters in the lung adenocarcinoma with meningioma, and glioblastoma.

5. Study of the molecular pathways of regulating ABC transporters in meningioma and glioblastoma.

- Identification of the components of the WNT signaling pathway that are significantly expressed in glioblastoma versus meningioma.
- Study to identify WNT signaling targets that can potentially affect both intra- and extra-parenchymal CNS tumors.

3 MATERIALS AND METHODS

3.1 Ethical statement

Brain tumor tissues

Brain tumor samples (**Table 1**) were collected at the Departments of Neurosurgery and Pathology, Clinical Centre, University of Pecs, Hungary. In accordance with the Declaration of Helsinki patients had given written informed consent and the project was approved by the Medical Research Council, Hungary (0194/16 (10833-/2016/EKU).

Lung tumor tissues

Twelve primary human lung AC samples were obtained after surgery and assessed by a certified lung pathologist at the University of Pecs, Hungary (**Table 2**). None of the patients were pre-treated with chemotherapeutic drugs before surgery. Patients provided written informed consent and the study was approved by the Ethical Committee of the University of Pecs and the Medical Research Council of Hungary (ETT-TUKEB 366/2015). All collected samples were treated anonymously following the guidelines and regulations of the 1975 Helsinki Declaration.

3.2 Patient samples

Twenty-two brain tumor samples were collected for this study. Samples with no clinical background (n=15) were excluded. Therefore, thirteen samples were analyzed including eight meningiomas, and five glioblastomas.

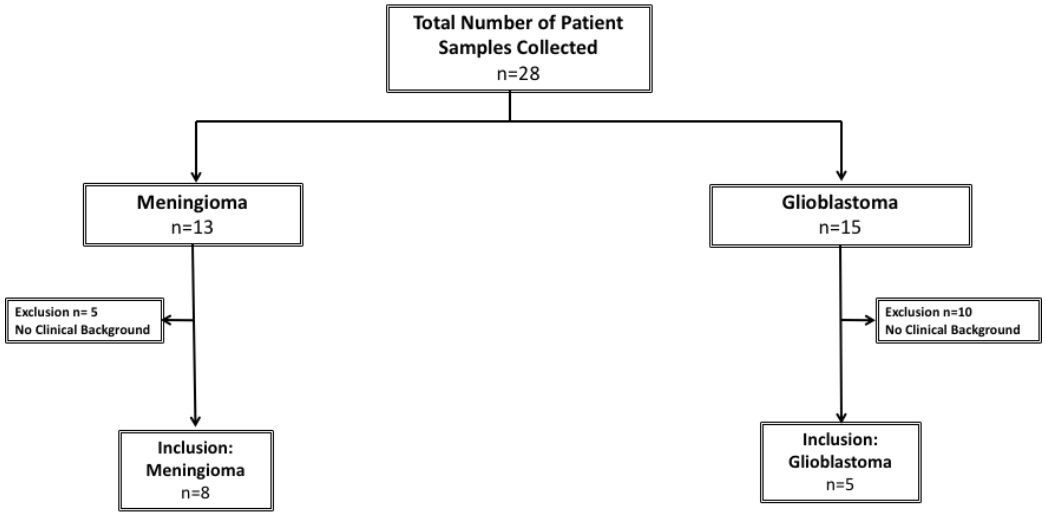

Figure 8. Brain tumor samples inclusion criteria flow chart.

Table 1. Summary of patient data-tumor type, age range, diagnosis, and therapy preceding surgery. Not applicable (N/A), negative (-).

Tumor Type	N°	Code	Age Range	IDH	Diagnosis	Radio- or Other Therapy before Surgery
MNG	1	M1	50–60	N/A	Grade I meningioma	-
	2	M2	70–80	N/A	Grade I meningioma	-
	3	M3	40–50	N/A	Grade I meningioma	-
	4	M4	70–80	N/A	Grade I transitional meningioma	-
	5	M5	60–70	N/A	Grade I meningioma brain invasion	-
	6	M6	40–50	N/A	Grade I meningioma	-
	7	M7	40–50	N/A	Grade I meningioma	+
	8	M8	70–80	N/A	Grade I meningioma	-
GBM	1	G1	60–70	(-)	Grade IV Glioma	-
	2	G2	70–80	(-)	Grade IV Glioma	-
	3	G3	60–70	(-)	Grade IV Glioma	+
	4	G4	40–50	(-)	Grade IV Glioma	-
	5	G5	60–70	(-)	Grade IV Glioma	-

Table 2. Adenocarcinoma samples. Patient data includes tumor type, mutation, gender, age, stage (TNM staging). Tumor-node-metastasis (TNM). T= refers to the size, invasion, and location of the tumors, N= the number of the lymph nodes that have tumors, M= refers to whether cancer has metastasized.

N°	Mutation	Histology	T	N	M	Age	Gender
1	EGFR/KRAS WT	AC	T2	N1	Mx	65	F
2	EGFR/KRAS WT	AC	T1	N1	Mx	69	M
3	EGFR MUTANT	AC	T2b	N1	Mx	73	F
4	EGFR MUTANT	AC	T1	N1	Mx	60	M
5	KRAS MUTANT	AC	T1	N1b	Mx	65	M
6	KRAS MUTANT	AC	T2b	N2	M0	62	F
7	KRAS MUTANT	AC	T1	N2	Mx	51	F
8	KRAS MUTANT	AC	T3	N2	Mx	57	F
9	KRAS MUTANT	AC	T2	N0	Mx	72	M
10	KRAS MUTANT	AC	T2	N2	Mx	62	M
11	KRAS MUTANT	AC	T2	N2	Mx	68	M
12	KRAS MUTANT	AC	T2	N1	Mx	59	M

3.3. RNA isolation and Reverse transcription

Total RNA was isolated from tumor samples using NucleoSpin RNA isolation kit (Macherey-Nagel, Düren, Germany). RNA concentration was measured by Nanodrop 2000 (ThermoFisher Scientific, Waltham, MA, USA). Reverse

transcription was performed using random primers and a high-capacity RNA to cDNA kit (ThermoFisher Scientific, Waltham, MA, USA). All generated cDNA samples were stored at −20 °C until used. Total RNA of five pooled normal human brain samples as well as total RNA of five pooled normal human lungs were purchased from a commercial source (BioChain Institute, San Francisco, CA, USA). All generated cDNA samples were stored at −20 °C until used.

3.4 Real-time quantitative polymerase chain reaction (qRT-PCR)

qRT-PCR reactions were carried out using Luminaris Color HiGreen qPCR master mix (ThermoFisher Scientific). Amplification was made by PikoREAL 96 PCR system (ThermoFisher Scientific). The reference genes were glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and/or β-actin (by taking the average of their Ct values, in both cases). The relative quantification (RQ) was calculated compared to gene expression levels of the normal tissues. The primer sequences are summarized in **Table 3**.

Table 3. List of gene specific primers used in qRT-PCR.

Target genes	**Accession number**	**Primers sequences**	**Amplicon size (bp)**
β -Actin	NM_001101.5		55
Forward		5'-GCGCGGCTACAGCTTCA-3'	
Reverse		5'-CTTAATGTCACGCACGATTTCC-3'	
GAPDH	NM_002046.7		189
Forward		5'-ATCCCTCCAAAATCAAGTGA-3'	
Reverse		5'-GGCTGTTGTCATACTTCTCA-3'	
FOXP3	XM_006724533.2		160
Forward		5'-AAGGACAGGTCAGTGGACAG-3'	
Reverse		5'-CGAAGACCTTCTCACATCCG-3'	
IDO1	NM_002164.5		136
Forward		5'-CCAAGAAACTGGAACTGCCT -3'	
Reverse		5'-CTGCAGTCTCCATCACGAAA-3'	
IL-10	NM_000572.3		200
Forward		5'-CCTGCCTAACATGCTTCGAG-3'	
Reverse		5'-GGTCTTGGTTCTCAGCTTGG-3'	
INF-γ	NM_000619.2		106

Forward		5'-GAATGTCCAACGCAAAGCAA-3'	
Reverse		5'-ACCTCGAAACAGCATCTGAC-3'	
CD27	>NM_001242.4		83
Forward		5'-TGCAGAGCCTTGTCGTTACAG-3'	
Reverse		5'-GCTCCGGTTTTCGGTAATCCT-3'	
CD163	XM_024449278.1		192
Forward		5'-GGACAGGGTTAGGGAGTCAT-3'	
Reverse		5'-TAAGCTGCTGGCAAAGAACA-3'	
CTLA4	NM_005214.5		117
Forward		5'-ATGTACCCACCGCCATACTA-3'	
Reverse		5'-CGAACTAACTGCTGCAAGGA-3'	
CD28	XM_011512194.2		134
Forward		5'-GCCTTGGCAGGAAACAAGAT-3'	
Reverse		5'-AGTCCTTTGTGAAGGGATGC-3'	
TGF- β	NM_000660.6		165
Forward		5'-GACATCAACGGGTTCACTACC-3'	
Reverse		5'-CGTGGAGCTGAAGCAATAGTT-3'	
CD4	NM_001195014.2		114
Forward		5'-TGCACCCTCATCTTCCTATCT-3'	
Reverse		5'-AGGAGAACTCCACCTGTTCC-3'	
PD-1	NM_005018.3		114
Forward		5'-CAGTTCCAAACCCTGGTGGT-3'	
Reverse		5'-GGCTCCTATTGTCCCTCGTG-3'	
PD-L1	NM_014143.4		200
Forward		5'-ATGGTGGTGCCGACTACAAG-3'	
Reverse		5'-GGAATTGGTGGTGGTGGTCT-3'	
CD19	XM_006721103.3		139
Forward		5'-CAGGGTCCCAGTCCTATGAG-3'	
Reverse		5'-TCTGGCCCATCGGGATTAT-3'	
CD56	NM_001242608.1		152
Forward		5'-TAGTTCCCAGCTGACCATCA-3'	
Reverse		5'-TGGCAGTCTGGTTCTCTACA-3'	
CD3	NM_000733.3		242
Forward		5'-ATGTCTGCTACCCCAGAGGA-3'	
Reverse		5'-GTTTTGTCCCCTTTGCCTGC-3'	
CD8	NM_001145873.1		74

Forward		5'-ACCCTTTACTGCAACCAC-3'	
Reverse		5'-TTGTCTCCCGATTTGACCAC-3'	
PAX5	NM_016734.3		147
Forward		5'-GTAGTCCGCCAGAGGATAGT-3'	
Reverse		5'-TCCAATTACCCCAGGCTTGA-3'	
CD70	NM_001330332.2		184
Forward		5'-GGCATCTACATGGTACACATCC-3'	
Reverse		5'-ACTTGACTTTGAGTCCCCAG-3'	
B7-1	NM_005191.4		108
Forward		5'-CAGGTGTTATCCACGTGACC-3'	
Reverse		5'-CCTTTTGCCAGTAGATGCGA-3'	
B7-2	NM_175862.5		100
Forward		5'-CACAGCAGAAGCAGCCAAAATG-3'	
Reverse		5'-CTTCAGAGGAGCAGCACCAGA-3'	
ABCB1	NM_001348945.2		93
Forward		5'-GCAGCTGGAAGACAAATACACAA-3'	
Reverse		5'-CCCAACATCGTGCACATCA-3'	
ABCG2	NM_001348989.2		126
Forward		5'-AACCTGGTCTCAACGCCATC-3'	
Reverse		5'-GTCGCGGTGCTCCATTTATC-3'	

3.5 WNT signaling arrays

Relative mRNA expression (RQ) of WNT signaling pathway genes were assessed in eight pooled MNG samples, five pooled GBM samples, and Five pooled normal human brain samples using Applied Biosystems™ TaqMan™ Array, Human WNT Pathway, Fast 96-well (Thermo Fisher Scientific Inc. TMO, Waltham, MA, USA). GAPDH, 18S, hypoxanthine phosphoribosyl-transferase 1 (HPRT1), and glucuronidase β (GUSB) were used as internal controls.

