Analysis of Glioblastoma's Peritumoral Brain Zone
Developing of Per-operative Analysis and New Therapeutic Targets

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1 STAGE OF THE RESEARCH

Our research topic is focused on the glioblastoma (GB), an aggressive primary brain tumor, and especially the study of its peritumoral brain zone (PBZ). The aim of our study is to developp per-operative analysis of GB-PBZ to guide the surgery and to develop targeted therapies for post-operative therapeutic management.

During the first part of my PhD, we studied the heterogeneity of the glioblastoma's peritumoral brain zone (GB-PBZ) using the different techniques used routinely to analyze tumor samples : radiology, histology, flow cytometry, genomic, transcriptomic, proteomic and cellular cultures. We identified a specific profile of the GB-PBZ, distinct from both the normal brain tissu and the glioblastoma's tumoral zone. This research will be the subject of a publication, currently being drafted.

We also identified in this study several alterations specific to the GB-PBZ. These alterations of the GB-PBZ profile will be the subject of further studies to define whether or not their expression may be a prognosis or therapeutic response marker, or a potential target for targeted therapies.

More, the proteomic study of the GB-PBZ confronted us to a methodological problem: the absence of a defined control sample for the proteomic analysis of brain tumor samples. This methodological problem was solved and was the subject of a publication (Lemée et al. 2013).

2 OUTLINE OF OBJECTIVES

The second part of my PhD will be focused on two main subjects: the development of per-operative analysis of the GB-PBZ to guide the surgical resection of the tumor, and the molecular analysis of the GB-PBZ to develop new therapeutic targets for GB treatment.

3 RESEARCH PROBLEM

The GB is the most frequent and aggressive primary tumor of the central nervous system with an incidence of 4.96/100.000/year, a poor prognosis and the absence of a curative treatment.

One of the leading causes of this poor prognosis is the systematical recurrence of the tumor, occurring in 90% of cases from the margin of the resection's cavity (Sherriff et al., 2013); (Petrecca et al., 2013). Few observations have been made on this peritumoral zone defined by the brain tissues outside the contrast enhancement on gadolinium T1 weighted MR-scan.

Most of them were post mortem observation and indicated that microscopic tumor infiltration extended a considerable distance beyond the low densities areas on CT scan or the high-intensity areas on T2 weighted MRI. This may offer a potential explanation to the tumoral recurrence around the resection's cavity (Yamahara et al., 2010); (Nagashima et al., 1999).

Infiltrating tumor cells have been isolated, by the culture in a serum-free medium, of biopsy specimens taken from the brain tissue surrounding the resection cavity (Glas et al., 2010). These cells display the alterations typical of GB, but can be distinguished from tumor cells isolated from the tumor core on the basis of their distinctive molecular marker profiles and responses to drug and irradiation challenges *in vitro* (Glas et al., 2010 #87).

Using a different type of culture medium, we have isolated, from the brain tissue surrounding GB, another unexpected population of cells from the stroma that we have named glioblastoma associated stromal cells (GASCs) (Clavreul et al., 2011). These cells do not display the genomic alterations typical of GB cells and resemble the cancer-associated fibroblasts (CAFs) described in the stroma of carcinomas.

These few studies showed that the peripheral brain zone, although macroscopically and

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radiologically considered as normal brain tissue, is in fact distinct at different levels from both the tumor zone of glioblastoma (TZ) and the normal brain (NB). A better understanding of the characteristics of the PBZ is critical to understand the mechanisms of GB recurrence, optimize the quality of the surgical resection and develop new therapies.

3.1 Per-operative Study of Glioblastoma's Peritumoral Brain Zone

The gross total resection of the GB is an important prognostic factor of overall survival and tumoral progression-free survival for the patient (Chaichana, 2013).

The difficulty of achieving a gross total resection in GB lays in the infiltrative nature of the tumor, with the presence a small tumoral infiltration in the surroundings of the tumor. To avoid this we cannot take security margins around the tumor during the resection, because of the major risk of aggravating the already impaired neurological status of the patient, without a significant benefit for him. In this condition, a macroscopically complete resection is the optimal surgical treatment we can propose.

Thus, the development of new techniques of neurosurgery, or the adaptation of the existing ones, to allow a fast, easy, accurate and reproducible analyse of the GB-PBZ during the surgery is essential to ensure a complete resection of the tumor without impairing the patient's neurological status. Technological advances in this field may be quickly transferred to clinic and will have a critical impact on the patients' therapeutic management, functional outcome and survival.

3.2 Development of New Therapeutic Targets

Despite the development of new therapies, the prognosis of GB remains poor with a mean survival of 15 months with an optimal therapeutic management. The identification and the validation of new therapeutic targets are essential for the future of adjuvant GB's therapy in an era of personalized medicine.

The future of GB therapy will be the development of targeted therapies for custom-made treatment after specific tumor profiling. One lead is the identification of targets for existing targeted therapies like Herceptin in Her2+ breast cancer or vemurafenib in B-Raf proto-oncogene V660E muted lung cancer and melanoma. The second option may be the identification of specific target for GB and GB-PBZ allowing the development of new targeted therapies through the use of lipidic nanocapsules (LNC). Different chemotherapeutic agents can be encapsulated in these particles and thus focused delivery will allow us to increase the dose of therapeutic agent without increasing the potential side effects of chemotherapy agents.

