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TESI DI DOTTORATO

The role of PD-L1 in biological behavior of intracranial meningiomas. Increased PD-L1 expression in meningioma recurrences compared to their primary presentation

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1.INTRODUCTION

1.1 MENINGIOMAS

Meningiomas are the most common group of adult central nervous system primary tumor, accounting for approximately 36.6% of all primary tumors of the central nervous system in the USA [1]. They are extra-axial tumors, deriving from arachnoid cap cells.

1.1.1 Epidemiology

The incidence of meningiomas is 7.4 per 100.000 individuals, the prevalence is 97,5 per 100.000 individuals, with approximately 18.000 new cases per year and 170.000 individuals living with this diagnosis in the U.S.A. [2].

Their incidence is age-related: it ranges from 0.14 cases every 100.000 individuals younger than 19 years, to 37.75 cases every 100.000 individuals aged between 75 and 84 years. The average age at diagnosis is 65 years [1].

The incidence is higher in the Africans and Americans compared to Caucasian and Asian populations. The female-male ratio is 3.15:1. Female predominance is thought to be partly related to endogenous sex hormone levels [3]. In fact, the increased risk in females occurs mainly before menopause. Interestingly, higher grade lesions are more frequent in the male gender.

1.1.2 Natural History

Usually (90% of cases) meningiomas present as a single lesion, while multiple forms are rare. The WHO classification distinguished meningiomas into three grades: most of them (70-80%) are classified as grade 1 and are usually associated with indolent behavior. On the other hand, the remaining 20-30% show a more aggressive attitude.

Among these, grade 2 meningiomas (20-25%) and grade 3 meningiomas (1-6%) are included [4].

Patients with benign, atypical and malignant meningiomas have 5-year overall survival of 88%, 86% and 50% and 10-year overall survival of 77%, 71% and 23%, respectively. Estimated overall 10-year survival for meningioma is 77.7% for young adults at the time of diagnosis (20-44 years) [1]. Median survival of patients with a malignant meningioma is 4 years and 1 month, despite aggressive therapeutic approaches.

Prognosis of higher grade meningiomas is much worse than that of grade 1: recurrence rate is 30-40 % for WHO grade 2 and 50-94% for WHO grade 3 meningiomas, meanwhile 5-year mortality are 21 % and 68 % respectively [2].

Although grade 1 meningiomas are known for their indolent behavior, there is a subset of them characterized by a more aggressive course, in which recurrence and progression despite gross total resection may happen. In literature the recurrence rate of grade 1 meningiomas ranges from 7 to 20 % [2].

Extracranial metastasization is extremely rare, occurring in 1 case per 1000, predominantly associated with grade 3 meningiomas. Rare cases of histologically benign meningiomas metastases occur more frequently after surgery.

Predictive prognostic factors for survival in patients with meningioma include the extent of surgical resection, histological grade, patient age and tumor location.

Depending on location and degree, meningiomas may have different mortality and morbidity values.

Recurrence

The best-known predictors for meningioma recurrence are WHO grade and extent of surgical resection [5].

The risk of meningioma recurrence is proportionally related to the histopathological grade and, among the same grade, to the mitotic count [6]. Normally, proliferation

index increases in proportion to grading. The mitotic index and Ki-67 proliferation index are approximately related to the rate of tumor growth.

Some studies suggest that meningiomas with a proliferative index (Ki-67) >4% have an increased risk of recurrence similar to that of atypical meningiomas, while those with an index >20% are associated with a risk of death similar to the risk of anaplastic meningiomas [7].

Despite this, an internationally recognized cut-off value is far to be determined. Currently, Ki-67 proliferative index is used as an addition to the standard WHO classification, rather than as an independent grade indicator.

Progression

Growth of residual tumor or transformation from a lower to a higher degree.

Multiple Meningiomas

Two or more synchronous or metachronous meningiomas in the same patient are called multiple meningiomas. Incidence is variable (1-16% of meningiomas), with a strong female predominance (ranging from 60 to 90%). They could be associated with neurofibromatosis 2; some familiar cases are described in families with no history of neurofibromatosis.

Multiple meningiomas may be secondary to a recurrence in the surgical site or to a postoperative dissemination through the cerebrospinal fluid (CSF) [8].

Some studies focusing on inactivation of X chromosome through Southern blot analysis show that meningiomas are monoclonal tumors [9], although some studies based on the use of PCR suggest that a small portion of them could be polyclonal [10].

The clonality of multiple meningiomas has also been studied focusing the inactivation of cromosome X and the analysis of the NF2 gene mutation in the same patients [11]. In these studies, most of the lesions of patients with >3 meningiomas, show to have

the same inactivated X chromosome or to carry the same NF2 mutation. These data provide strong evidence of clonal origin of multiple meningiomas in most patients.

1.1.3 Pathology

Most meningiomas are globular, encapsulated tumor, well delimited, lobulated, with a large area of adhesion to the dura.

Regardless of grade, meningiomas frequently invade adjacent anatomic structures (especially dura or dural sinuses), even though this possibility and the extent of infiltration are greater in the more aggressive subtypes.

Some meningiomas invade adjacent cranial bones, where they can induce a characteristic hyperostosis, which is highly suggestive of bone invasion. Meningiomas can encase cerebral arteries, but infrequently infiltrate the arterial wall. They can also infiltrate the skin and extracranial compartments. The adjacent brain is very often compressed, but rarely invaded.

The distribution of intracranial meningiomas is approximately as follows: convexity (35%), parasagittal (20%), sphenoid ridge (20%), intraventricular (5%), tuberculum sellae (3%), infratentorial (13%), and others (4%) [8].

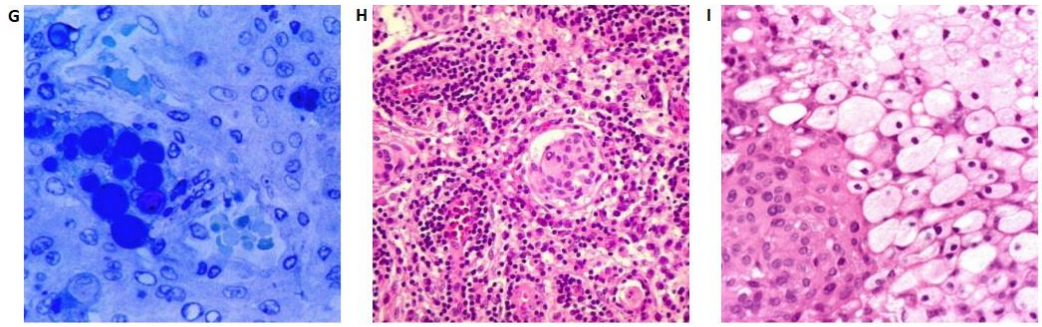
In some sites, especially along the sphenoidal ring, meningiomas so called "en plaque," can grow as a flat, carpet-like mass, taking the shape of the underlying bone.

Atypical and anaplastic meningiomas tend to be larger and often have areas of necrosis.

Last WHO classification of tumors of the central nervous system (2016) defined 15 histological sub-types of meningiomas: meningothelial, fibrous, transitional, psammomatous, angiomatous, microcystic, secretory, lymphoplasmacyte-rich, metaplastic, chordoid, clear cell, atypical, papillary, rhabdoid, anaplastic (malignant). [4]

- meningothelial: characterized by medium size epithelioid tumor cells forming lobules, some of which are partly delimited by thin collagen septa.
- fibrous: consisting of spindle cells organized in parallel bundles and intertwined in a collagen-rich matrix.
- microcystic: characterized by cells with thin and elongated processes that enclose microcysts and create a spider web-like background.
- psammomatous: it contains a predominance of psammomatous bodies compared to tumor cells.
- angiomatous: characterized by numerous blood vessels, which often constitute a large portion of the tumor mass.
- transitional: it presents a mixed growth pattern, with meningothelial and fibrous areas and transition characteristics.
- secretory: characterized by the focal presence of epithelial differentiation, which manifests itself in the formation of intracellular lumens containing eosinophilic secretions positive to PAS (Periodic Acid Schiff), called pseudopsammomatosis bodies.
- lymphoplasmacyte-rich: characterized by a chronic inflammatory lymphoplasmacytic infiltrate, which often obscures the meningothelial component.
- metaplastic: it has a focal or diffuse mesenchymal component; this component can be bone, cartilaginous, lipomatous, mixoid and xantomatose, singularly or in combination.
- chordoid: histologically resembles chordoma, in fact, we can observe cords or trabecole of eosinophilic cells, in the context of an abundant mucoid matrix.