3.6 Hematoxylin-Eosin staining

Five μm thick tissue sections were stained in Mayer's hematoxylin solution (Sig-ma-Aldrich, St. Louis, MO, USA) for 10 min, washed, then exposed to 0.25% acetic acid and eosin solution. Sections were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Images were taken using an Eclipse Ti-U inverted microscope (Nikon Inc., Tokyo, Japan).

3.7 Immunohistochemistry

Five µm thick slides were cut from formalin-fixed, paraffin embedded tissue blocks corresponding to the surgical samples (GBM and MNG) used for qRT-PCR. First, the slides were rinsed in heated xylene and were washed with a descending dilution series of ethanol (97%–80%–70%–50%) to remove paraffin. After deparaffinization the slides were rehydrated in distilled water and DAKO antigen Target Retrieval Solution (DAKO, Agilent, Santa Clara, CA, USA) at 97 °C for 20–30 min. Endogenous peroxidase activity was blocked for 15 min with Tris Buffer Saline (TBS, pH 7.4) containing 3% H_2O_2. Slides were washed three times with TBS containing Tween 20 (TBST) (0.05%, pH 7.4). Pre-blocking was carried out with 3% bovine serum albumin (BSA) in TBS for 20 min before overnight incubation with the appropriate primary antibody at 4 °C. Slides were then washed with TBS three times. The reactions were visualized using Envysion System (DAKO). For nuclear counterstaining, hematoxylin staining was performed. Finally, slides were mounted with Faramount Aqueous Mounting Medium (DAKO). Histological evaluation was performed with the help of Panoramic MIDI digital slide scanner (3DHistech, Budapest, Hungary). The number of positive cells was assessed per mm^2 except for the CD68 positive cell count that was assessed per 0.08 mm^2. Image analysis was performed using the ImageJ software with the IHC toolbox plug-in. The list of antibodies and dilutions are summarized in **Table 4**.

3.8 Immunofluorescent staining

Five µm thick slides were cut from formalin-fixed, paraffin embedded tissue blocks corresponding to the surgical samples used for qRT-PCR. After deparaffinization and antigen retrieval the sections were pre-blocked with 5% BSA in TBST for one hour before applying the primary antibodies anti-CD19 and anti-CD45 for overnight incubation at 4 °C. CD19 and CD45 were detected using an anti-mouse IgG Alexa Fluor 488 (1:200) and anti-rabbit IgG Alexa Fluor 555 (1:200) secondary antibodies (ThermoFisher Scientific), respectively. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (ab142859) (1:1000) (Abcam Plc., Cambridge, UK). Images were obtained using an Olympus IX-81 (OLYMPUS Corporation,

Tokyo, Japan) fluorescence microscope. The list of antibodies and dilutions are summarized in **Table 4**.

Table 4. List of antibodies

Antibody	Clone	Source	Isotype	Source	Dilution
Anti-CD4	4B12	Mouse	IgG1, kappa	Thermo	1:20
Anti-CD8	C8/114B	Mouse	IgG1, kappa	Thermo	1:50
Anti-CD3	Polyclonal	Rabbit	/	Dako	1:400
Anti-CD45	2B11+ PD7/26	Mouse	IgG1, kappa	Dako	1:400
Anti-CD19	EPR5906	Rabbit	IgG	Abcam	1:500 - 1:1000
Anti-CD79	JCB117	Mouse	IgG1, kappa	Dako	1:200
Anti-PAX5	Polyclonal	Rabbit	/	Thermo	1:50
Anti-CD68	PGM1	Mouse	IgG3, kappa	Dako	1:200
Anti-PD1	NAT105	Mouse	IgG1, kappa	Abcam	1:50
Anti-PDL1	22C3	Mouse	IgG1	Dako	1:50
Anti-LEF1	EP310 clone	Rabbit	IgG	CellMarque	1:25 - 1:100

3.9 Statistical analysis

Statistical analysis was performed using the SPSS software version 20 (IBM, USA). Grouped data were presented as logRQ ± technical error. Non-grouped data are presented as 1/dCt individually and average ± standard error of the mean (SEM) using one-way and two-way analysis of the variance (ANOVA). $p < 0.05$ was considered as significant. Figures were generated using the GraphPad Prism 8 software (2018, GraphPad Software, Inc., USA).

4. RESULTS

4.1 Variable infiltration of T, B, NK and macrophage cells into meningioma and glioblastoma

Based on qRT-PCR analysis of cell surface markers, both MNG and GBM brain tumor samples have shown a slightly increased $CD45^+$ white blood cell (WBC) infiltration compared to normal brain (**Figure 9A**). To identify the main cell types within the WBC population, screening of the expressions of $CD3^+$ T-cell, $CD56^+$ NK and $CD19^+$ B-cell markers were performed (**Figure 9A**). The transcript levels of the T-cell marker CD3 in MNG were found to be significantly higher (**Table 5**) than in the normal brain, while in the GBM samples CD3 expression was not different (**Figure 9A**). The NK cell marker CD56 was significantly reduced in all MNG samples compared to both normal brain and GBM (**Figure 9B**). In both MNG and GBM samples the mRNA levels of CD19 B-cell marker were not different from the normal brain (**Figure 9A**). IHC supported the initial findings, as the tested individual MNG samples had generally higher protein expression of the CD3 T-cell marker than what was detected in GBM (**Figure 9B**). Neither tumors, nor infiltrating $CD45^+$ lymphocytes have stained positive for CD19. In certain areas of GBM sections some congregation of $CD19^+CD45^+$ double positive cells were detected, whereas such areas were not found in MNG section (**Figure 9C**). The negative staining for CD79a and the expression of paired box 5 (PAX5) support the lack of B cells in both tumors (**Figure 10**).

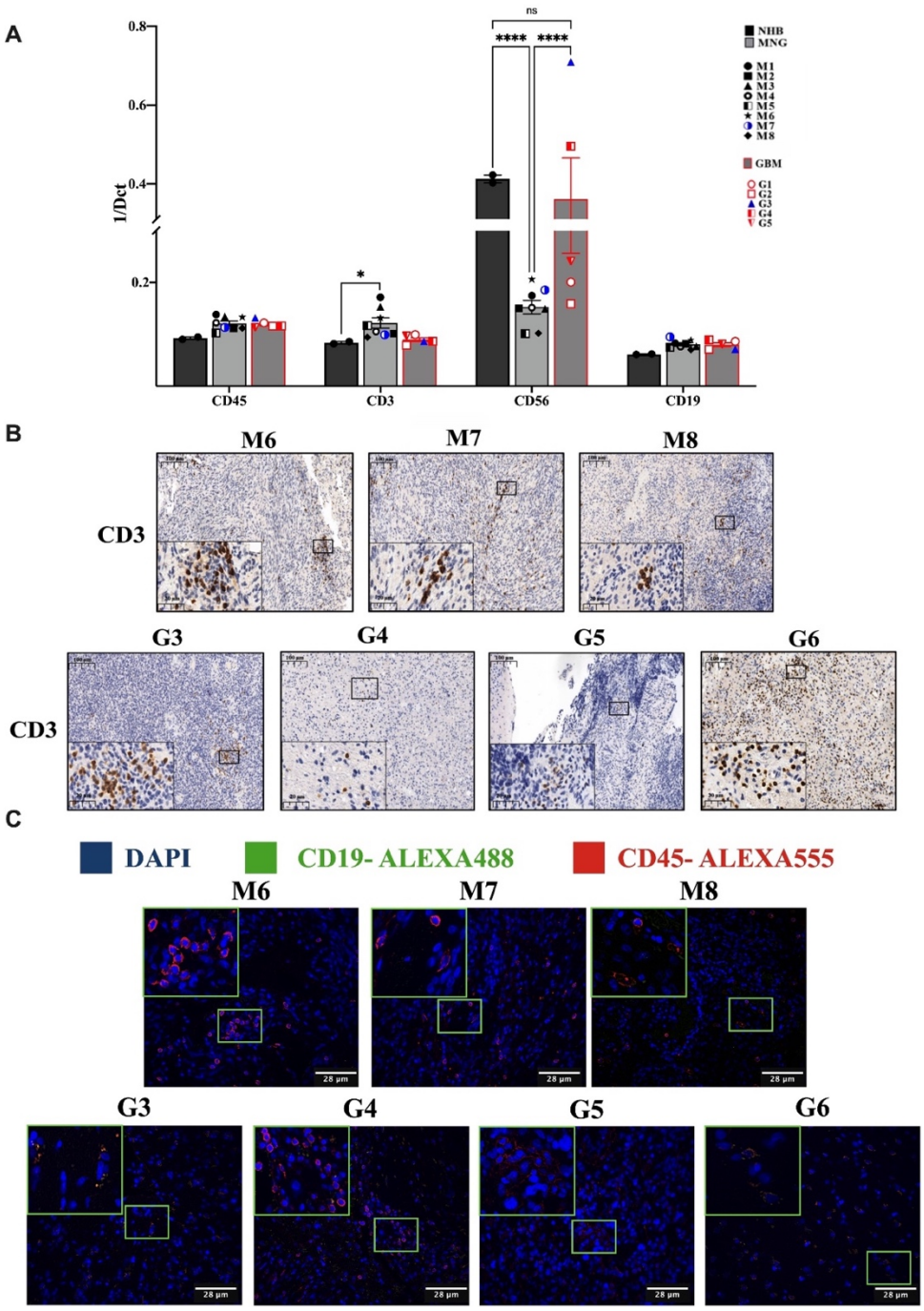

Figure 9. Infiltration of immune cell populations into MNG and GBM. (**A**) mRNA levels of the general leukocyte antigen CD45 and general immune cell subpopulation $CD3^+$ T-cell, $CD56^+$ NK and $CD19^+$ B-cell markers in MNG (n=8) and GBM (n=5). Data are presented as 1/dCt individually and as average ± SEM. Significant changes are marked as *, **, *** and **** ($p < 0.05$, $p < 0.001$, $p < 0.0002$ and $p < 0.0001$, respectively). (**B**) IHC staining of $CD3^+$ T-cell population in both brain tumor types (GBM and MNG), magnification ×20 and ×40, size bar 100 and 20 µm, respectively. (**C**) immunofluorescence staining of the general leucocytes marker CD45 and of CD19 in MNG (n=3) and GBM (n=4), magnification ×40, size bar 28 µm. Only red staining can be detected in MNG and yellow (overlapping red and green) in some GBM samples.

