Players in glioma progression

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2017

Citation for published version (APA):

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CHAPTER 8

Summary and General Discussion
This doctoral thesis presents the analysis and tools for genomic exploration in glioma cell compartments, particularly tumor and immune cells. This section summarizes the findings in each chapter and further discuss them, focusing on future perspectives.

**Summary**

Malignant brain tumors are highly aggressive cancers. Their diffuse forms are infiltrative neoplasms, invading the surrounding normal tissue and hampering surgical resection. Among gliomas, glioblastomas (GBMs) are the most frequent and aggressive subtype (Ostrom et al., 2015). Studies on the heterogeneity—both intertumoral and intratumoral—of GBMs showed that these brain tumors, although histologically similar, GBMs are a heterogeneous diseases regarding both its cells and its genetic alterations (Dunn et al., 2012; Filbin and Suvà, 2016). This conclusion was only possible due to advances in large-scale molecular analysis through next generation sequencing (NGS) over the last decade. Molecular alterations predicting patients’ response to treatment, overall survival and clinical outcome have been proposed and new GBM subtypes: proneural, classical and mesenchymal were identified (Brennan et al., 2013; Cancer Genome Atlas Research Network et al., 2015; Ceccarelli et al., 2016; Parsons et al., 2008; Phillips et al., 2006; Stieber et al., 2014; Verhaak et al., 2010). In Chapter 2, we explored the differential expression of genes associated with glioma stem cells (ID4, SOX4 and OCT-4) and showed their association with a worsening on the overall survival of GBM patients in the advent of conjoint hyper expression of these markers. In Chapter 3, we applied NGS technology to classify a Brazilian cohort of GBM samples. We also aimed to assess the correlation of our molecular findings using a more feasible proteomic immunohistochemistry-based approach. Our results, however, indicate the need for a genetic approach to further classify GBMs, particularly the mesenchymal subtype, as the IHC approach failed to do so. In Chapter 4, we explored the role of a family of transcription factors, inhibitors of differentiation (IDs) in gliomas from different origins (astrocytic and oligodendrocytic) and grades (I-IV), as well as the different GBM subtypes classified in Chapter 3. We showed an association between IDs and the proneural subtype of GBM, as well as their usefulness in differentiating between astrocytomas and oligodendrogliomas. The response to treatment between astro- and oligodendrogliomas varies, with the latter proving to be more sensitive to the standard
of care and to present a better prognosis (recently reviewed by Otani et al., 2016), highlighting the importance to differentiate both tumors. With the aim of analyzing the different cell compartments of glioma, we investigated the immune myeloid cell compartment, which comprises up to 30% of brain tumors (Roggendorf et al., 1996). In Chapter 5, we describe a protocol for ex vivo isolation of pure populations of microglia and myeloid infiltrates from the CNS, based on mechanical dissociation followed by FACS-sorting. In Chapter 6, we describe the use of this methodology to isolate human microglia from cortical post-mortem tissue. We then identified the human microglia transcriptome and assessed how these cells are affected by aging. Aside from stipulating a core of genes associated with human microglia identity, we demonstrated that genes related to actin modulation are affected during aging, possibly hampering cell motility. In Chapter 7, we reported the differences found in the transcriptome of glioma and normal human microglia, as well as the differences between microglia derived from lower grade gliomas and glioblastomas. We identified a transcriptional network of regulators related to cell proliferation and motility processes, as well as related to extracellular matrix, which genes presented overexpressed among the most malignant subtype of GBM (mesenchymal subtype). Our findings propose a role for microglia in GBM invasiveness and highlight their potential to be an optional complementary treatment target as a step forward to a precision medicine.

General discussion and future perspectives

GBM sub classification and its applicability

Diffuse gliomas, which include astrocytomas, oligodendrogliomas and glioblastomas, (Louis et al., 2016) are invasive CNS tumors. Complete surgical resection of these tumors is hence very difficult to achieve. The presence of residual tumor cells results in recurrence and malignant progression, albeit at different intervals. Some of lower grade tumors will either recur or progress to a GBM within months, while others will remain stable for years; the same is true for GBMs, with recurrence occurring at different rates (Cancer Genome Atlas Research Network et al., 2015; Ceccarelli et al., 2016; Foote et al., 2015; Kamoun et al., 2016). The determinant factors for one behavior or another are not yet completely understood. For GBMs, despite surgery, radiotherapy
and temozolomide chemotherapy, patient median survival is of 14.6 months, where only 10.7% of the patients is disease-free after two years (Stupp et al., 2005).