- clear cells: often lacking a characteristic growth pattern, characterized by round or polygonal cells with clear cytoplasm, rich in glycogen (PAS+); e usually a deposition of interstitial and perivascular collagen coexist.
- rhabdoid: characterized mostly by rhabdoid cells: swollen cells with eccentric, open chromatin, prominent nuclei, and often with prominent eosinophilic paranuclear inclusions.
- papillary: defined by the presence of a pseudopapillary pattern that constitutes the majority of the tumor.
- anaplastic: shows a malignant cytology (reminiscent of a carcinoma or melanoma) and/or high proliferative activity (≥20 mitosis for 10HPF). Often large areas of necrosis are present.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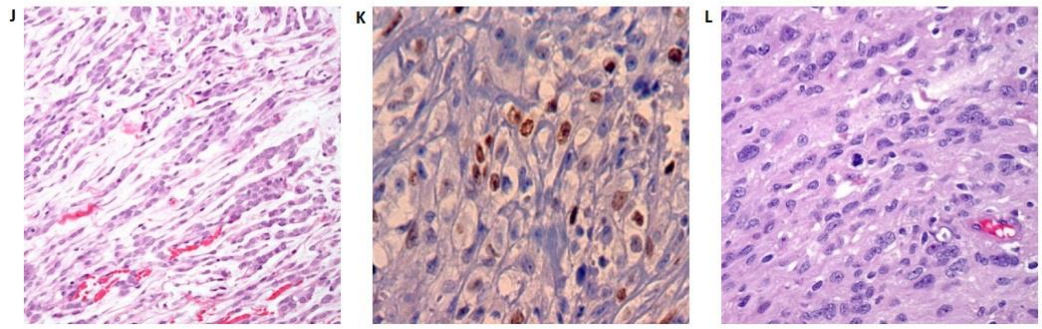
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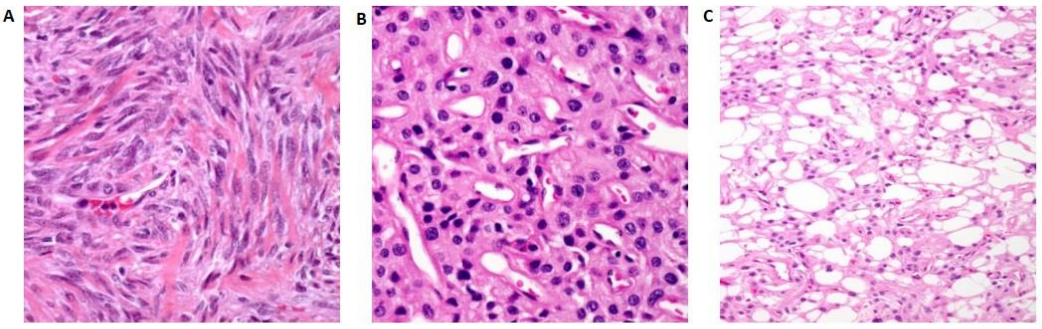
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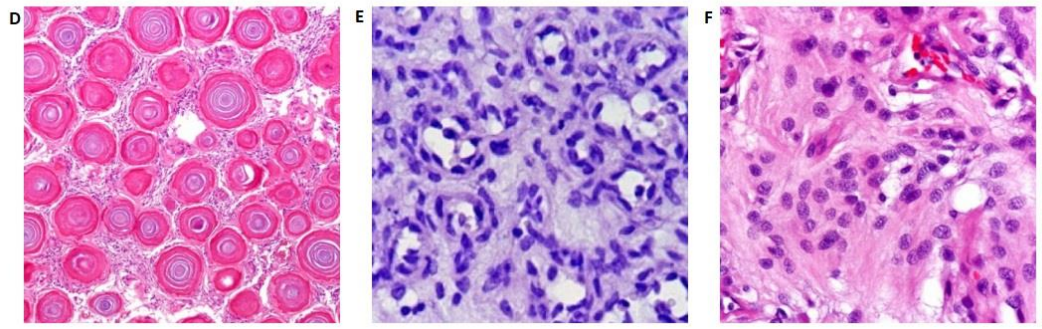
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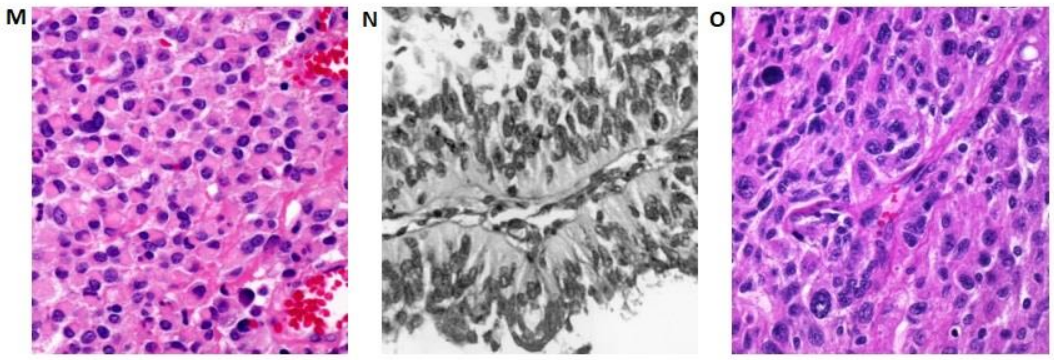


Figure 1. ***Histological types*** **[12]**

A: meningothelial; **B:** fibrous; **C:** microcystic ; **D:** psammomatous; **E:** angiomatous; **F:** transitional; **G:** secretory; **H:** lymphoplasmacyte-rich ; **I:** metaplastic; **J:** chordoid; **K:** clear cell; **L:** atypical; **M:** rhabdoid; **N:** papillary; **O:** anaplastic.

Most of the subtypes behave in a benign way, but four distinct histological variants are more likely to recur and determine a more aggressive clinical course and are therefore classified by the WHO as grade 2 (chordoid and clear cell) and grade 3 (rhabdoid and papillary histology).

The immunohistochemistry (IHC) marker most commonly used to identify meningioma is the epithelial membrane antigen (EMA), although more recent studies have shown that the somatostatin 2A receptor (SST2A) is more sensitive [13]. EMA is less reliable in atypical and anaplastic lesions. On the other hand, the somatostatin 2A receptor is less specific, being also expressed by neuroendocrine tumors.

Positivity to vimentin has been found in all meningiomas but is not specific.

Positivity to the S100 protein is very common in fibrous meningiomas but is not as widespread as it usually is in schwannomas.

In addition, 70-80% of meningiomas are receptor positive for progesterone and, to a lesser extent, for the estrogen receptor [14]. The expression of the progesterone receptor is inversely proportional to the degree; its complete negativity seems to be associated with a worse prognosis [15]. In fact, practically all grade 3 meningiomas are negative for the progesterone receptor.

Meningiomas are divided into three grades, based on histopathologic criteria and the risk of recurrence and aggressive growth. Most of them (70-80%) are classified as grade 1 (called "benign") and are associated with indolent behavior. The remaining 20-30%, on the other hand, show a more aggressive attitude and these include grade 2 meningiomas (20-25%) and grade 3 meningiomas (1-6%) [4].