Table 5. Quantification of infiltrating immune cells in mm^2 tissues. $CD3^+$, $CD4^+$ and $CD8^+$ positive cells/mm^2 in MNG and GBM IHC stained tissues.

Code	Freeze	Localization	Comment	CD3	CD4	CD8	CD4/CD8
M6	Yes	Left posterior fossa	Focally lymphocytes	210	12	40	0.3
M7	Yes	Right frontal and orbital	Tumor cells focally CD4	150	18	25	0.72
M8	Yes	Over left sphenoidal bone	Tumor cells focally CD4	54	9	9	1
G3	Yes	Left temporal	Focally lymphocytes	30	6	6	1
G4	Yes	Left occipital	-	9	3	2	1.5
G5	Yes	Right centrum semiovale	-	60	27	7	3.857143
G6	Yes	Left temporal	-	120-600	180	50	3.6

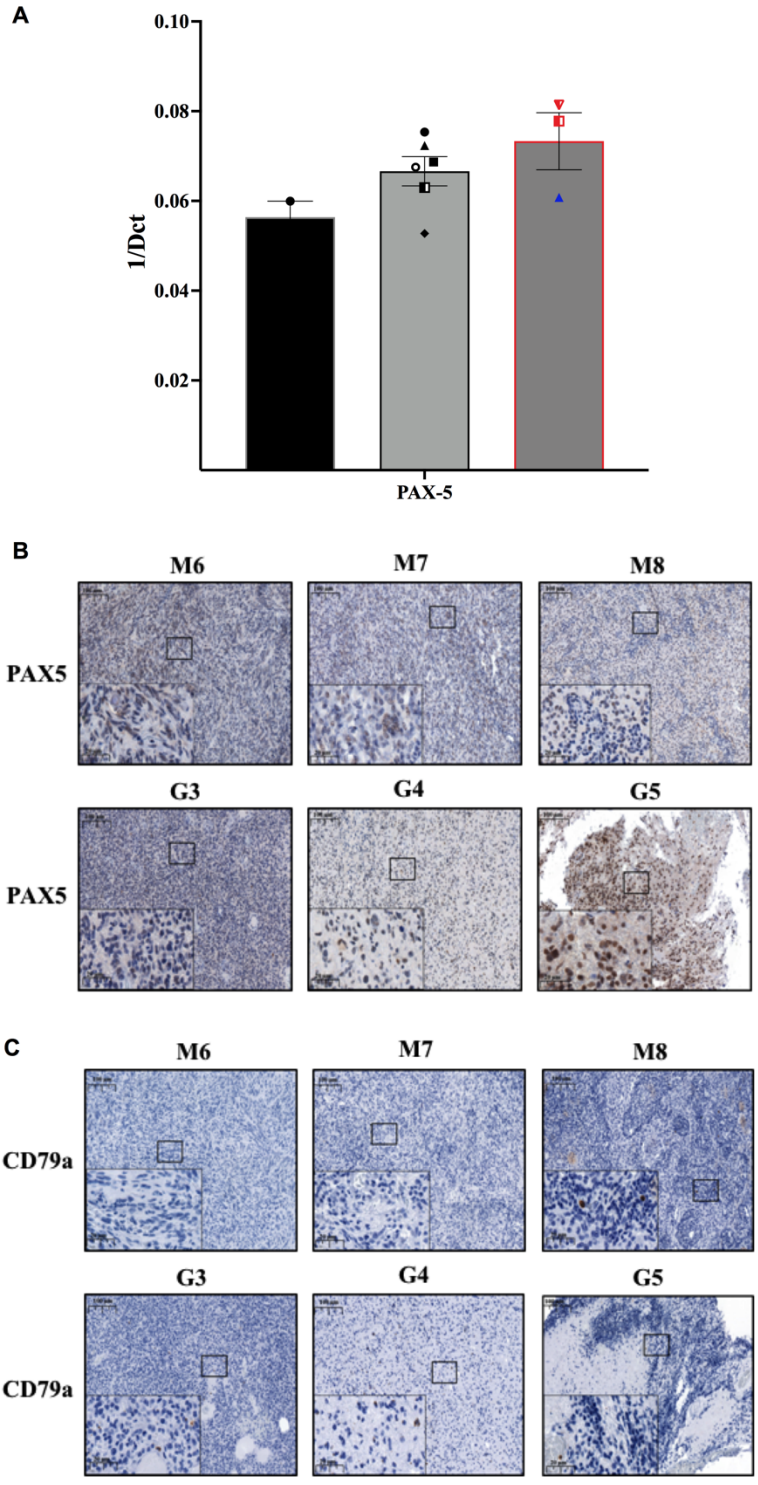

Figure 10. Expression levels of CD79a and PAX5. **(A)** mRNA expression levels of PAX5 in MNG and GBM samples. Data presented as 1/dCt individually and as average ± SEM. **(B, C)** IHC staining of PAX5 and CD79a in MNG and GBM in tumors, magnification ×20 and ×40, size bar 100 and 20 μm, respectively.

4.2. The immune microenvironment is actively suppressive in both meningioma and glioblastoma

Initial screening showed that the immune microenvironment appeared different at T and NK cells levels in MNG and GBM. These findings, at first glance might explain some of the characteristically different behavior of the tumors, indicating a more active tumor suppressive microenvironment in MNG. The cytotoxic T-cell marker (CD8) was markedly increased in MNG (**Table 5**) compared to normal brain (**Figure 11A**) and significantly higher than in GBM samples. The co-stimulatory molecule, CD28 [187] which is essential for T-cell activation was also present in both tumor types but only detected at a significantly higher level in MNG compared to normal brain (**Figure 11A**). The pro-inflammatory and anti-tumor INF-γ mRNA levels were also slightly increased (**Figure 11B**) in both MNG and GBM samples compared to normal controls. Although the cytotoxic ($CD8^+$) T-cells were only found in certain areas of the studied tumor samples (**Figure 11C**), their higher presence in MNG samples suggested a potentially successful immune checkpoint intervention for MNG as the CD8 ration to CD4 was higher in MNG than in GBM (**Table 5**).

Further analysis of T-cell markers revealed that the $CD4^+$ helper T-cell (Th) marker message levels were slightly elevated in both tumor types along with the regulatory T cell (Treg) marker forkhead box P3 (FOXP3) (**Figure 11A**). The presence of the $CD4^+$ T-cells was also supported by IHC (**Figure 11D**). In contrast to the $CD8^+$ T-cells marker, which was localized to specific tumor areas, evenly distributed CD4 staining was detected (**Figure 11C, D**) in both types of brain tumors. This indicates that the presence of $FOXP3^+CD4^+$ Th cells throughout both tumor tissues were mostly immuno-suppressive Treg cells.

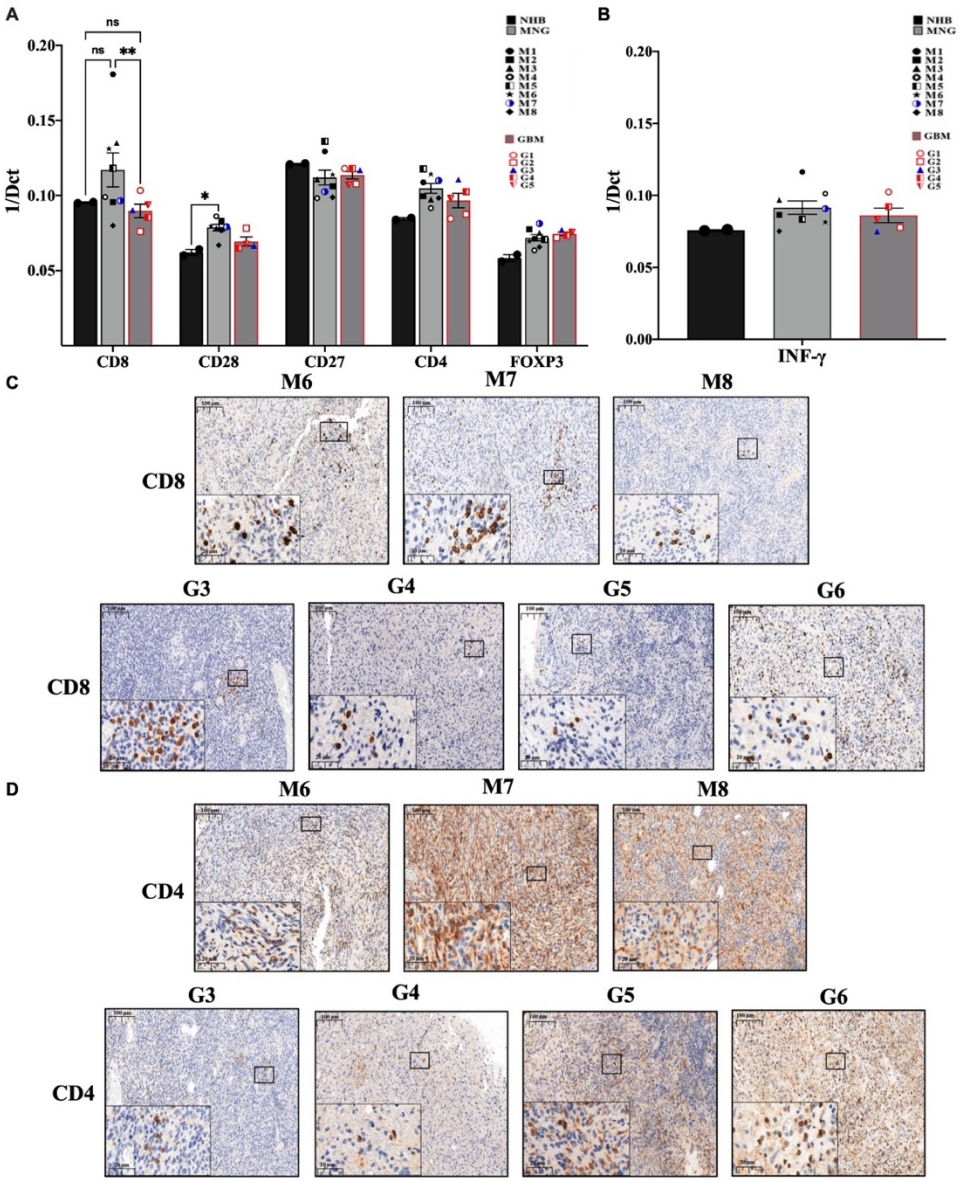

Figure 11. Evaluation of the immune profile of MNG and GBM. (**A**) mRNA expression levels of CD8 cytotoxic T-cells and CD4 Th cells, CD28 as costimulatory signal transducer for T-cells survival and activation, as well as FOXP3 marker characteristic for Treg cells. (**B**) the anti-tumor INF-γ mRNA expression levels were evaluated in both brain tumor microenvironments. Data are presented 1/dCt individually and as average ± SEM. Significant changes are marked as * and ** ($p < 0.05$ and $p < 0.001$, respectively). (**C**) IHC staining of CD8 was performed in MNG (n=3) and GBM (n=4) samples, magnification ×20 and ×40, size bar 100 and 20 µm, respectively. (**D**) IHC staining of CD4 cells in MNG and GBM, magnification ×20 and ×40, size bar 100 and 20 µm, respectively.