The advent of next generation sequencing and large-scale molecular analysis in the last decade has revealed that molecular alterations predict patients’ response to treatment, overall survival and clinical outcome. A new light was shed on the high level of GBM heterogeneity and new subclassifications have emerged. For GBM, several studies have singled out specific determinant mutations of the main, newly identified subtypes: proneural, neural, classical and mesenchymal (Brennan et al., 2013; Cloughesy et al., 2014; Parsons et al., 2008; Phillips et al., 2006; Stieber et al., 2014; Verhaak et al., 2010).

We have performed a somatic mutation analysis in a GBM cohort utilizing a customized gene panel for next-generation sequencing (Chapter 3). This gene panel included all coding regions of the target genes, as well as their splicing regions. The analysis of our results made clear that the identification of GBM subtypes is not possible by targeting point mutations alone, particularly in the case of the mesenchymal subtype. The identification of mesenchymal subtype of GBM is important because it demands an aggressive treatment to improve the poor prognosis (Phillips et al., 2006; Verhaak et al., 2010). Although patients with classical and mesenchymal GBM subtype display similar overall survival, the latter also present resistance to treatment. By implementing the correct approaches to identify GBM subtypes, new discoveries leading to better clinical approaches and precision medicine might be developed.

Studies on glioma progression could also benefit from GBM subclassification. In Chapter 4, we analyzed a family of genes, inhibitors of differentiation (IDs), associated to specific low-grade gliomas and to the proneural GBM subtype. As prognostic markers, this family of genes could help guide treatment course in the long run, as proneural GBMs were also shown to progress to a mesenchymal GBM profile (Segerman et al., 2016). More recent studies have focused on the identification of genetic profiles for low-grade gliomas (Cancer Genome Atlas Research Network et al., 2015; Ceccarelli et al., 2016), also concluding that genetic status was more reflective of the disease subtypes than was histologic class. The genetic pathways leading to glioma progression are progressively being unraveled, providing possible intervention targets. Although still costly and sometimes laborious, genetic profiling has proven its value as an overall survival predictor and to the understanding of glioma biology.
Immune microenvironment in the treatment of gliomas

Innate immune cells, such as microglia are major components of the glioma microenvironment. There are conflicting studies regarding the role of such cells in tumor progression. While some claim better outcomes for patients with high levels of immune cells, either by peripheral infiltration or from resident cells in the tissue, many others have assessed the same phenomena to be related to a poorer prognosis (reviewed by Fridman et al., 2012). Such divergence in results seems to arise from the different functional and activation states that innate immune cells can adopt within the same tumor at different time points. Considering their ability to respond readily to stimuli, changes in microenvironment can lead to both anti- or pro-tumoral responses. Immune evasion, one of the hallmarks of cancer (Hanahan, 2014), is characterized by the ability tumor cells have to manipulate the immune system via secretion of cytokines and growth factors. This process may promote tumor progression and escape from destruction, and it is crucially dependent on the crosstalk between these two types of cells. In the scope of glioma, in addition to target highly heterogeneous tumor cells, also targeting non-neoplastic cells, particularly microglia and iTAMs, seems an attractive gambit to overcome the mechanisms associated with recurrence and therapeutic resistance. New insights on how the immune cells respond to tumor signals and which molecules they release to support tumor growth and progression will prove valuable.

New perspectives for cancer treatment have risen with the onset of immunotherapy-based treatments. For gliomas, however, there are several issues to be resolved, the main of which is the blood-brain barrier (BBB) which prevents CNS entry of certain macromolecules and hampers drug delivery (Preusser et al., 2015). Nonetheless, there are immune checkpoints in the crosstalk between glioma cells and leucocytes that can be approached. The first of which is the uptake of antigens released by tumor cells by APCs (microglia and iTAMs), as well as the initial changes in phenotype these cells undergo. The early interaction between APCs and tumor cells has been particularly difficult to track in human gliomas so far. The process is followed by migration of APCs to lymph nodes and presentation of antigens to T cells. When T cells infiltrate the brain, their interaction with tumor cells and tumor supportive microglia/iTAMs results in the release of immunosuppressive factors, leading to tumor cell immune evasion instead of immune destruction.