WHO grade	*Criteria*	*Frequency*
Grade 1	- Low mitotic count, <4/ 10 HPF - No brain invasion - 9 histological sub- types: menigothelial, fibrous, transitional, psammomatous, angiomatous, microcystic, secretory, lymphoplasmacyte-rich, metaplastic	80-85%
Grade 2	- Mitotic count 4-19/ 10HPF Or - Brain Invasion Or - ≥ 3 of 5 specific histological characteristics: • necrosis • macronucleoli • hypercellularity • small cell with high index n/c • solid sheets Or - Specific histology: clear cell or chordoid	15-20%
Grade 3	- Mitotic count >20/ 10 HPF Or - Specific histology: papillary or rahbdoid	1-2%

Table 1. Frequency and WHO 2016 graduation criteria of meningiomas

1.1.4 Genetics

Meningiomas, as all neoplasms, are characterized by clonal proliferation.

Some meningiomas are associated with chromosomal deletions, which tend to be multiple in high-grade meningiomas, up to determine a picture of chromosomic instability. In particular the deletion with consequent inactivation of NF2 on chromosome 22 is one of the predominant features in sporadic tumors [16]. Other deletions that have been frequently identified are in the 14q, 1p, 6q, and 18q regions [17].

There are also family syndromes that predispose to the development of meningiomas, the most frequent being neurofibromatosis type 2 (NF2), an autosomal dominant condition [18]. Other predisposing syndromes are Li-Fraumeni syndrome, Gorlin syndrome, Von Hippel-Lindau ma-can, Cowden syndrome and multiple neoplasms of the endocrine system type 1 (MEN1).

NF2 gene

The Neurofibrin 2 (NF2) gene, which encodes the Merlin cytoskeletal protein, is the first gene characterized as a meningioma tumorigenesis driver [5].

NF2 gene mutations have been identified in several meningiomas associated with neuro-fibromatosis type 2 (NF2) and in 60% of sporadic meningiomas [16]. In many cases, mutations are small insertions, deletions or nonsense mutations (i.e. they create stop codons).

The frequency of NF2 mutation varies depending on tumor histotype. Fibroblastic and transitional meningiomas, (mostly convexity meningiomas), very often are NF2 mutated [19].

Non-NF2 oncogenic drivers

Recently, non-NF2 oncogenic drivers were identified, like POLR2A, TRAF7, KLF4, AKT1, FOXM1, SMARCB1, SMARCE2, and SMO genes. Consequently, different

pathways, like RNA polymerase, proapoptotic E3 ubiquitin ligase, PI3K, Wnt signaling, SWI/SNF chromatin remodeling complex and the Hedgehog are implicated in meningiomas tumorigenesis and progression [5].

1.1.5 Etiology and Risk Factors

-IRRADIATION

To date, the main environmental risk factor identified for meningioma is exposure to ionizing radiation (IR). In fact, an increase in the incidence of meningioma in survivors of the atomic explosion has been observed [20].

Another study carried out in Israel between 1948 and 1960, showed that children who received radiotherapy for Tinea Capitis have a relative risk of almost 10 times higher for the development of neoplasia [21].

The most recent case-control study of 200 patients with meningioma reported that patients who received full-mouth radiographs had a significantly increased risk of meningioma (OR 2.06, IC 95% 1.03, 4.17) although there was no dose-response relationship (P for trend = 0.33) [22].

- HORMONES

An association between hormones and meningioma risk is suggested by the increased incidence in women and progesterone receptor expression in most cancers.

Researchers have tried to understand whether the use of exogenous hormones such as oral contraceptives (OC) and/or hormone replacement therapy (HRT) was associated with an increased risk of meningioma.

However, numerous studies have shown that there is no real correlation between the use of oral contraceptives and the onset of meningiomas [23,24,25].

- BREAST CANCER

A possible association between breast cancer and meningioma has been examined in several studies [26, 27, 28], including that of Custer et al. which took into account data from the Washington State cancer registry [28]; the relative risks observed in existing studies vary between 1.5 and 2.0. Most of these studies, however, are based on relatively small sample size. Probably, there is no real causal relationship between these two tumors, but it should be hypothesized they share certain risk factors such as the female sex.

- HEAD TRAUMA

Some case-control studies report a correlation between head trauma and meningioma, both for males and females [29, 30], while other studies do not report it [31].

- MOBILE PHONE USE

Whether cell phone use can somehow be associated with the possible development of meningioma remains a matter of general interest.

To date, there is little evidence of association; moreover, the follow-up time is relatively short and, in some cases, the measurement of usage time is not very precise [32].

1.1.6 Imaging

The most widely used imaging method for the diagnosis of meningioma is magnetic resonance imaging (MRI).

On T1-weighted MRI, 60% of meningiomas are isointense and 30% are mildly hypointense compared with gray matter. On T2-weighted images, the tumors are isointense (50%) or mildly to moderately hyperintense (40%).

Hyperintensity on T2-weighted images suggests higher water content, denoting a meningothelial meningioma, a vascular meningioma, or an aggressive meningioma. However, it is suggestive of an easily aspirated tumor during surgery.

Low signal intensity within the tumor may often be due to calcification or to vascular flow voids, a distinction sometimes difficult to make

Meningiomas usually enhance intensely and uniformly after the injection of gadolinium, with typical dural tail enhancement.
These tumors also tend not to respect the dural boundary, which is a distinctive feature not typical of other neoplasms [34].

Calcifications are frequent findings and can be better seen at CT [8].
Perilesional edema is thought to be vasogenic, and probably related to meningioma secretion of vascular endothelial growth factor (VEGF), rather than a result of direct mass effect or venous invasion [35].
Peritumoral cerebral edema is occasionally prominent, particularly in certain histological variants or in high grade meningiomas [36].
No clear radiological criteria distinguish WHO grade I and grade II meningiomas, meanwhile anaplastic meningiomas are often more irregularly shaped and display a higher relative cerebral blood volume than lower grade ones.

Zhang et al analyzed the relationship between MRI features and pathological diagnosis d for each WHO grade I meningioma subtype. They found that angiomatous meningiomas were the most easily identified subtype, followed by meningothelial meningiomas. Difference in MRI features were seen between these two histotypes. No obvious difference was observed among the mixed, fibroblastic, and psammomatous meningiomas [37].

Diffusion weighted imaging (DWI)

Reduced water diffusivity has been correlated with more aggressive tumor behavior and is sometimes seen with higher grade meningiomas (atypical/anaplastic) and recurrence [38].

Perfusion MRI

This technique permits to differentiate between dural-meningeal supply and pial-cortical supply.

Benign meningiomas typically derive their blood supply from the external carotid via dural branches (dural-meningeal supply).

As the meningioma enlarges, it may parasitize pial branches from the brain parenchyma (pial-cortical supply).
A high volume of pial-cortical supply (as opposed to dural-meningeal supply) usually predicts an aggressive meningioma with a higher tendency of recurrence [34].

Angiography

Normally, conventional angiography is performed for pre-operative endovascular embolization with the goal to minimize the blood loss intraoperatively [34].

The classic angiographic appearance is characterized by a prolonged homogeneous vascular blush beginning in the late arterial phase and continuing into the late venous phase, with slow washout [39].
Exceptions are en plaque meningiomas, especially those associated with the planum sphenoidale, clinoid, and floor of the anterior cranial fossa, that are generally poorly vascularized [8].

Meningioma is supplied by the normal meningeal arterial supply to the meninges of the tumor site.

Diagnostic angiography can also identify important information for the surgeon such as potential occlusion of a dural sinus adjacent to the tumor and the pattern of collateral venous drainage around such an occlusion [39].

1.1.7 Treatment

Surgery is the main treatment, and, in case of complete resection, it could represent a definitive cure. The more complete the resection, the less chance there is of recurrence.

As a matter of fact, not all meningiomas grow exponentially, they may grow also linearly, or not grow at all [40]. Therefore, not for all meningiomas there are surgical indications.

Reganchary and Suskind wrote "Some die from meningiomas, others with them. A neurosurgeon's role is to recognize these two sets of populations and give the benefit of surgery to those who need it and spare those who do not" [41].