IHC of the tumor-associated macrophage (TAMs) marker CD68 further supported an immunosuppressive microenvironment (**Figure 12A**). Both tumor types were strongly and evenly positive for CD68 [188]. qRT-PCR analysis of another TAM marker CD163 strongly supported the initial observation (**Figure 12B**), as both MNG and GBM expressed CD163 message levels way above the detected levels in the

normal brain. Amongst the known functions of TAMs are the expression of IL-10 and transforming growth factor β (TGFβ) that suppress the T-cell mediated anti-tumor immune response [189]. The anti-inflammatory TGFβ and IL-10 were expressed at greater levels in both MNG and GBM compared to the normal brain (**Figure 12C**), which indicates the presence of active TAMs. As both cytokines are involved in creating the immune-suppressive environment by the inhibition of the polarization of naïve T-cells into Th1 and NK cells, the low level of NK cell marker CD56 in MNG was supported by the increased message levels of the above-mentioned cytokines (**Figure 9**). Additionally, IL-10 is known to be over-expressed by both $CD163^+$ TAMs and immunosuppressive Treg ($CD4^+$ $FOXP3^+$Treg) cells. As the Treg marker FOXP3 message levels were higher in both tumor types than in the normal brain controls, the results indicate an actively immunosuppressive microenvironment in both brain tumor types. Although cytotoxic T-cell levels in MNG were higher than in GBMs, and the expression of the co-stimulatory CD28 was also present in both tumor types, the mRNA levels of CD27, a member of the TNF receptor superfamily and co-stimulatory immune checkpoint molecule for activated T-cell survival was increased compared to CD28 (**Figure 11B**). As CD27/CD70 interaction promotes lymphocyte apoptosis, it is likely that activated immunosuppressive lymphocytes persist in both MNG and GBM. CD27 also aids differentiation of plasma cells from B cells if CD27 can interact with its ligand CD70.

As TAMs can directly suppress T-cell function by the induction of PD-L1 [190] and B7-homolog expression [191], the expression of immune checkpoint therapy targets were tested. Interaction of PDL-1 with programmed cell death protein 1 (PD-1) and B7 with CTLA-4 can block T-cell activity, respectively, and lead to suppression of cytotoxic T-cell activation.

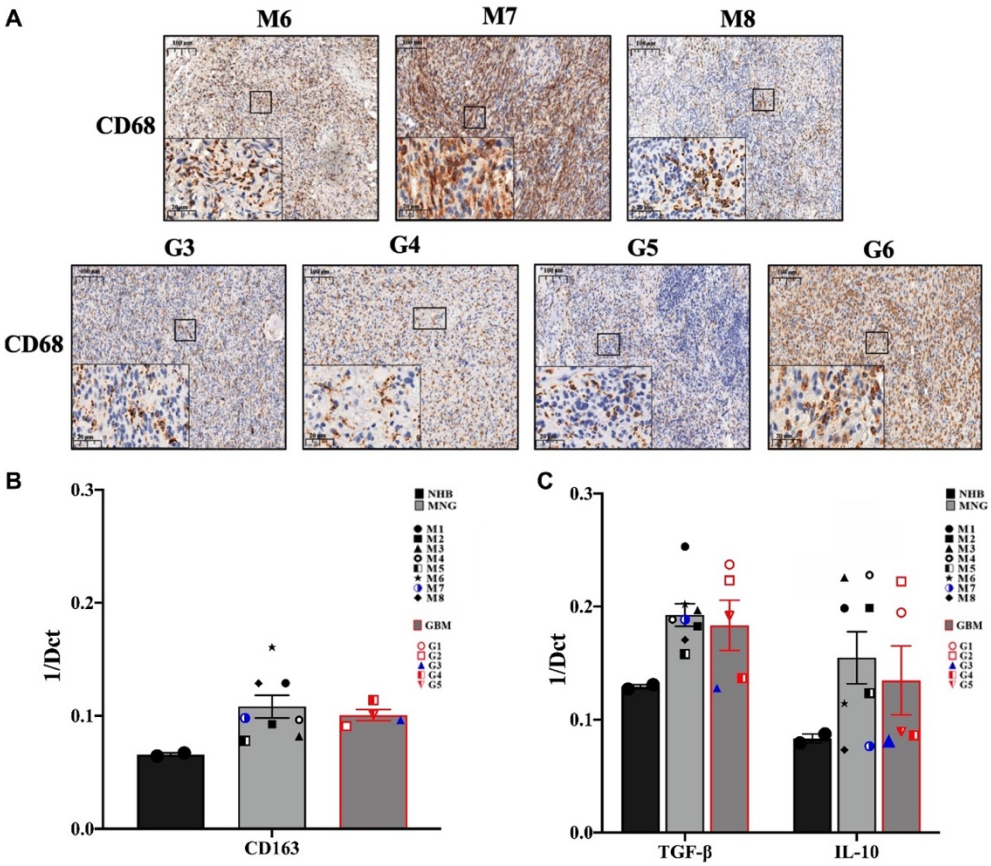

Figure 12. TAMs and immunosuppressive cytokine expressions in MNG and in GBM. (**A**) IIHC staining of CD68 indicates the higher protein expression of TAM in both tumors, magnification ×20 and ×40, size bar 100 and 20 µm, respectively. (**B**) CD163 m-RNA expression levels which represents TAM marker in MNG and GBM. (**C**) mRNA expression levels of anti-inflammatory cytokines IL-10 and TGFβ in MNG and GBM samples. Data are presented as 1/dCt individually and as average ± SEM. Significant changes are marked as * ($p < 0.05$).

4.3 Immune checkpoint targets in CNS tumors

The immune checkpoint targets (PDL1-PD1, B7-CTLA4) in MNG and GBM were different. qRT-PCR analysis in some individual cases showed nearly 100-fold increase of PD1 in MNG compared to normal brain control. Some individual MNG samples also showed at least 3-fold increase in PD1 expression, compared to GBM and normal controls (**Figure 13A**). In contrast, PDL1 message levels were slightly increased by mRNA detection and PDL1 protein was not detectable in either tumor types (**Figure 13A**). As PD1 is found on T-cells, but its ligand PDL1 was not detected at increased levels in either tumor types compared to normal controls. The above results indicate that simple inhibition of the PDL1-PD1 immune checkpoint is highly unlikely to be effective in tumor elimination.

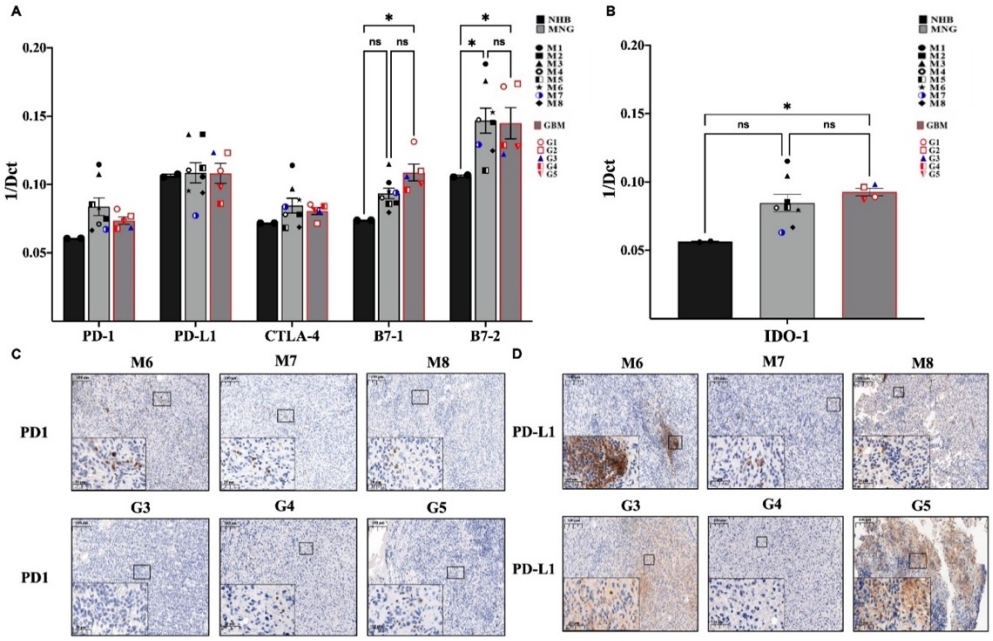

Figure 13. Immune checkpoint molecules that mediate immune therapy response in MNG and GBM. (**A**) mRNA expression levels of PD-1, PD-L1, CTLA-4, B7-1, and B7-2 in MNG and GBM patient samples. (**B**) mRNA expression levels of indoleamine 2,3-dioxygenase-1 **(**IDO-1) in MNG and GBM. Data are presented as 1/dCt individually and as average ± SEM. Significant changes are marked as * ($p < 0.05$). (**C**, **D**) both MNG and GBM samples were stained for PD-1 and PD-L1, magnification ×20 and ×40, size bar 100 and 20 µm, respectively.

Levels of CTLA-4, a strong inhibitor of T lymphocyte co-stimulation, were higher than in normal controls. The ligands of CTLA-4, B7-1 (CD80) and B7-2 (CD86), were expressed at higher levels in both MNG and GBM than in normal brain control. Based on this data it can be assumed that T lymphocytes are inactive in both brain tumor microenvironments, due to CTLA-4 induced inhibition that competitively binds B7-1 and B7-2 (**Figure 13A**). Additionally, indoleamine 2,3-dioxygenase 1 (IDO1) was vastly increased in both types of tumor microenvironments (**Figure 13B**). It is well studied that the metabolic product kynurenine generates and enhances the activities of $CD4^+$ $FOXP3^+$ Treg cells and myeloid-derived suppressor cells, as well as promote angiogenesis indicating a suppressed immune microenvironment in both GBM and MNG.

Table 6. Quantification of TAMs in mm2 tissue area. $CD68^+$ positive cells/0.08 mm^2 in MNG and GBM IHC stained tissues. Macrophages are positive (*), lymphocytes

show strong positivity in one focus (**), weak staining in tumor cells (*), moderate to strong staining in tumor cells (**).