The most successful immunotherapy drugs have targeted the mid to late phases of immune checkpoints, namely the presentation of antigens to T cells and the
interactions between activated T cells and tumor/myeloid cells. By further and thoroughly exploring early immune checkpoints, such as the early interaction between tumor cells and APCs, new potential complimentary targets may be revealed.

**Paving the way for glioma-microenvironment interaction studies: human microglia profile in homeostasis and glioma**

The human CNS immune cells transcriptome is necessary to address their functional changes upon stimuli from glioma cells. While there are several reports on mouse microglia gene expression profiles, both under physiological and disease conditions (recently reviewed by (Hambardzumyan et al., 2016; Prinz and Priller, 2017), reports on human microglia transcriptome data were still scarce due to several reasons.

The first challenge was to achieve an isolation protocol preserving homeostatic features of microglia and CNS infiltrative cells. In Chapter 5, we describe such protocol with adapted improvements from previous publications (Becher and Antel, 1996; Melief et al., 2012; Olah et al., 2012). Subsequent flow cytometry and gene expression analysis showed that isolated cells through our developed protocol retained their steady state features.

The next challenge was related to what kind of brain sample to use as starting point to profile microglia. Recent studies on human microglia have reported the transcriptome findings in cells derived from either epilepsy or tumor surgery, and in a restricted number of samples (Bennett et al., 2016; Spaethling et al., 2017; Zhang et al., 2016b). In Chapter 6, we present the characterization of an extensive cohort of human microglia derived from postmortem cortical brain tissue. We compared the human microglia to a mouse microglia transcriptome profile generated in our groups and to the previously reported human and mouse microglia datasets. The analysis of these datasets using the same bioinformatics pipeline allowed to elucidate the (dis)similarities between microglia profile between these species. Recent studies have reexamined the validity of mouse models to study microglia in aging and neurodegenerative conditions (Smith and Dragunow, 2014). As our cohort comprised individuals with ages ranging from late thirties to over 100 years old, our dataset also allowed to address what age-related changes occur in human microglia during aging and to what degree they overlap with mouse microglia aging and priming signatures.
We established a core human microglia gene signature and the functional properties associated with these genes by gene ontology analysis. As expected, many significantly enriched terms associated with the innate immune activity of microglia, like ‘immune response’, ‘defense response’, cytokine production’ were present. The profile was also enriched for ‘phagocytosis’, ‘cell migration’, ‘cell motility’, confirming that human microglia are the immunocompetent and phagocytic cells of the CNS that express a wide range of immune receptors and ligands, equipped to respond to a wide variety of pathogen- and damage-associated molecules. Interestingly, although human microglia seem to possess a highly activated pathway for proliferation with genes involved in cell cycle highly expressed, this pathway seems to be dependent on extracellular signals. This data corroborates the recently reported findings regarding the turnover rate of microglia in both mouse and humans (Askew et al., 2017), in which the authors concluded that microglia cell levels are maintained throughout adult life in a tightly regulated mechanism alternating between proliferation and apoptosis.

Microglia-specific transcriptional regulators were also identified in the human microglia core genes. These include transcription factors required for microglia ontogeny SPI-1 (or PU.1) and IRF8 (Kierdorf et al., 2013), along with CIITA, a positive regulator of MHC-II gene transcription; TRIM22, a transcription activator induced by interferon; MNDA, an interferon target gene; IRF5, a factor modulating inflammatory responses; TAL1, a transcription factor associated with microglia aging (Wehrspaun et al., 2015); and IFI16, an interferon gamma inducible gene. A large number of members of the core microglia signature were regulated by these transcription factors, suggesting their critical role in human microglia identity.

An extensive overlap between the transcriptome of human and mouse microglia was found, however, few genes were exclusively represented in human microglia, without any mouse orthologues. Despite the absence of any specific associated biological pathways, significant roles as host defense and modulation of immune responses can be attributed to these human-exclusive genes. This might indicate a possible evolutionary role for these genes in the immune development of the brain.