Many asymptomatic, incidentally discovered meningiomas can be managed by observation using annual clinical and MRI tests, after an initial observation interval of 6 months.

When a treatment is needed, surgery is the first choice.

In fact, the vast majority of patients can be cured by surgery alone, particularly patients with WHO grade I tumors in favorable locations (e.g., convexity meningiomas, and easily accessible skull-base meningiomas).

Extent of resection is classified by the Simpson grade (table 2), which relies on the surgeon's assessment during surgery, and is an important prognostic factor for risk of tumor recurrence.

Simpson Grade	Definition
0	For convexity meningiomas, Al-Mefty advocated that an additional 2-cm margin of dura be remover other than Simpson 1
1	GTR of tumor, dural attachment, and abnormal bone
2	GTR of tumor, coagulation of dural attachment
3	GTR of tumor without resection or coagulation of dural attachments, or extradural extensions (e.g., invaded or hyperostotic bone)
4	Partial Resection
5	Biopsy

Table 2- Simpson Grading

Radiotherapy is often useful to increase local control, especially if surgery alone seems insufficient.

With radiotherapy it is generally possible to obtain a tumor stabilization, i.e. an inhibition of neoplastic growth, but rarely meningiomas show a reduction in size [18].

Radiotherapy (radiosurgery or fractioned radiotherapy) could be employed in case of residual disease in grade I meningiomas, left voluntarily to lower the morbidity of surgery.

Radiotherapy is mandatory in all cases of subtotal resection of WHO grade II meningiomas and in all cases of WHO grade III meningiomas, irrespective to extent of resection [42].

Controversy exists about the utility and necessity to add adjuvant radiotherapy in case of complete removal [43].

Moreover, radiosurgery might be the first option in small WHO grade I meningiomas in specific location; patients with WHO grade I meningioma who cannot undergo surgery can be treated by fractionated radiotherapy or radiosurgery [42].

By contrast, systemic treatment has thus far only had a minor role in the management of meningiomas.

In fact, to date there are no approved drug treatments for meningiomas; the use of treatments such as hormone-therapy and chemotherapy have shown minimal benefit in some clinical trials [3, 44].

Thanks to different technologies, such as cytogenetic analysis, sequencing and immunohistochemistry, new potential molecular drug targets have been identified [45].

Several clinical trials are under investigation, evaluating the efficacy of chemotherapies, such as trabectedin, and novel molecular agents targeting Smoothened, AKT1, and focal adhesion kinase in patients with recurrent meningiomas [46].

1.2 PD-1 / PD-L1

PD-1

The programmed cell death protein 1, also known as PD-1 or CD279 (differentiation cluster 279), is a surface protein belonging to the superfamily of immunoglobulins that plays a role in regulating the immune system response by promoting self-tolerance and suppressing T-cell activity. This process prevents the onset of autoimmune diseases, but at the same time can also prevent our immune system from eliminating cancer cells [51].

PD-L1

The programmed death ligand 1 (PD-L1) also known as differentiation cluster 274 (CD274) or homologous (B7-H1) [38]. This ligand is a type 1 transmembrane protein that has been hypothesized to play an important role in suppressing the adaptive arm of the immune system during special events such as pregnancy, tissue allografts, autoimmune diseases and other pathological states such as hepatitis.

The binding between PD-L1 and PD-1 transmits an inhibitory signal that reduces the proliferation of antigen-specific T cells in the lymph nodes, while reducing the apoptosis of regulatory T cells.

PD-1/PD-L1 inhibitors are therefore drugs that, by preventing the binding between these two molecules, hinder the inhibition of immune system activity against cancer cells [51].

1.2.1 ROLE OF PD-L1 IN MENINGIOMAS

PD- L1 expression, together with regulatory T-reg cells, is supposed to contribute to the immunosuppressive tumor microenvironment. According to the study conducted by Du et al. [2], PD-L1 mRNA expression levels are related to the aggressive phenotype of the tumor and increase with the degree of meningioma.

Within the anaplastic meningioma, in fact, a decrease in CD4+, CD8+ and PD-1+ T cells is observed, with a concomitant increase in FoxP3+ (T-Reg) T-cells. This immune mechanism, also observed in other types of cancer, is associated with the evasion of the immune system mediated by the tumor.

To date only few cases have been reported in the literature demonstrating the effects of checkpoint inhibitors in patients with meningioma.

The first clinical report suggesting that antibodies targeting PD-1 are effective in treating meningioma described a patient treated with anti PD-1 antibodies for lung cancer and whose concomitant known intracranial meningioma has significantly decreased in size after such treatment [52].

Ian F et al. described a case of an important anticancer immune response to nivolumab in a patient with an MSH2-deficient meningioma, evidenced by dramatically increased infiltrating CD8+ T cells and a durable therapeutic response [53].

To date, 4 clinical trials of treatment of meningiomas with anti-PD-1 and anti-PD-L1 are ongoing [45].

Drug (Trade Name)	Target	Trail ID
Immunotherapies		
Avelumab (Bavencio)	PD-L1	NCT03267836
Nivolumab (Opdivo)	PD-1	NCT02648997
Nivolumab + ipilumumab (Yervoy)	PD-1	NCT03267836
Pembrolizumab (Ketruda)	PD-1	NCT03279692 NCT03016091
Targeted Small Molecules		
Evereolimus	mTOR	NCT01880749
Evereolimus + bevacizumab (Avastin)	mTOR/ VEGFR	NCT02648997
Evereolimus + octreotide (Sandostatin)	mTOR/ SST2	NCT02333565
AZD2014 (Vistusertib)	mTOR	NCT03071874 NCT02831257
Trametinib (Mekinist) + Alpelisib (Piqray)	MEK/ PI3K	NCT03631953
Tazemetostat	EZH2	None to date
Tumor Treating Field		
NovoTTF – 110A (Optune)	N/A	NCT01880749
Optune + bevacizumab (Avastin)	N/A	NCT01880749
PRRT		
Cu-64-SARTATE	SST2	NCT03936426
LU177-DOTATE (Lutathera)	SSR	NCT03971461

Figure 2. Experimental Approaches for Treatment of Meningioma

1.2.2. PDL1 PATHWAY

PD-1/PD-L1 pathway controls the induction and maintenance of immune tolerance within the tumor microenvironment. The activity of PD-1 and its ligands PD-L1 are responsible for T cell activation, proliferation, and cytotoxic secretion in cancer to degenerating anti-tumor immune responses.

PD-L1 is usually expressed by macrophages, some activated T cells and B cells, DCs and some epithelial cells, particularly under inflammatory conditions. In addition, PD-L1 is expressed by tumor cells as an "adaptive immune mechanism" to escape anti-tumor responses. It has been demonstrated that IFN-γ causes PD-L1 upregulation in ovarian cancer cells, which is responsible for disease progression, whereas IFN-γ receptor 1 inhibition can reduce PD-L1 expression in acute myeloid leukemia through the MEK/extracellular signal-regulated kinase (ERK) and MYD88/TRAF6 pathways Inhibition of PKD2 activity inhibits the expression of PD-L1 and promotes a strong antitumor immune response. NK cells secrete IFN-γ through the Janus kinase (JAK)1, JAK2 and signal transducer and activator of transcription (STAT)1 pathways, increasing the expression of PD-L1 on the surface of the tumor cells. Studies on melanoma cells have shown that IFN-γ secreted by T cells through the JAK1/JAK2-STAT1/STAT2/STAT3-IRF1 pathway may regulate the expression of PD-L1.

PD-L1 has been also shown to exert non-immune proliferative effects on a variety of tumor cell types. For example, PD-L1 induced epithelial-to-mesenchymal transition (EMT) and stem cell-like phenotypes in renal cancer cells, indicating that the presence of the intrinsic pathway of PD-L1 promotes kidney cancer progression.