Code	CD68	CD68	PD1	PDL1	PAX5	CD79a
M6	100	High	12	0%**	0	0
M7	100	High	9	<1%	0*	0
M8	40	Low	<1	<1%*	0*	2
G3	40	Moderate	<1	<1%	30	1
G4	40	Moderate	<1	0%	0*	2
G5	40	Moderate	18	-	0**	1
G6	100	High	90	-	-	-

4.4 ABCB1 and ABCG2 are differently expressed in glioblastoma and meningioma compared to primary human adenocarcinoma

Drug transporters expression was measured in different primary tissues; MNG and GBM compared to normal brain and lung AC compared to normal lung control, by qRT-PCR. Non-diseased lung mRNA was used as normal control for the lung tissue and normal brain mRNA for the brain tumors. AC patient samples were clustered based on mutation as follows: where neither EGFR or KRAS gene mutations were detected wild type (WT), and where ABCB1 levels were very high, while ABCG2 mRNA levels were undetectable. In the EGFR mutant/KRAS WT and the KRAS mutant/EGFR WT patient groups, both ABCB1 and ABCG2 were detected. In fact, ABCB1 levels were higher in the presence of KRAS mutation. Overall, in lung AC primary tumors, ABCB1 was the dominant transporter, and the expression levels of ABCG2 were evidently lower than ABCB1 or even undetectable (**Figure 14A**). Recent studies [143] have shown, that both ABCB1 and ABCG2 play a key role in the regulation of drug penetration of the BBB. In the CNS tumors, however, neither MNG nor GBM expressed significantly higher levels of ABCB1 than the normal brain tissue (**Figure 14B**). In MNG ABCG2 expression was slightly higher than in GBM or normal brain, but the increase was non-significant and only affected some individual patient samples (**Figure 14B**). Both ABCB1 and ABCG2 mRNA expression levels were barely detectable in GBM (**Figure 14B**). Interestingly, MNGs, slow-growing tumors, are highly express MDR1 P-glycoprotein (P-gp), which considered as another defense system against chemotherapy [140].

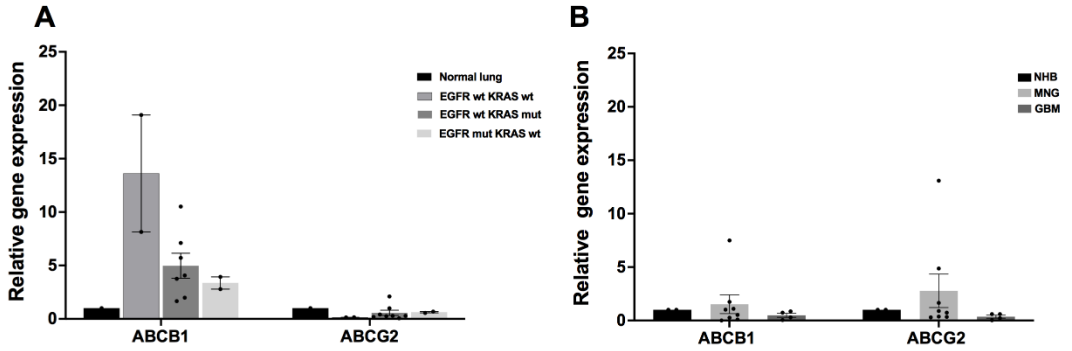

Figure 14. Drug transporter analysis of primary lung AC and two different brain tumor samples. (**A**) relative mRNA expression (RQ) levels of ABCB1 and ABCG2 drug transporters in AC patients (*n*=12) with different mutational background (EGFR wild type/KRAS wild type; EGFR mutant/KRAS wild type, EGFR wild type/KRAS mutant). (**B**) the expression levels of both drug transporters ABCB1 and ABCG2 in MNG (*n*=8) and GBM (*n*=4). mRNA expression is relative to normal tissue. Data are presented as average ± SEM.

4.5 WNT microenvironment in glioblastoma and meningioma

Recent studies confirmed that the WNT/β-catenin pathway is crucially important in the regulation of ABC transporters [148]. The WNT microenvironment is also responsible for ABC transporter expression in the brain, but certainly regulates ABCB1 and ABCG2 expression in the BBB [143].

Out of eighty-two WNT signaling-related genes (**Figure 15**), forty-one of them were deregulated. Quantification analysis of the WNT ligands revealed overexpression of WNT2B, WNT5B, and WNT6 in MNG. In GBM, WNT4, WNT7A, WNT9B, and WNT16 were upregulated whereases, they were downregulated in MNG. The above data indicates that both GBM and MNG have different WNT microenvironments. Additionally, kringle containing transmembrane protein 2 (KREMEN2) is highly expressed in GBM, while it is not expressed in MNG. Emerging evidence suggests that the high expression of KREMEN2 in tumors is linked to a poor patient outcome [192]. Reported for the first time, the member of the DKK family, DKK2 was upregulated in MNG and downregulated in GBM. DKK2 is known to act as either agonist or antagonist of WNT/β-catenin signaling, depending on the cellular context and the presence of the co-factor KREMEN2. Furthermore, WNT pathway TaqMan analysis identified the increased expression of LEF transcription factor in both tumors compared to normal brain. Both LEF1 and the TCF

family of transcription factors are activated by the WNT/β-catenin pathway and can lead to transcription of downstream target genes. LEF1, a key effector of WNT/β-catenin signaling, regulates intra-tumoral heterogeneity, signifying a widespread interplay between this WNT signaling-related transcription factor and GBM driver pathways. It was previously unknown, that the mRNA levels of LEF1 was also highly expressed in MNG compared to the normal tissue. As LEF1 is a potential marker for malignant transformation, its expression was markedly higher in GBM samples compared to both MNG and normal brain (**Figure 16A**). Further analysis was performed to identify the protein expression of LEF1 in both tumors. Apart from destabilized vessels at tumor sites and from chemotherapy-induced vessel injury [193], endothelial progenitor cells (EPC) that are needed for neovascularization of neoplastic diseases can migrate from the bone marrow and contribute to the blood supply of the tumor. Such EPC-s can be positive for Tie-2, Sca-1, CD31 and CD45 [194]. Double staining of CD45/LEF1 using IHC revealed great variability in the expression of LEF1 in individual MNG cases (**Figure 16B**).

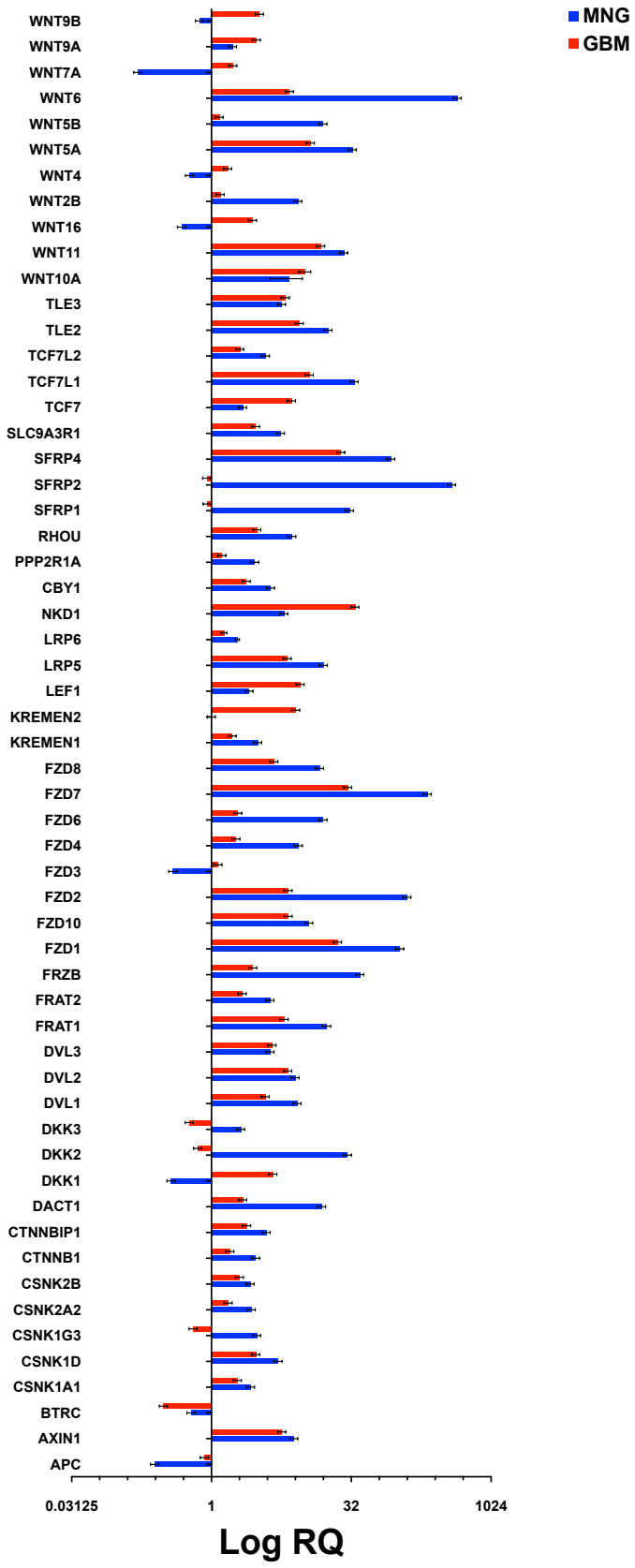

Figure 15. WNT microenvironment analysis of primary MNG and GBM samples. Relative mRNA expression levels of WNT signaling pathway genes in MNG and GBM compared to the normal brain (data were generated from pooled samples *n*=8, *n*=5, and *n*=5, respectively). WNT signaling TaqMan data presented as logRQ ± technical error.