Microglia are highly ramified and with motile processes that constantly survey their immediate surroundings (Davalos et al., 2005; Nimmerjahn et al., 2005). Gene expression changes in these processes, like integrins and actin (de)polymerization and remodeling were observed in the microglia profile when age was considered as a variable. Purinergic receptors and their downstream signaling are implicated in
chemotaxis and part of the microglia sensome (Ferrari et al., 2016; Hickman et al., 2013). Movement of the fine microglial processes to sense the environment and initiate chemotaxis is primarily governed through the activation of P2Y12 receptors. P2RY12, is an established microglia marker (Butovsky et al., 2014; Hickman et al., 2013). This gene was also downregulated with aging in our cohort, further highlighting the impairment of microglia motility with aging.

Genes varying with age related to inflammation/priming as previously reported in mouse aging models (Holtman et al., 2015; Raj et al., 2015) were not observed in the present study. Instead, important immune regulators participating in cell adhesion were detected. In our analysis, human and mouse microglia transcriptome similarities were mostly associated to a disequilibrium in cell adhesion and motility related pathways.

Another noteworthy result from our analysis of human microglia was that postmortem delay (PMD), ranging from 4-24h interval, had no effect on the transcriptome, corroborating previous reports that PMD does not correlate with RNA quality or integrity (Chevyreva et al., 2008; Durrenberger et al., 2010; Ervin et al., 2007). This confirmation is highly relevant allowing the use of this source of human specimens for future studies.

In Chapter 7, we demonstrated the differences found between glioma and normal microglia, as well as the comparison between microglia derived from lower grade gliomas (LGG) and from glioblastomas (GBM). We compared our tumor microglia to a set of normal microglia samples from the study in Chapter 6. The comparison to post-mortem brain derived microglia permitted to avoid the already known bias of comparisons to microglia derived from epilepsy cases.

Tumor microglia showed an expected heterogeneity in their gene expression, just as heterogeneity is demonstrated in tumor tissue (Filbin and Suvà, 2016). These results led us to speculate that different activation signaling pathways occur within the same tumor sample. Microglia are subject to a number of signals from different clones of tumor cells, driving different responses along the progression of neoplasia, increasing its tumorigenicity. One alternative to elucidate how human microglia respond to stimuli from cells in each of glioma niches is to focus on single cell sequencing analysis. Such studies have been conducted on glioma cells in recent works (Meyer et al., 2015; Patel et al., 2014; Tirosh et al., 2016), revealing important mechanisms of drug response, growth, and differentiation potential related to specific
genetic alterations. Using the same approach to microglia would elucidate their definitive role in tumorigenesis and progression.

The biological pathways associated with the differentially expressed genes in tumor versus normal microglia were not restricted to inflammation related pathways, neither to the M1/M2 paradigm (Glass and Synowitz, 2014). Prominent changes in proliferation, cell cycle control and motility, including extracellular matrix related pathways were also detected. It also seems that, even if markers previously identified in non-neoplastic microglia are still expressed, tumor microglia undergo such drastic changes upon glioma stimuli, that the “quiescence” markers are supplanted by others.

With stringent criteria, we determined the core genes for both GBM and LGG microglia, and through independent component analysis, we were able to assess the differentially expressed genes according to the molecular stratification of the analyzed gliomas. A great portion of signature genes from LGG microglia were also present in GBM microglia, but at higher levels. This increased expression might reflect the progression in malignancy as previously described (Hambardzumyan et al., 2016), reinforcing the hypothesis that microglia play a crucial role in the progression of gliomas.

An explicitly inflammatory phenotype was not detected in tumor microglia. Previous murine studies (a Dzaye et al., 2016; Sielska et al., 2013; Zhang et al., 2016a) revealed that microglia display an inflammatory profile – either pro- or anti-inflammatory – upon interaction with glioma cells, and speculation on how each of these phenotypes promote or hamper tumor growth have been made. However, our dataset depicted a balance in the expression of chemokines and secreted factors that characterize both phenotypes, not permitting any determination of a specific inflammatory profile for human microglia along glioma progression.