1.2.3. miRNA ANALYSIS IN MENINGIOMAS

Noncoding RNAs refer to RNAs that have no protein coding ability, and the most studied types are microRNAs (miRNAs) with a length of about 22 nucleotides. miRNAs are a class of small single-stranded RNAs that post-transcriptionally modulate gene expression by binding to the mRNA 3-untranslated region (3′ UTR) of target genes, causing mRNA degradation or repression of translation.

Currently, it is well-known that miRNAs can be aberrantly expressed in various human cancers as a result of which the majority of studies on miRNAs have focused on their function as oncogenes or tumor suppressors. As it happens in various tumors, the expression of miRNAs was recently proposed as possible key regulators with a significant effect on meningioma biology, irrespective of their histopathological degree, the identification of which would allow a more accurate prediction of their behavior. Recent studies focused and identified sets of miRNAs deregulated in benign and high-grade meningiomas but a few miRNA signatures able to predict meningioma recurrences far to be recognized.

Furthermore, the role of miRNAs in the regulation of PD-1 and PD-L1 was investigated, and a small amount of miRNA have been hypothesized to play an role in tumor immune escape, leading to the development of microenvironments conducive to tumor growth and progression. For instance, several studies have currently focused on the relationship between miR-155-5p and PD-L1 in cancer. In human dermal lymphatic endothelial cells, miR-155-5p was able to affect the kinetics of PD-L1 and reduce its expression upon interferon (IFN)-c and TNF-a treatment via directly binding to the 3'-UTR of PD-L1. However, in lymphoma cells, miR-155- 5p could positively regulate the transcriptional activity of PD-L1 and inhibit CD8+ T cell function via the PD1/PD-L1 pathway to enhance the immune tolerance of tumor cells. Also in lung adenocarcinoma, miR-155-5p was hypothesized to be involved in the immune response. The cross-talk between miR-155 and PD-L1 was thought to provide a new mechanism for inflammation-associated tumorigenesis and suggested a potential use for miR-155-5p and PD-L1 in lung adenocarcinoma therapy.

2. AIM OF THE STUDY

The aim of our study better is to understand the role of PD-L1 signaling in different meningiomas, correlating PD-L1 expression to tumor grade, clinical behavior and recurrences.

3. MATERIALS AND METHODS

3.1. Patients' selection

We retrospectively evaluated 104 samples from 77 patients with a diagnosis of meningioma, underwent surgery from 1998 to 2020 in the Neurosurgery Department of San Martino Policlinico Hospital - IRCCS, Genoa.

This is a selected case study, as more recurrent and/or high-grade meningiomas were chosen for the purposes of the study: for this reason, the ratio of grade 1, grade 2 and grade 3 meningiomas to non-recurrent meningiomas does not reflect that reported in Literature.

The demographic, clinical and health care data of our patients' cohort were collected from the electronic patient records.

We defined tumor recurrence based on growth of residual tumor or new appearance of disease according to postoperative neuroimaging and clinical assessment.

All cases have been classified or re-classified according to histological type, morphological characteristics and WHO 2016 grade.

The case history was reviewed by examining the slides obtained from material fixed in 10% neutral buffered formalin, included in kerosene, sectioned at 3 µm and stained with hematoxylin-eosin, from the archives of the O.U. of Pathological Anatomy of the IRCCS Policlinico San Martino of Genoa. The cases have been reassessed blind (with two other experienced pathologists) and reclassified according to WHO 2016 criteria. For each case, and their eventual recurrences, the most suitable inclusion block for the execution of immunohistochemistry was chosen. From these blocks uncolored sections at 3 µm were obtained, then deparaffined and then rehydrated.

3.2. PD-L1 analysis

Immunohistochemistry was performed with monoclonal anti PD-L1 antibodies (Monoclonal Mouse Anti-Human PD-L1 CLONE 22C3) at a 1:50 dilution in 0.05 mol/L tris-HCl, 0.015 mol/L sodium azide, 1% bovine albumin serum, pH 7.2, using the

BenchMark Ultra (VentanaMedical System) automatic color-immunochlorine. The antibody provides a 32-minute incubation at 37°.

The recovery of the antigenicity was performed by heating the PAD using an EDTA - Borated buffer at pH 8 for 64 minutes at 100°C.

The antibody binding was highlighted with the OptiView DAB kit (Roche-Ventana) using a polymer and the avidin-biotin-peroxidase complex.

After immunostaining, the slides were contrasted with hematoxylin and finally mounted with an automatic mount.

The preparations obtained were evaluated on the basis of the percentage of stained cells and the pattern of immunostaining.

To evaluate PD-L1 expression the cut-off value of 1% has been adopted, a threshold already identified as discriminating in lung cancer [56]:

- positive ≥1% (dot, granular, membrane)
- negative <1%

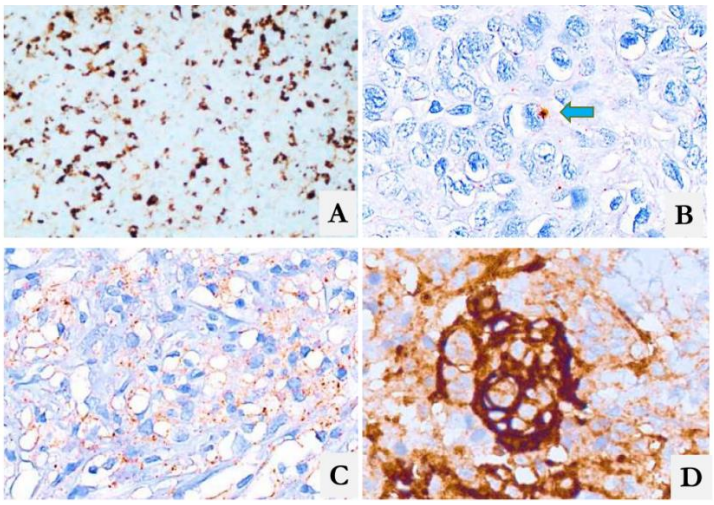


Figure 3. **Immunohistochemical expression of PD-L1. A) positive control on lymph node; B) positivity in dot; C) granular positivity; D) membrane positivity.**

Two tests were used for the statistical analysis of the data obtained:

- Fisher Exact Probability Test: non-parametric statistical analysis test with two nominal dichotomous variables for small samples;

- Wilcoxon Signed - Rank test: non-parametric hypothesis test used to compare two related samples, paired samples or repeated measurements of a single when the distribution of the population difference cannot be assumed to be normal.

3.3. miRNAs expression analysis

MicroRNAs (miRNAs) are non-protein-encoding small RNAs of approximately 22 nucleotides in length that regulate target gene expression at the post-transcriptional level. Collectively, miRNA genes are one of the most abundant classes of regulatory genes in mammals, and deregulated miRNAs play an important role in human diseases such as cancer.

A cohort of 22 meningioma patients divided between low (grade I) and high grade (grade II and III) was retrospectively selected. The total RNA including miRNAs was extracted from paraffinated sections, using FFPE miRNeasy kit (Qiagen), quantified with Nanodrop and evaluated for fragmentation on Tape Station 2200 (RNA ScreenTape). RNA was processed using Nanostring digital molecular barcoding technology and Human v3 miRNA Panel (798 miRNA). The expression profile of miRNAs was carried out using nSolver and Rosalind softwares (Nanostring).

The analysis of significant miRNAs target mRNAs was carried out in advance through various bioinformatics web-tools (Rosalind- Nanostring and DIANA Tools).

4. RESULTS

4.1. Meningioma Patients Cohort

We retrospectively collected and analyzed 104 samples from 76 patients who underwent surgery for meningioma, 51 females (67 %) and 25 males (33%).

Age at diagnosis ranged from 18 to 78 years old (median age 59,5 years old).

This was a selected case study, the cohort was enriched for recurrent and/or high-grade meningiomas for the purposes of the study.

Twenty out of 76 patients (26, 3%) had a recurrent disease (1 or more recurrences).

4.2 Meningioma samples reclassification according to WHO 2016 Classification

In order to make the cohort of patients homogeneous, all 104 samples were reclassified according to WHO 2016 criteria.