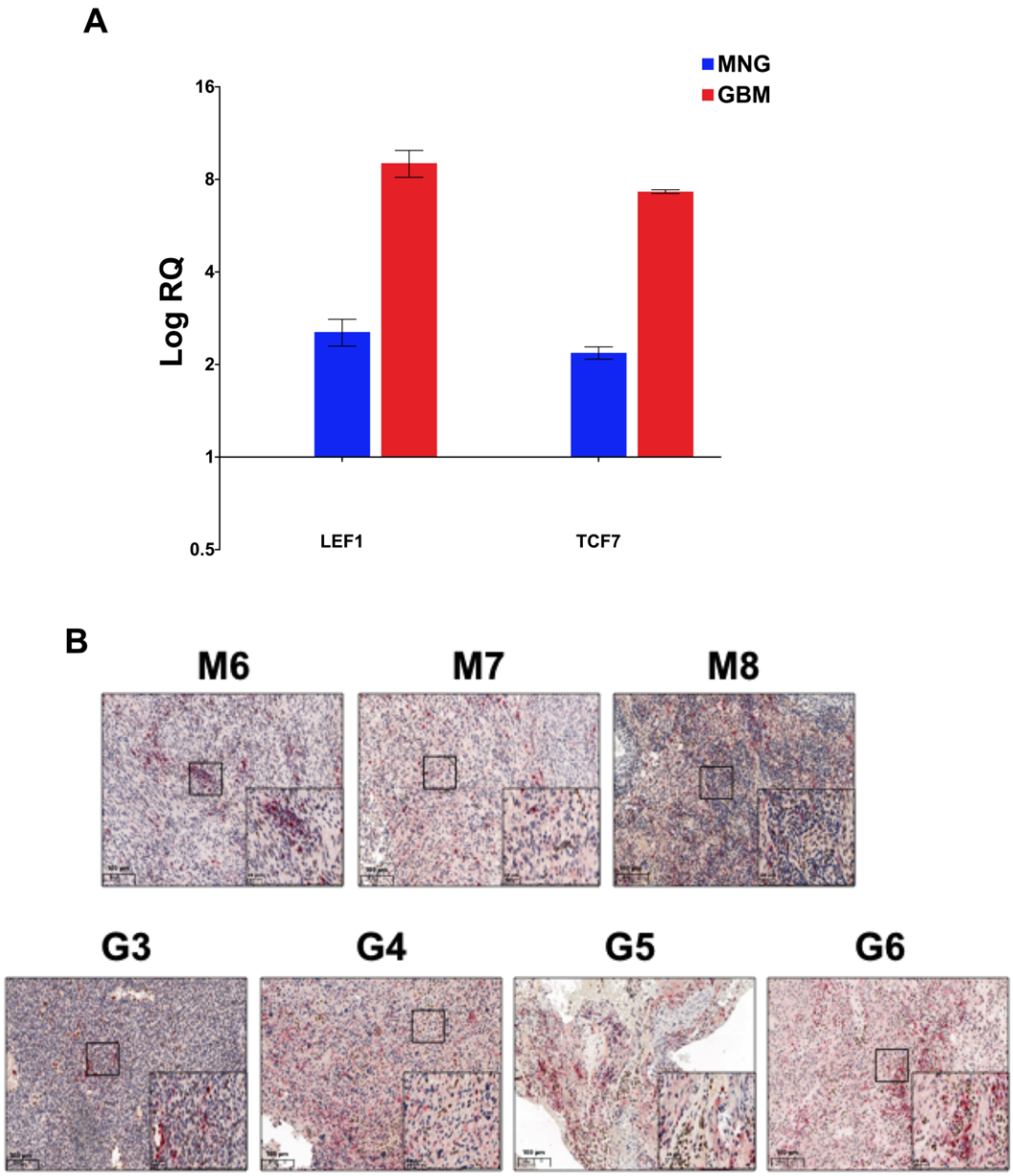

Figure 16. Evaluation of the expression levels of LEF1 in MNG and GBM. (**A**) mRNA expression level of LEF1 in MNG and GBM compared to the normal brain tissue (data were generated from pooled samples *n*=8, *n*=5, and *n*=5, respectively), data are presented as LogRQ ± technical error. (**B**) IHC CD45 (red) /LEF1(brown) dual staining in individual MNG and GBM cases, magnification ×20 and ×40, size bar 100 and 20 μm, respectively.

Some MNG cases had sparse $CD45^+$ white blood cells which were negative for LEF1. Semi-quantitative analysis revealed that the expression of the LEF1 in GBM was strongly associated with the $CD45^+$ positive cell and perivascular cells in areas of microvascular proliferation (MVP) (**Table 7**).

Table 7. Quantification of the expression of LEF1 (Histoscore (H-score) and percentage of positive cells) in MNG and GBM cells. H-score is assessed by counting 100 cells (0= non staining, 1= weak, 2= medium, 3= strong staining). The range of possible scores was from 0-300). White blood cells showed strong positivity (**), heterogeneous (*).

Code	LEF1		$CD45^{+}$-LEF1
	H-score	**% positive**	
M6	41	28%*	Negative
M7	9	8%	Weak, partial
M8	86	62%*	Weak, negative
G3	22	16%	strong in MVP
G4	57	22	Strong in MVP
G5	39	20%**	Strong in MVP
G6	43	32%	Moderate-strong in MVP

5. DISCUSSION

Better understanding of the microenvironments of various types of tumors is essential to improve therapies. It is especially important in CNS tumors, as despite all efforts, CNS tumors are hard to treat effectively. Neurosurgery, even if the brain tumor is operable, might lead to memory loss, speech and mobility problems. If the tumor is inoperable, radio and chemotherapy are the remaining alternative treatments, as previously described (**section 1.5**). Although chemotherapy invariably involves TMZ, only about 50% of GBM patients respond to such treatment [195]. Unfortunately, TMZ also has cytotoxic effects on normal brain cells that leads to various adverse effects. Moreover, MNGs are rarely responsive to TMZ [196].

Theoretically, the immune system should be able to eliminate brain tumors. Not surprisingly, oncologists were hopeful that immune checkpoint antibodies would revolutionize therapy. Unfortunately, the results were controversial [197], [198]. To understand the reasons why immune checkpoint therapies often fail, several studies have investigated the immune microenvironment of GBMs [199], but not MNGs. Although the two tumor types couldn't be more different histologically, the clinical problem remains the same: how to debulk or remove a tumor completely without damaging the brain tissue. Hence, we compared the immune markers of the highly aggressive GBMs and the slow growing neoplasm MNGs with each other and the normal brain. Initially, we focused on two immune checkpoint targets, PD1 and CTLA-4, as monoclonal antibodies developed to target these two checkpoints, and they are mostly successful against several tumor types [109], [111], [200], [201].
Ipilimumab, the anti–CTLA-4 antibody was approved by the FDA in 2011 against melanoma [109]. While the first anti-PD1 antibody, nivolumab was approved in 2014 for treating patients with advanced NSCLC. CTLA-4 is a protein receptor expressed by activated T-cells to provide control for the immune system over T-cell activities. CTLA-4 has about 30% homology with CD28 (T-cells co-stimulatory protein), and both molecules competitively bind to B7-1 (CD80) and B7-2 (CD86) ligands on the surface of APCs [202]. Therefore, the expression of CTLA-4 receptor ligands were also tested. In the periphery, tissues control T cells via expression of PD-L1 as PD-L1 is the ligand for PD-1 an additional inhibitory co-receptor that is expressed on the surface of T-cells and their interaction maintains peripheral tolerance [113]. Certain tumors exploit this system to evade the immune system by expressing high levels of

PDL1 [113]. PD-1 and CTLA-4 have extensively researched immunotherapy targets, but clearly, their inhibition in brain tumors does not result in the desired effect. Based on our data, it is hardly surprising as neither GBM, nor MNG express the ligands for PD-1 or CTLA-4. In the absence of immune suppression activating ligands, immune checkpoint monoclonal antibodies cannot have the expected therapeutic effect. The low level of NK cell marker, $CD56^+$ explains the lack of efficacy of NK cell targeting therapy [203]. CD56 is the archetypal phenotypic marker of NK cells but can actually be expressed in other cell types, therefor other NK markers should be applied (CD16, CD161), however those are also not exclusive NK-specific markers [204]. Additionally, even the presence of NK cells does not ensure their activated state as tumor-infiltrating immune cells such as DCs, suppressive or tolerogenic macrophages, and Treg cells can interfere with NK cell activation, either through secretion of immunosuppressive cytokines or by interfering with receptor expression [205], [206]. The lower level of CD56/NCAM in MNG samples were initially surprising, however, an early article draw my attention to the differences between low grade and high grade meningiomas. Based on that information low grade meningiomas express low levels of NCAM [207].

For instance, TGF-β is documented as a key inhibitory cytokine of NK cells which limits the number and anti-metastatic function of NK cells and is highly expressed in the studied tumors. While T-cells are present in both tumor types, $CD8^+$ cytotoxic T-cells are only in abundance in MNG providing the false impression that MNG could be targeted with immune checkpoint therapy. The initial observation, however, is misleading.

The microenvironment in GBM and MNG are highly immunosuppressive as $CD68^+$ and $CD163^+$ anti-inflammatory M2 type TAMs infiltrate both GBM and MNG. TAMs, which secrete anti-inflammatory and immune suppressive cytokines (e.g., TGF-β and IL-10), enhance the expansion of immune suppressive $CD4^+$ T-reg cells inhibiting the functions of $CD8^+$ cytotoxic T and NK cells. Additionally, both tumor types express IDO [208] (**Figure 16**). IDO is a heme-containing enzyme that catalyzes the first and rate-limiting step in the kynurenine pathway, which is the O_2-dependent oxidation of L-tryptophan to N-formylkynurenine. INF-γ that is highly expressed in both GBMs and MNGs, stimulates tissue macrophages to produce a higher level of IDO-1, which via alteration of cytokine levels, inhibits the proliferation of effector T-cells. The immune-suppressive role of IDO-1 was supported previously by studies

using Trp metabolites that induce the differentiation of Treg cells and increase apoptosis of effector T-cells through inhibiting the mechanistic target of rapamycin complex 1 (m-TORC1) [209], [210].

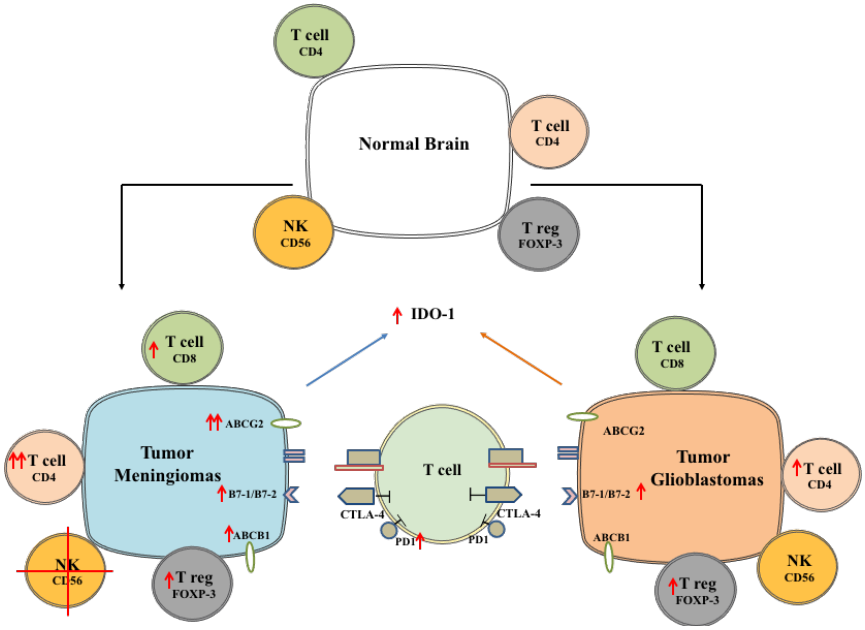

Figure 17. Schematic summary of the characteristic immune and molecular microenvironments of intra-and extra-parenchymal CNS tumors. Adapted from our publication [211].

Currently, several IDO-1 inhibitors, including epacadostat, navoximod, and BMS-986205 [212], are under clinical evaluation and the results are promising using IDO inhibitors in combination with anti-PD1 in preclinical models of GBM [213]. Another study performed in a GBM mouse model using anti-PD-1, anti-CTLA-4, and IDO-1 inhibitor combination showed a dramatic improvement in therapy of the disease [214].

Another emerging target of cancer immunotherapy is the CD27-CD70 pathway [215]. While the CD27–NFκ B pathway is mediated by TRAF2, the CD27 –c-Jun kinase pathway is mediated by TRAF5, resulting in survival, proliferation, and differentiation signals. The cytoplasmic tail of CD27 can also bind to Siva when caspase-mediated apoptosis is induced. The interaction of CD27 receptor on the surface of T-cells with CD70 on tumor cells can induce apoptosis in lymphocytes [216]. Recent studies have provided evidence that anti-CD27 combined with PD1/PD-L1 inhibitors can reactivate $CD8^{+}$ T-cells [217], that might offer an additional treatment for brain tumors. Although the low levels of CD27 in both investigated

tumor types, further investigation might provide further, potentially more effective treatment approaches.

Overall, activation or inhibition of the immune system depends on the balance between co-stimulatory and co-inhibitory pathways. In aggressive tumors, the immune system is often suppressed which secures the survival of the abnormal cells. It appears that combination therapy is necessary to overcome the strongly immune-suppressive brain tumor milieu. Using the appropriate immune checkpoint inhibitors in combination with IDO-1 inhibitors might be an alternative treatment for the inoperable brain tumors, refractory MNGs, and chemoresistant GBMs.