Our analysis revealed that tumor microglia express high levels of genes related to extracellular matrix (ECM) remodeling, such as fibronectin (FN1), tenascin-C (TNC) and thrombospondin-1 (THBS1), along with invasiveness-related genes as ANXA2. The proteins encoded by these genes have been previously associated to glioma tumorigenicity and invasiveness (Blandin et al., 2016; Colin et al., 2006; Kling et al., 2016; Maule et al., 2016; Serres et al., 2014; Van Obberghen-Schilling et al., 2011; Xia et al., 2016). However, these previous studies focused on tumor cells, while the present analysis identified microglia as presenting high expression levels for these genes.

Among GBM subtypes, genes encoding ECM proteins were most abundant in mesenchymal subtypes, associated to the highest invasive rate and the worst prognosis
These genes highly expressed in microglia are attractive targets for treatment options to prevent invasion of the surrounding CNS parenchyma by tumor cells. Microglia are extremely mobile cells, needing to migrate to exert their function, both in homeostatic and pathological conditions. It is interesting then, that the main biological processes affected in microglia in both aging – a physiological process – and by glioma were related to motility, migration and invasion.

Functional studies focusing on the early changes that human microglia undergo upon interaction with glioma will further elucidate the results presented herein. Recent reports have advanced in the culturing of human microglia, allowing for newer approaches on the manipulation of these cells. It is now possible to derive microglia-like cells from both iPS and embryonic stem cells, and culture them for longer periods (Muffat et al., 2016; Rustenhoven et al., 2016), creating new opportunities for assessing the crosstalk between tumor cells and microglia.

**Final considerations**

We explored the current genetic approaches for glioma studies, and demonstrated the relevant role of molecular profiling. We had also expanded our study to the innate immune compartment, first analyzing a pure population of non-neoplastic cohort of microglia and further comparing them to glioma-derived cells. Our data sheds new light on the transcriptome changes that human microglia undergoes upon both aging and glioma stimuli towards tumor progression. These findings will guide the next step studies to further deepen the knowledge on this domain.

Many aspects regarding the players in glioma progression remain to be elucidated. We showed here that heterogeneity within the tumor is not restricted to cancer cells, but also to microglia. Other cells from the tumor microenvironment, such as endothelial cells, astrocytes and pericytes, could also present such intrinsic populational differences. Single-cell sequencing analysis of diverse cell types could further aid understanding how they affect / are affected by glioma cells. Recent report on mass cytometry used for single cell analysis in solid tumors (including glioma) demonstrated the possibility to isolate several populations from the same human sample (Lee latian et al., 2017). Along with next generation sequencing analysis, these would be elegant approaches to tumor heterogeneity.

A recent molecular study using a large cohort of human glioma samples revealed discrete pathways in tumor progression to be associated with epigenetic
mechanisms of control (Ceccarelli et al., 2016). Epigenetic changes, such as histone modifications and chromatin-remodeling complexes, play a role in gene transcription following stimuli, from either pathogens, injuries or tumor cells. This phenomenon involves a series of proteins that “read”, “write” and “erase” epigenetic signals, either by inherited characteristics or by stimuli response (Mehta and Jeffrey, 2015). These changes have been included in glioma classification and are now considered crucial for determining therapeutic strategies (recently reviewed by (Reifenberger et al., 2016). In myeloid cells, these changes occur within hours and are tightly associated with the microenvironment, as observed by studies using mouse microglia and tissue macrophages (Gosselin et al., 2014; Lavin et al., 2014) and, more recently, human macrophages differentiated from blood monocytes (Schmidt et al., 2016). Studies on human microglia or iTAMs epigenetic changes upon stimuli are still lacking. Functional co-culturing experiments with glioma and microglia cells would allow to assess these early changes.

Another aspect to be assessed is whether microglia from different regions in human brain display different transcriptional profiles. In mice, this has been previously shown (Grabert et al., 2016), as well as for human total brain tissue (Hawrylycz et al., 2012; Oldham et al., 2008). Considering different tumor can rise in different regions of the brain, understanding what is the “quiescent” profile of the local immune cells would help explore how these cells would respond to tumorigenesis.

The scientific field is experiencing an era in which the discoveries made today can reach clinical application much faster than in previous times. Cancer biology research has expanded its interest from just looking into tumor cells, but also focusing on the microenvironment. The results of such approach will much benefit future and innovative treatment options.
REFERENCES