This reclassification produced grade changes in 19 cases (18.3%) and in particular up-grade in 10 cases (52.6%) and down-grade in 8 cases (42.1%).

After re-classification, 55 samples were WHO grade 1 (G1), 42 samples were WHO grade 2 (G2) and 7 samples were WHO grade 3 (G3).

Original Diagnosis	**WHO 2016**		
	G1	G2	G3
G1 N. 54	N. 47	N. 7	N. 0
G2 N. 45	N. 8	N. 34	N. 3
G3 N. 5	N. 0	N. 1	N. 4
Total	N. 55	N. 42	N. 7

Table 4. **Grade variation between original diagnosis and reclassification according to WHO 2016**

Several criteria define the WHO 2016 meningioma grading system. In Table 5 and Table 6 it has been evaluated the contribution that each feature provided to define grade 2 and grade 3 respectively.

The microscopic characteristics that most frequently contributed to the definition of Grade 2 meningiomas were:

- Mitotic count ≥4 /10 HPF (62%), among the independent factors;

- Hypercellularity (88%), solid sheets (67%) and macronucleoli (64%) among the lesions and elementary factors.

The independent factors that most frequently contributed to the definition of grade 3 were:

- Mitotic count ≥ 20 / 10HPF (57%);

- The frank anaplasia / sarcomatoid histology (43%).

	G2 microscopic features	Present N.	(%)	Absent N.	(%)	Total
Independent factors	≥ 4 mitotic figures/10 HPF	26	62	16	38	42
	Brain invasion	15	36	27	64	
	Clear cell histology	5	12	37	88	
	Chordoid histology	0	0	42	100	
	hypercellularity	37	88	5	12	
	Necrosis	17	40	25	60	

Elementary lesions (at least 3/5)	Solid sheets	28	67	14	33	
	Small cells	3	1	39	99	
	Macronucleoli	27	64	15	36	

Table 5. **Contribution of elementary lesions to grade (G2 cases only)**

Indipendent Factors	Present N.	(%)	Absent N.	(%)	Total
≥ 20 mitotic figures /10 HPF	4	57	3	43	7
anaplasia/sarcomatoid histology	3	43	4	57	
Papillary	0	0	7	100	
Rhabdoid	1	14	6	86	

Table 6. **Contribution of elementary lesions to grade (G3 cases only)**

4.3 PD-L1 expression in meningiomas classified by grade (WHO 2016)

Of the 104 samples, 103 were available for PD-L1 expression analysis.

Fifty of the 103 samples examined were PD-L1 positive (48,5%) of which 19 grade 1 (38%), 26 grade 2 (52%) and 5 grade 3 (10%). (Table 7)

The analysis of PD-L1 expression among meningioma classified by grade showed that 35,2 % of G1 samples were PD-L1 positive versus the 61,9 % of G2 samples versus 71,4 % of G3. (Table 7)

Therefore, the prevalence of PD-L1 positivity in grade 1,2 and 3 meningiomas showed a statistically significant direct correlation between these two features (p=0,0176).

	G1 (54 samples)	**G2 (42 samples)**	**G3 (7 samples)**
PD-L1 ≥1 N. (%)	**19**(35,2)	**26** (61,9)	**5** (71,4)
PD-L1 <1 N. (%)	**35** (64,8)	**16** (38,1)	**2** (28,6)

Table 7. PD-L1 expression in meningioma samples classified by grade

Fisher Exact Probability Test: p= 0.0176

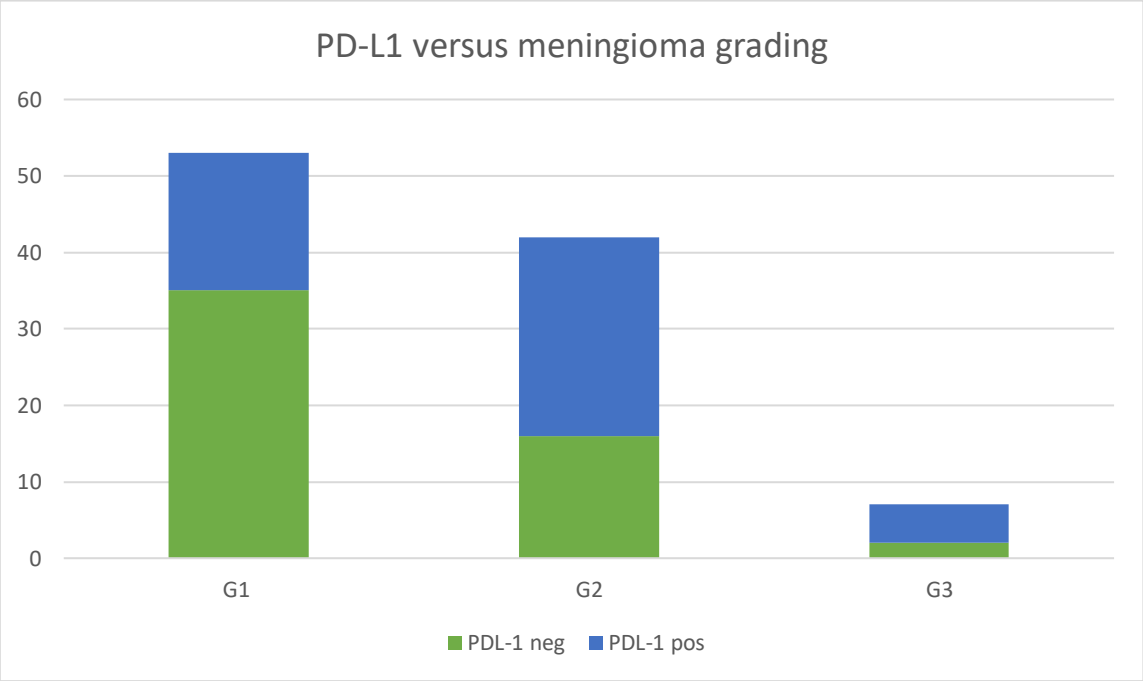


Figure 4. ***PD-L1 expression according to WHO 2016 meningioma grading***

4.4 PD-L1 *expression in recurrent meningiomas*

Of the 76 patients, 20 had one or more recurrence. Among this group, 11 were low grade (G1) and 9 were high grade meningiomas (8 G2 and 1 G3) (Table 8).

Two of 20 patients were PD-L1 positive at primary presentation (2 and 3% of expression respectively), 11 were found positive at first recurrence (range of PD-L1 expression 1-70%).

The prevalence of PD-L1 positive patients were significantly higher at recurrence (11/20) than at primary presentation (2/20), regardless of the grade (p= 0,003).

The average expression of PD-L1 at first diagnosis was 1.047 cells +/case, while in recurrences it was 8.238; therefore the expression of PD-L1 increased significantly in recurrences, regardless of the grade (p=0.00298).

Analyzing patients with meningiomas grade 1 at primary presentation, we found that 6 of 11 patients were PD-L1 positive at first recurrency (range of PD-L1 expression 1-10%), 7/11 were positive considering also the second recurrency.
Five of 11 patients G1 at primary presentation maintained the same grade at recurrency (not progressed-NP group), while 6 of 11 have progressed (P group) to G2 in 5 cases (5/6) and to G1 in 1 case (1/6).
With the limitation of small cohort of patients, we did not find any difference in the change of PD-L1 expression (negative versus positive expression) among the NP and the P groups (3/5 of NP patients and 3/6 of P patients became PDL-1 positive at recurrency).

Among the high grade recurrent meningiomas (8G2 e 1 G3 at primary presentation), any case had PD-L1 positive expression at diagnosis. Six of 9 cases were PD-L1 positive at recurrency (range of PD-L1 expression 2-70%). Two of 9 cases have progressed to relapse and both were PD-L1 positive at recurrency.