Many tumors are resistant to chemo- and immunotherapy due to the complexity of their microenvironments. Alteration of the immune microenvironment strongly correlates with the aberrant WNT molecular microenvironment. WNT signaling is one of the main oncogenic pathways that have been found to act on the tumor microenvironment and leads to immune evasion [218]. Based on the WNT microenvironment analysis, WNT5A was nearly three-fold higher in MNG than GBM. It is well known that in certain tumors WNT5A signaling is a significant event in tumor progression, via promoting the synthesis of the anti-inflammatory cytokines such as IL-10 [219]. Recently, an immunomodulatory role of WNT/β-catenin signaling in tumor microenvironment has been further considered [220]–[222]. Deregulation of WNT ligands, receptors, and pathway modulators would not only result in increased invasion, proliferation, and metastatic potential but would also significantly affect the clinical outcome of the disease [223]. Additionally, aberrant WNT signaling leads to chemoresistance via modulation of the expression of ABC drug transporters. In lung squamous cell carcinoma (SCC), WNT5A downregulates the ABCB1 and ABCG2, which are involved in chemotherapy resistance and appear to be upregulated by WNT/β-catenin signaling when the cells are treated with the chemotherapeutic agent cisplatin [123]. WNT7A acts via activation of the canonical WNT/β-catenin to induce cell proliferation in endometrial carcinoma [224]. Screening of the WNT signaling pathway showed that WNT7A was expressed more than ten-fold in GBM compared to MNG. Whereas SFRP2 level was significantly (over four -fold) higher in MNG than in GBM. SFRP2 has a role in suppressing the activity of WNT7A, leading to inhibition of cell division. Our study showed an overexpression of WNT6 in benign MNG (over sixty-fold) than GBM. WNT6 was recently reported as a novel oncogenic

target in GBM [225]. Therefore, since WNT6 is overexpressed in MNG, it might be a potential marker aiding in the rational treatment decision for these tumors. WNT16 was found to be about six-fold higher in GBM compared to MNG. It was reported previously that WNT16 is associated with poor overall survival in patients with glioma [226].

Our data shows similar findings with the results published in the literature concerning the expression of DKK1 in GBM [227], [228] and DKK3 in MNG [229]. Additionally, our results revealed that the expression levels of KREMEN2 were present at a similar level in MNG and in the normal brain while, its expression was increased in GBM. The present study was the first to report a higher expression level of DKK2 in MNG than DKK1 and DKK3. It is known that DKK2 acts as an inhibitor of WNT/LRP6 signaling in the presence of KREMEN2 [230], which might explain the role of DDK2 as an agonist of WNT/β-catenin signaling in MNG. Similar expression levels of DKK3 were reported in the endothelial cells of MNG previously [229].

The canonical WNT/β-catenin signaling is one of the most frequently activated pathways that implicated in the pathogenesis of a variety of tumors. Above, we identified aberrant expression of the WNT ligands and signaling members in MNG and GBM. LEF1 is a critical mediator of the WNT/β-catenin signaling, which interacts with β-catenin to regulate WNT target gene expression such as axin2 and naked cuticle1 (NKD1). NKD1, has a well-known nucleo-cytoplasmic role and it is negative feedback regulator of the canonical WNT signaling pathway [231], [232]. Since TCF/LEF are identified as critical effectors, their functions have been under intensive investigation in cancer biology.

In previous studies elevated LEF1 expression was observed in 71% of GBM samples and is considered as a potential marker for malignant transformation [233]. In our study, elevated mRNA expression levels of LEF1 were detected in both GBM as well as in MNG. However, while LEF1 positivity was observed in WBC of GBM vessels, increased LEF1 presence was detected in the tumor cells in MNG. In GBM, endothelial cells acquire transformation into mesenchymal stem cell (MSC)–like cells, driving tumor resistance to cytotoxic treatment via upregulating a distinct target genes including LEF1 [234]. Not surprisingly, the LEF1 is highly expressed in radioresistance GBM cells [235]. Although the WNT signaling pathways are infrequently mutated in GBM, these pathways are often aberrantly activated and

enhance the cancer stem cell-like phenotypes of GBM cells. Furthermore, a few reports have suggested that WNT signaling plays a crucial role in dampening antitumor immune response in the tumor microenvironment [236]. Within T cells, WNT/β-catenin appears to inhibit T-cell activation [220]. It was described in lung cancers that the canonical WNT pathway can induce ABCB1 and ABCG2 drug transporter expression [123]. In GBM ABC transporter expression is stage dependent [237]. It is also known that TMZ is a weak substrate of ABCB1which also explains the developing drug resistance to TMZ over time.

6. CONCLUSION

Overall, the altered immune- and WNT molecular microenvironments not only result in increased proliferation but also in tumor resistance against radio-immuno- and chemotherapy.

The balance between co-stimulatory and co-inhibitory pathways determines activation or inhibition of the immune system. In aggressive tumors, such as GBM, the immune system is often suppressed which ensures the survival of the tumor cells. It appears that combination therapies are essential to overcome the strongly immune-suppressive brain tumor environment. Using IDO1 inhibitors in combination with the appropriate immune checkpoint inhibitors might be an alternative treatment for both parenchymal and extra-parenchymal therapy-resistant brain tumors.

Although further studies are essential, the difference between MNG and GBM are clear. MNG patient samples have no NK cells, so even targeted therapy using specific antibodies would not be able to activate NK cells that carry FcγR as there aren't any NK cells to recognize the antibody and eliminate the tumor cell. Even macrophages are likely to be of TAMs. The $CD8^+$ cytotoxic T-cells are present in MNG and they also express PD1, however, the tumor is negative for PDL1. Therefore, immune checkpoint inhibitors would not likely to have any effects. The presence of a large number of $CD4^+$ Th cells, $CD68^+$ TAMs, and IDO1 point to the immunosuppressive tumor milieu. Furthermore, MNG samples have shown deregulation in distinct genes causing alteration in WNT/β-catenin signaling. Our data reveal for the first time that the MNG tumor cells overexpressed LEF1. This might explain the somewhat increased levels of ABCB1 and ABCG2 in MNG indicating reduced ability to respond to chemotherapeutic agents. Despite MNG samples are defined as slow growing neoplasms, they show a clear tendency to recurrence after resection.

Although GBMs have a normal level of NK marker, have shown a restricted level of cytotoxic T-cell level than normal and no PD1 staining. Meanwhile, there is an abundance of $CD4^+$ Th cells, $CD68^+$ TAMs, and significantly elevated levels of IDO1. Moreover, both brain tumor types have significantly increased B7-2 (CD86) expression, which is the ligand of CTLA-4 on T-cells. CTLA-4 has shown a role in turning down T-cell activity. Although CTLA-4 is there on all T-cells, its level is not higher than in the control in either MNG or GBM, it can also lead to a complex

immunosuppressive signal. The immunosuppressive $CD4^+$ Treg cells that are in abundance in both MNG and GBM do not express CTLA-4, which can lead to continuous activation of the immunosuppressive $CD4^+$ Treg cells. Furthermore, in GBM, WBCs have shown alteration in the expressions of the LEF1, which potentially increase the invasion of the tumor and significantly affect therapy. Deregulation in WNT/β-catenin signaling guarantee the molecular microenvironment that supports and maintains the malignancy of GBM. Apparently, WNT/β-catenin signaling modulates both the immune and molecular microenvironment of each tumor differently leading to resistance.

7. SUMMARY

Tumor microenvironment is the key regulator of carcinogenesis which not only controls the progression of tumor but also the tumor response to various treatment modalities. To date, the survival rates of patients diagnosed with CNS tumors have not improved significantly. Generally, MNG is a low-grade or benign brain tumor, originating from the non-glial tissues of the CNS while GBM is the most aggressive malignant CNS neoplasm. The primarily available option to treat MNG or GBM is still surgery, and even if the tumor is operable, the process might lead to major side effects. Radio- and chemotherapy are the remaining treatment options for inoperable cases and recurrent tumors.

The complexity of CNS tumors can also be detected at the levels of aberrant immune- and molecular microenvironments where WNT signaling plays a very important role and can modulate both. Additionally, tumor behavior is mediated by the microenvironment against radio-, immuno- and chemotherapy. Immunotherapy, although is a novel and efficacious treatment option in several other malignancies, is mostly ineffective in neuro-oncology. The reason behind it is likely to be the immunosuppressive property of the brain tumor. Study of the immune microenvironment of both MNG and GBM has proved to be similar. The immune-suppressive cells including Treg and TAMs were highly elevated in both tumor types. Moreover, the cytokine environment like IL10 and TGFβ that support Treg differentiation and the increased presence of IDO also drive the development of the immunosuppressive microenvironment. The presence of immune cells is also support the lack of immune protection. GBM patient samples for example have just a normal level of CD56+ NK cells while, MNG samples do not. They have no NK cells compared to normal control. Despite extensive research, molecular pathogenesis remains poorly characterized in brain tumors. The WNT-molecular microenvironment has a great effect on tumor progression and behavior against immune- and chemotherapy. LEF1 is a WNT/β-catenin nuclear effector, which activates downstream target genes. IHC double staining analysis revealed that GBM patient cases showed greater positivity of LEF1 in infiltrating WBC. However, in MNG patients the positivity of LEF1 was mostly detected in tumor cells. Thus, this might explain the high levels of ABCB1 and even more elevated levels of ABCG2 in MNG. The balance between suppressed and activated status of tumor immune

microenvironment is decided by the variety of signaling pathways. WNT/β-catenin signaling cascade is one of the critical pathways that regulate the anticancer immune response. It leads to the subsistence of Tregs and maintains the immune suppressive microenvironment, through upregulation of anti-inflammatory cytokines.

Immune modulatory treatments in combination with IDO1 and WNT/β-catenin inhibitors might even become alternative therapy via targeting tumor microenvironment of relapsed, multiple and/or malignant MNG or chemo-resistant GBM.

8. Összefoglalás

A karcinogenezis kulcsfontosságú szabályozója a tumor mikrokörnyezet, amely nemcsak a daganat progresszióját szabályozza, hanem a daganat válaszát a különböző kezelési módokra is. Mind a mai napig a központi idegrendszeri daganatokkal diagnosztizált betegek túlélési aránya nem mutatott jelentős javulást. A meningioma (MNG) általában egy alacsony grádusú vagy jóindulatú agydaganat, amely a központi idegrendszer nem gliális szöveteiből származik, míg a glioblastoma (GBM) a legagresszívebb rosszindulatú központi idegrendszeri daganat. Az MNG vagy GBM terápiája elsődlegesen továbbra is a műtéti eljárás, de még abban az esetben is, ha a daganat műthető, maga a folyamat súlyos mellékhatásokhoz, nem várt eseményekhez vezethet. A visszatérő daganatok és az inoperábilis daganatok esetében a sugár- és kemoterápia az egyedüli kezelési lehetőség.