	Primary presentation	Recurrence 1	Recurrence 2	Recurrence 3	Primary presentation	Recurrence 1	Recurrence 2	Recurrence 3
Case	Grade				PD-L1 (%)			
1	1	1			0	0		
2	1	1			0	1		
3	1	2			0	0		
4	1	2	2	2	0	0	1	1
5	1	1			0	0		
6	1	1			0	3		
7	1	2			0	1		
8	1	2			0	1		
9	1	1			0	0		
10	1	3			2	0		
11	1	2	2	2	3	10	10	10
12	2	2	1		0	0	0	
13	2	2	3		0	2	2	
14	2	2			0	0		
15	2	2			0	40		
16	2	2			0	10		
17	2	2			0	15		
18	2	3			0	10		
19	2	3			0	70		
20	3	2			0	0		

Table 8. Grade distribution and PD-L1 *expression (%) in primary and recurrent meningiomas*

Wilcoxon Signed – Rank Test: p=0,003

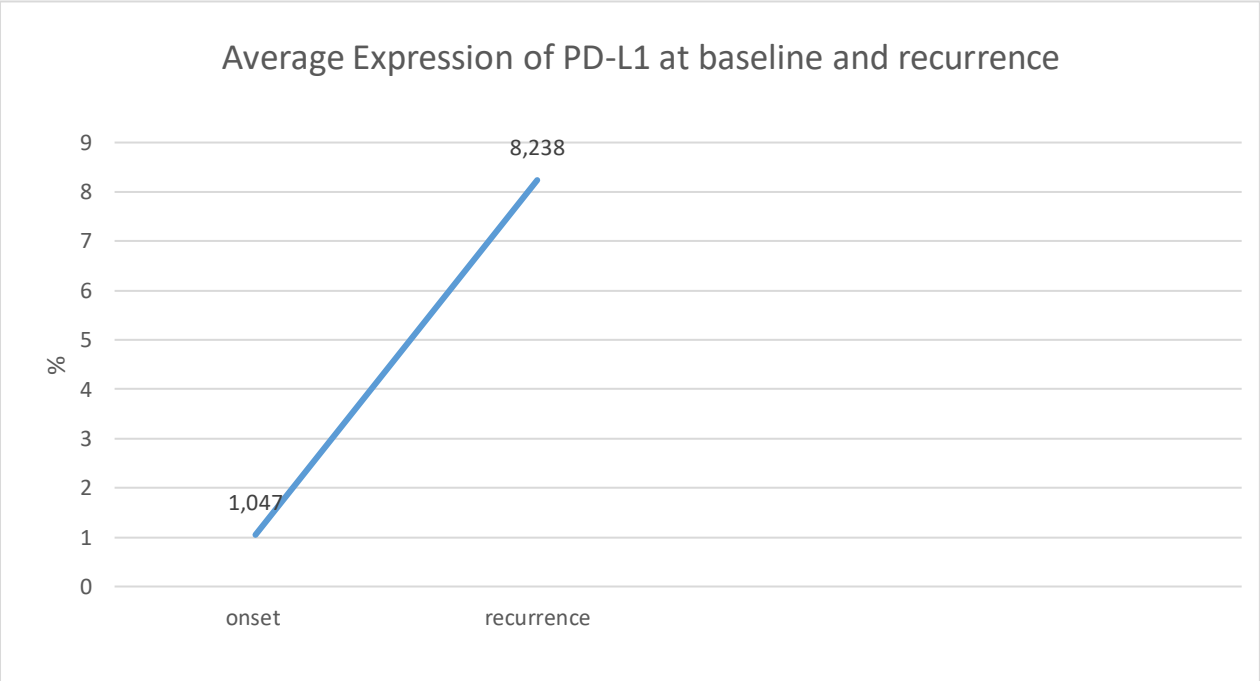


Figure 5. PD-L1 expression trend in primitive cases and relapses

4.5 Clinical and anatomopathological features at primary presentation in PD-L1 positive and PD-L1 negative recurrent patients.

We evaluated whether PD-L1 positive recurrences had different clinical or anatomopathological characteristics at primary presentation compared to PD-L1 negative recurrences.

At recurrence 77 % of female patients had PD-L1 expression versus 16.7 % of male patients (p= 0,04).

We did not find any statistically significant correlation between age, time to recurrence or tumor site and PD-L1 expression in recurrent patients. However, all parafalcine meningiomas were PD-L1 positive at recurrence (5/5 cases).

We did not find any correlation between all the analyzed anatomopathological features (number of mitosis, hypercellularity, solid sheets and macronucleoli) and PD-L1 expression at recurrence.

The expression of PD-L1 was evaluated in a subpopulation of 34 grade 1 subjects with at least 5 years of follow up. Comparing the non-recurrent cases (10/34) with recurrent cases (24/34), it was found that the expression of PD-L1 did not predict the probability of recurrence in cases (Table 11).

PDL1 expression %	**No recurrence N. (%)**	**Recurrence N. (%)**
<1	20 (59)	9 (26)
≥1	4 (11,8)	1 (3)
Total	**34**	

Table 9. Evaluation of the correlation between recurrence of G1 cases and PD-L1 expression

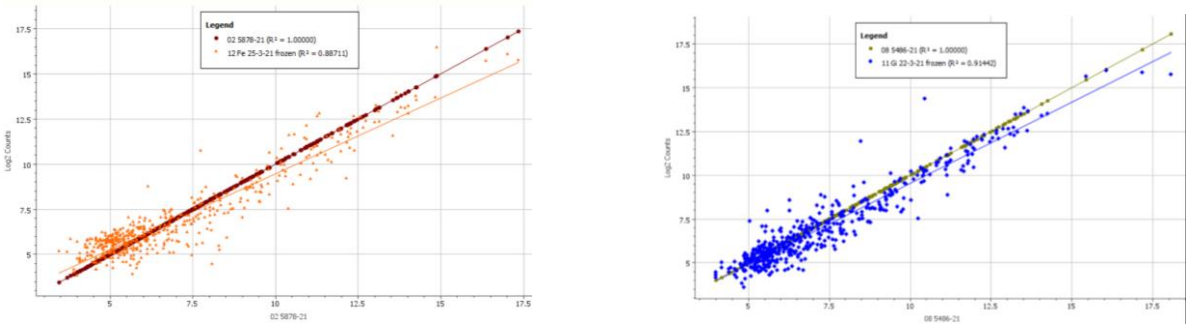
4.6 miRNA analysis

The MiRNome was analyzed in 24 RNA samples from 22 meningiomas (11 grade I and 11 grade II). In parallel, 2 tissue samples were analyzed in duplicate, using both FFPE tissue and cryopreserved tissue. RNA samples were isolated from 2 tissue sections (10 µm) and subjected to subsequent quantification and quality control / fragmentation. The range of RNA obtained was 100.8-827 ng / µl (median 408.45 ng / µl) while the RNA Integrity Number (RIN) range was 1.7-6.6 (median 2.5) .

One sample was excluded as it resulted an outlier due to the unsuccessful ligation process.

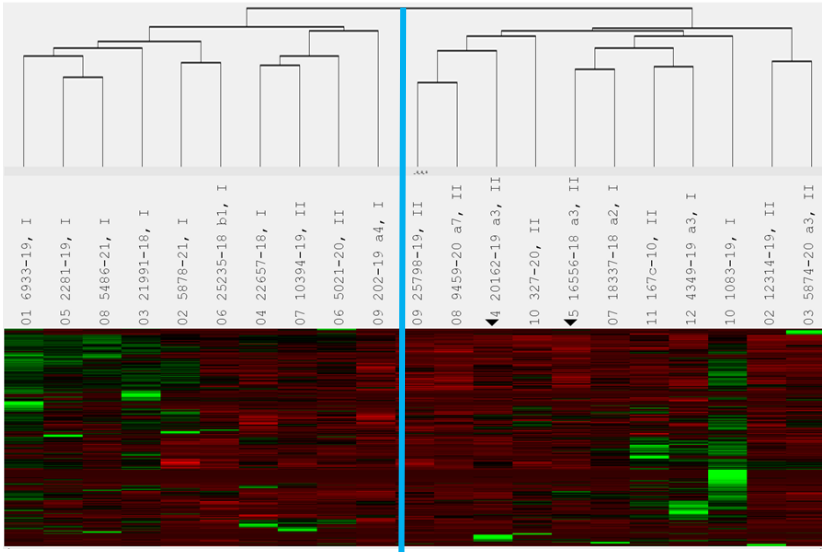
The analysis of the miR expression was carried out using the nSolver 4.0 software (Nanostring) coupled with R statistical analysis and data normalization (relative to the counts obtained for each sample). The two samples analysed in duplicate (FFPE and cryopreserved) provided concordant data (r2 = 0.88 and r2 = 0.91) (Figure 6). Then, the subsequent analysis was performed only using data from FFPE tissue.