A központi idegrendszeri daganatok komplexitása a rendellenes immun- és molekuláris mikrokörnyezetek szintjén is kimutatható, ahol a WNT jelátvitel nagyon fontos szerepet játszik, és mindkettőt modulálni tudja. Ezenkívül a tumor válaszreakcióját a saját mikrokörnyezete határozza meg a sugár-, immun- és kemoterápia közben. Az immunterápia, számos egyéb rosszindulatú daganat esetében újszerű és ugyanakkor hatékony kezelési lehetőséget biztosít, azonban a neuro-onkológiában többnyire hatástalan. Ennek oka valószínűleg az agydaganat immunszuppresszív tulajdonságában rejlik. Az immun-mikrokörnyezet vizsgálatakor az MNG és a GBM tulajdonságaikat tekintve hasonlónak bizonyult. Az immunszuppresszív sejtek, beleértve a regulatív T-sejteket és a tumor-asszociált makrofágokat, mindkét tumor típusban jelentősen emelkedett értéket mutattak. Ezenkívül az a citokin-környezet, mint például az IL10 és a TGFβ, amelyek támogatják a regulatív T-sejtek differenciálódását, továbbá az IDO fokozott jelenléte, szintén az immunszuppresszív mikrokörnyezet kialakulását teszik lehetővé. Ugyanakkor az immunsejtek jelenléte szintén az immunvédelem hiányát támogatja. A GBM betegmintákban például normális a CD56 + NK (természetes ölő sejtek, “Natural Killer”, NK) sejtek szintje, míg az MNG mintákban nem kimutatható, nincsenek NK sejtjeik a normál kontrollhoz képest. A széleskörű kutatások ellenére az agydaganatok molekuláris patogenezise továbbra sem tisztázott. A WNT molekuláris mikrokörnyezetnek fontos szerepe van a tumor progressziójában, valamint az immun- és kemoterápiára adott válaszreakcióban is. A LEF1 egy olyan

WNT/β-katenin nukleáris effektor, amely aktiválja a downstream célgéneket. Az immunhisztokémia kettős festési elemzéséből kiderült, hogy a GBM-esetek nagyobb LEF1 pozitivitást mutattak. MNG-s betegeknél azonban az LEF1 pozitivitását leginkább a tumorsejtekben mutatták ki, amely megmagyarázhatja az ABCB1 emelkedett szintjét és az ennél is magasabb ABCG2 szintet is. A tumor immun mikrokörnyezetének szuppresszált és aktivált állapota közötti egyensúlyt a jelátviteli utak változatossága határozza meg. A rákos megbetegedéssel szembeni immunválasz kialakításáért a WNT/β-katenin jelátviteli kaszkád felelős, mely a gyulladáscsökkentő citokinek szabályozásán keresztül T-sejt reguláción át az immunszuppresszív mikrokörnyezetet.

Az immunmodulációs kezelések IDO1 és WNT / β-catenin inhibitorokkal kombinálva, a visszatérő, többszörös és / vagy rosszindulatú MNG vagy kemo-rezisztens GBM tumor mikrokörnyezetét célozva alternatív terápiaként szolgálhatnak.

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10. Publications

The thesis is based on the following publications

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Poster presentations related to this thesis

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12. APPENDIX

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Brief Report

Is an Immunosuppressive Microenvironment a Characteristic of Both Intra- and Extraparenchymal Central Nervous Tumors?

Amina Soltani [1,2], Bela Kajtar [3], El Husseiny Mohamed Mahmud Abdelwahab [1,2], Anita Steib [4], Zsolt Horvath [5], Laszlo Mangel [6], Luca Jaromi [1,2] and Judit E. Pongracz [1,2,*]

1 Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, University of Pecs, 7624 Pecs, Hungary; aminasoltani8@gmail.com (A.S.); elhusseiny.mohamed@pte.hu (E.H.M.M.A.); jaromi.luca@pte.hu (L.J.)
2 Szentagothai Research Centre, University of Pecs, 7624 Pecs, Hungary
3 Medical School and Clinical Centre, Department of Pathology, University of Pecs, 7624 Pecs, Hungary; kajtar.bela@pte.hu
4 Humeltis Ltd., 7624 Pecs, Hungary; anita.steib@humeltis.com
5 Medical School and Clinical Centre, Departments of Neurosurgery, University of Pecs, 7623 Pecs, Hungary; horvath.zsolt@pte.hu
6 Oncotherapy, Medical School and Clinical Centre, University of Pecs, 7624 Pecs, Hungary; mangel.laszlo@pte.hu
* Correspondence: pongracz.e.judit@pte.hu

Abstract: AbstractsIn spite of intensive research, the survival rates of patients diagnosed with tumors of the central nervous system (CNS) have not improved significantly in the last decade. Immunotherapy as novel and efficacious treatment option in several other malignancies has failed in neuro-oncology likely due to the immunosuppressive property of the brain tissues. Glioblastoma (GBM) is the most aggressive malignant CNS neoplasm, while meningioma (MNG) is a mainly low grade or benign brain tumor originating from the non-glial tissues of the CNS. The aim of the current preliminary study is to compare the immune microenvironment of MNG and GBM as potential target in immunotherapy. Interestingly, the immune microenvironment of MNG and GBM have proved to be similar. In both tumors types the immune suppressive elements including regulatory T cells (Treg), tumor-associated macrophages (TAM) were highly elevated. The cytokine environment supporting Treg differentiation and the presence of indoleamine 2,3-dioxygenase 1 (IDO1) have also increased the immunosuppressive microenvironment. The results of the present study show an immune suppressive microenvironment in both brain tumor types. In a follow-up study with a larger patient cohort can provide detailed background information on the immune status of individual patients and aid selection of the best immune checkpoint inhibitor or other immune modulatory therapy. Immune modulatory treatments in combination with IDO1 inhibitors might even become alternative therapy for relapsed, multiple and/or malignant MNG or chemo-resistant GBM.

Keywords: meningioma; glioblastoma; immune microenvironment; immune suppression

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1. Introduction

In 2019 about 87,000 people were diagnosed with primary brain tumors in the United States alone. An estimated 26,000 cases were malignant and 61,000 cases were so called benign [1]. In the current study, we focused on the immune microenvironments of two main types of brain tumors with unrelated histology and origin, namely glioma and meningioma. We compared their immune microenvironments to evaluate the potential use of currently available immune therapies.

Histologically the two tumor types that were selected for the study couldn't be more different. The malignant gliomas originated from glia cells (astrocytic, ependymal and oligodendrocytic types) and are categorized as low-grade gliomas (LGG grades I and II) and high-grade gliomas (HGG grades III and IV) [2,3]. Glioblastomas (GBM), the most

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KRAS and EGFR Mutations Differentially Alter ABC Drug Transporter Expression in Cisplatin-Resistant Non-Small Cell Lung Cancer

Luca Jaromi [1,2], Veronika Csongei [1,2], Monika Vesel [1,2], ElHusseiny Mohamed Mahmud Abdelwahab [1,2], Amina Soltani [1,2], Zsofia Torok [1,2,3], Gabor Smuk [4], Veronika Sarosi [3] and Judit Erzsebet Pongracz [1,2,*]

1 Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, University of Pecs, 2 Rokus Str, H-7624 Pecs, Hungary; jaromi.luca@pte.hu (L.J.); csongei.veronika@pte.hu (V.C.); monika.avdicevic@gmail.com (M.V.); elhusseiny.mohamed@pte.hu (E.M.M.A.); aminasoltani8@gmail.com (A.S.); torok.zsofia@pte.hu (Z.T.)
2 Wnt-Signalling and Biotechnology Research Group, Szentagothai Research Centre, University of Pecs, 20 Ifjusag Str, H-7624 Pecs, Hungary
3 Department of Pulmonology, Internal Medicine, The Medical School and Clinical Centre, University of Pecs, 12 Szigeti Str, H-7624 Pecs, Hungary; sarosi.veronika@pte.hu
4 Department of Pathology, The Medical School and Clinical Centre, University of Pecs, 12 Szigeti Str, H-7624 Pecs, Hungary; smuk.gabor@pte.hu
* Correspondence: pongracz.e.judit@pte.hu; Tel.: +36-72-536-000 (ext. 29250) or +36-30-435-7944

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Abstract: Lung carcinoma is still the most common malignancy worldwide. One of the major subtypes of non-small cell lung cancer (NSCLC) is adenocarcinoma (AC). As driver mutations and hence therapies differ in AC subtypes, we theorized that the expression and function of ABC drug transporters important in multidrug resistance (MDR) would correlate with characteristic driver mutations KRAS or EGFR. Cisplatin resistance (CR) was generated in A549 (KRAS) and PC9 (EGFR) cell lines and gene expression was tested. In three-dimensional (3D) multicellular aggregate cultures, both ABCB1 and ABCG2 transporters, as well as the WNT microenvironment, were investigated. ABCB1 and ABCG2 gene expression levels were different in primary AC samples and correlated with specific driver mutations. The drug transporter expression pattern of parental A549 and PC9, as well as A549-CR and PC9-CR, cell lines differed. Increased mRNA levels of ABCB1 and ABCG2 were detected in A549-CR cells, compared to parental A549, while the trend observed in the case of PC9 cells was different. Dominant alterations were observed in LEF1, RHOU and DACT1 genes of the WNT signalling pathway in a mutation-dependent manner. The study confirmed that, in lung AC-s, KRAS and EGFR driver mutations differentially affect both drug transporter expression and the cisplatin-induced WNT signalling microenvironment.

Keywords: NSCLC; AC; KRAS; EGFR; ABC drug transporters; WNT signalling

1. Introduction

Lung carcinoma (LC) is still the most common malignancy and one of the leading causes of cancer-related death worldwide [1]. Despite significantly improved treatment modalities, five-year survival of LC barely exceeds 18% primarily due to late diagnosis [2–4]. The two main lung cancer types are small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Approximately 12 out of 100 LC patients are diagnosed with SCLCs (12%), while the larger proportion (88%) with NSCLCs. NSCLC has three common types: squamous cell carcinoma (SCC), adenocarcinoma (AC) and large cell carcinoma (LCC). Treatment of patients diagnosed with AC is based on specific marker mutations, including Kirsten rat sarcoma viral oncogene homologue (KRAS), epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), proto-oncogene 1, receptor tyrosine kinase

Statement on the originality of the PhD thesis and papers

Undersigned name: Soltani Amina
Birth name: Soltani Amina
mother's maiden name: Soltani Abbassia
place and date of birth Biskra, Algeria, 1993.03.13

Today I submitted my PhD thesis entitled: Characteristics of The Immune and Molecular Microenvironment of The Intra-and Extra-Parenchymal Tumors of The Central Nervous System

To Doctoral School of Biology and SportBiology

Name of supervisor: Prof. Dr. Pongrácz Judit E.
Name of co-supervisor: Dr. Járomi Luca

In addition, I declare my doctoral dissertation submitted in the present case

- had no submitted other doctoral school (not domestic nor abroad university) earlier,
- my application to doctoral process is not rejected within two years,
- I had no unsuccessful doctoral procedure the last two years
- my doctorate degree has not cancelled within five years,
- my thesis is an independent work, I have not presented another person's intellectual property as it would be mine, the references are clear and complete, the false or falsified data are not used in my dissertation.

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Signature of candidate