Figure 6



MiRNA expression was detected on 21 normalized cases, with a range of 259-421 miRNA expressed (median 300, cut-off> 50 count). The "supervised" heatmap of the 21 samples clustering according to the algorithm of the nSolver software is shown in Figure 7, with annotation of meningiomas in grade I and II. The distribution clearly shows a "cluster A" of 10 patients, 8 of them with a diagnosis of grade I meningioma. In that cluster, an increased expression of miRNA is more evident than in grade II meningiomas mostly grouped in "Cluster B" (8 cases of grade II, 3 cases of grade I). Regarding grade II meningiomas, there seems to be a trend of low diffuse expression of studied miRNAs.

Figure 7



cluster A **cluster B**

The two cluster of patients with grade I and II meningiomas were analysed in terms of fold change of expressed miRNAs. More than 100 expressed miRNAs were statistically different (p <0.05, t-test). These data were further "adjusted" using the FDR (False Discovery Rate) parameter, which us to be more stringent and avoid false positive results as much as possible. After correction with FDR, 24 miRNAs were statistically significant: hsa-miR-100-5p, hsa-miR-24-3p, hsa-miR-214-3p, hsa-miR-423-5p, hsa-miR-199a-5p, hsa-miR-574-3p, hsa-let-7e-5p, hsa-let-7b-5p, hsa-miR-99a-5p, hsa-let-7a-5p, hsa- miR-199a-3p + hsa-miR-199b-3p, hsa-miR-1271-5p, hsa-miR-98-5p, hsa-miR-193a-3p, hsa-miR-656-3p, hsa-miR- 23c, hsa-let-7c-5p, hsa-miR-125b-5p, hsa-miR-186-5p, hsa-miR-23b-3p, hsa-miR-155-5p, hsa-miR-216b-5p, hsa-miR-10a-5p and hsa-miR-140-5p. From the fold change analysis, it emerged that all of the 24 miRNAs were down-regulated in grade II meningiomas (range -1.55 -6.25).

4.7 PD-L1-related miRNA analysis

Some miRNAs found to be down-modulated in Grade II have already been correlated with the PD-1 / PD-L1 pathway in literature. In particular, miR-155 would tag the PD-L1 gene and its inhibition would result in an increased expression of PD-L1. Similarly, down-regulation of miR-140-5p and miR-574-3p have also been associated with over-expression of PD-L1 in various tumors.

5. DISCUSSION

Our results confirmed that PD-L1 is expressed by a significant proportion of meningiomas and that there is a significant correlation between PD-L1 expression and meningioma grade. These results are in line with other studies in literature [2,47,48,49].
The role of immune checkpoints expression in the solid tumors' microenvironment is currently recognized as a major factor in tumor-induced immunosuppression and escape of the cancer- innate immune response.
In particular, we know that also meningiomas, among solid tumors, can express and upregulate PD-L1 expression.
PD-L1 expression, together with regulatory T-reg cells, is supposed to contribute to the immunosuppressive tumor microenvironment [2].

To our knowledge, this is the first report of increased PD-L1 expression in meningioma recurrences compared to their primary presentation, regardless of their grade.

PD-L1 expression in recurrences may represent the immunosuppressive shift in the tumour microenvironment. It is known that PD-L1/PD-1 signaling pathway can inhibit the activation of T lymphocytes and enhance the immune tolerance of tumor cells and is therefore crucial for tumor's immune escape mechanism [54].
This mechanism may play a major role of in the formation and growth of meningioma recurrence.

This finding needs to be validated in larger prospective studies but may suggest that recurrences are characterized by a different genomic and mutational profile. Moreover,

the expression of PD-L1 may be a biomarker of immunotherapy activity. This can be an innovative treatment option in this patient population when standard local treatments are not feasible anymore.

In our study, PD-L1 expression did not predict recurrence in WHO grade I cases nor the progression in the whole cohort.

Nevertheless, in literature the prognostic role of PD-L1 expression in meningiomas is controversial.

In fact, Du et al did not report an association between PD-L1 expression and outcome in their cohort of patients whereas Han et al correlate PD-L1 expression with poor prognosis in grade II and III meningiomas [2, 47].

Another study by Karimi et al reported that PD-L1 positivity was predictive for tumor progression in WHO grade I meningiomas [49].

Despite our patient cohort was more similar to that of Han et al, our results were closer to those by Du et al.

We investigated the link between PDL-1 status at recurrence and clinical or pathological features at primary presentation. We only found that PDl-1 was more expressed in female patients than in male patients.

Other clinical and anatomopathological features did not correlate with PDL-1 status at recurrence. However, we noticed that all parafalcine meningiomas were PDL-1 positive at recurrence (5/5 cases).

Obviously, due to the small number of patients, it is not possible to evaluate the real meaning of this finding. However, in literature is reported that radiotherapy could be associated to an increased PDL-1 expression in meningioma patients [47]. Therefore, our finding could be explained with the higher tendency to do an adjuvant radiotherapy after surgical removal of parafalcine meningiomas. In fact, due to potential sagittal sinus invasion, surgical exeresis is almost ever subtotal [55,56].

A distinct immune composition between convexity and skull base meningiomas has been described, with a prevalent myeloid cell (mast cells and neutrophils) population in convexity meningioma and a prevalent gamma-delta T cells population in skull base meningiomas [57].

The difference in infiltrating immune cell population may explain the different expression of PD-L1 in parafalcine location.

Current literature suggests that there might be key regulators with a significant effect on meningioma biology irrespective of their histopathological degree, the identification of which would allow a more accurate prediction of their behavior. The expression of miRNAs was recently proposed as just such a predictor. These noncoding small RNAs can act as oncogenes or tumor suppressors in various tumors. Our MiRNome analysis permitted to find a small cluster of miRNA signatures that differentiated grade I meningiomas from grade II. This is the first time that such difference was individuated. Additionally, some miRNAs found to be down-modulated in Grade II have already been correlated with the PD-1 / PD-L1 pathway in literature. In particular, miR-155 would tag the PD-L1 gene and its inhibition would result in an increased expression of PD-L1. Similarly, down-regulation of miR-140-5p and miR-574-3p have also been associated with over-expression of PD-L1 in various tumours[58,59,60]. It should be hypothesized that the relationship between specific miRNA downregulation and PDL1 overexpression in malignant and recurrent meningiomas may play a crucial role in tumor behavior and may have an impact on tumor-microenvironment interaction.

6.CONCLUSIONS

To our knowledge, this is the first report of increased PD-L1 expression in meningioma recurrences compared to their primary presentation, regardless of their grade.

This mechanism may play a major role in the formation and growth of meningioma recurrence.

The high prevalence of PD-L1 expression in the meningioma population and its correlation with grade and clinical behavior may be an important tool to tailor therapeutical strategies and follow up, in particular in high grade and recurrent cases.

Moreover, a miRNA-based model could potentially serve as a novel predictor of meningioma recurrence, thus help in determining an optimal postoperative surveillance regime to identify patients who may benefit from early retreatment. Combining the model with molecular mechanisms governing meningioma-microenvironment interaction, such as PDL1 expression and associated signaling pathways, might help to identify clinically distinct meningiomas and better target their treatment.

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