4 STATE OF THE ART

4.1 Glioblastoma's Therapeutic Management

GB is the most frequent and aggressive primary tumor of the central nervous system. The mechanisms of recurrence remain unclear but the infiltration of the PBZ by GB cells may be considered as the explanation of the high rate of local recurrences.

The gold standard for GB's therapeutic management is a surgical gross total resection followed by radiotherapy and chemotherapy with temozolomide, according to the protocol described by Stupp (Stupp, 2009). However, even with this optimal treatment, the prognosis remains poor with a progression-free survival of 7 months and a mean survival of 15 months.

4.2 Per-operative Study of the Peritumoral Brain Zone

As mentioned before, the quality of the surgical resection of the GB is critical for its therapeutic management making the realization of per-operative quality controls of the surgical resection essential.

Actually, the gold standard of GB surgery is to perform a macroscopically complete resection, stopping the surgery when no tumoral tissue is left in the surgical cavity, without taking any security margin. But if the complete resection of the tumor jeopardizes the neurological status of the patient, a subtotal resection is performed instead. Numerous techniques have been used to attempt to develop a better per-operative control of the resection's quality.

The actual techniques used in per-operative GB-PBZ study are the per-operative imaging and the per-operative fluorescence. Per-operative imaging consist in the realization during the surgery of a brain scanner using a CT- or a MR-scan to ensure the complete resection of the tumor. Due to the cost

of a dedicated scanner or MRI in the operating room, this technique is still confidential and only available in a few centers.

The use of per-operative fluorescence allows to see with a specific light the tumoral cells stained in red, after the ingestion of a 5-amino levulinic acid analog by patients 5 hours prior to surgery. This simplify the completion of the surgical resection by showing clearly where are located the tumoral remnants on the wall of the resection's cavity. This technique is still under evaluation in France and is actually the object of a national clinical trial (RESECT).

4.3 Therapeutic Targets in GB

Actually, there are no validated or available drugs targeted therapies available for GB treatment. GBs are currently under a specific study to identify potential targets for known and commercially available targeted therapies in AcSE study.

For now, we didn't dispose of a specific marker of GB or GB-PBZ tumoral tissue for target specific delivery of chemotherapeutic agents.

5 METHODOLOGY

5.1 Per-operative Study of the Glioblastoma's Peritumoral Brain Zone

In the search of new means to study the GB-PBZ, we will study several techniques routinely used in medical practice in specialities other than the Neurosurgery.

5.1.1 Second Harmonic Imaging Microscopy and Two Photon-excited Fluorescence

First, we decided to study two modern techniques of microscopy: the Second Harmonic Imaging Microscopy (SHIM) and the Two Photons-Excited Fluorescence (TPEF). SHIM is an efficient non-destructive method to study in vivo tissues without involving molecular excitation. TPEF is a technique similar to the immunofluorescence, but less susceptible to lead to phototoxicity and photobleaching. SHIM reacts specifically to non-centrosymmetric molecules such as collagen, myosin and microtubules. This is an interesting lead because normal brain cells do not produce collagen whereas GB cells produce collagen in their extracellular matrix using it as a support for cell migration.

TPEF is a living imaging fluorescence that allows deep study of tissue up to 1mm. TPEF can excite fluorescent dyes with far less toxicity than the other fluorescent methods and has a high definition thank to a strong suppression of background signal due to infrared photon absorption.

Preliminary Study. In collaboration with the Dr Denis Gindre, of the Department of Physics of the University of Angers, we did a preliminary study on paraffin embedded sections of brain tumor to assess the feasibility of the study.

The obtained images showed a differential SHIM signal between the different brain zones, with the absence of SHIM signal in the central tumoral necrosis, a strong signal in tumoral zone and an intermediary signal in the GB-PBZ (Figure 1). These data are in favour of the continuation of this study on a wider panel of brain tumor samples.

Adaptation of the Technique. However, before continuing in scanning the samples, we will have to solve a problem specific to the brain tumor and the SHIM, the representativity of the sample.

Indeed, the SHIM and TPEF techniques allow the scanning of a square of 100x100 µm. This is an insufficient size to obtain a representative SHIM and TPEF profile of a GB sample, which is by definition a heterogeneous tumor with foci of necrosis and tumoral proliferation.

Dr. Gindre and one of his PhD student are currently creating a program which will scan random squares of 100x100 µm of the tumor sample under different polarization angle to create an mean image of the SHIM and TPEF signals of the lesion in order to obtain a representative SHIM and TPEF profiles of the lesion.

Creating an Atlas. The next objective, when the acquisition of a representative SHIM and TPEF profiles of the studied samples will be validated, will be to create an atlas of the SHIM and TPEF profiles of the different types of normal brain and brain tumor samples, using a collection of brains' paraffin embedded sections with an histologically confirmed diagnosis.

The constitution of the atlas of SHIM and TPEF signals of brain tumors samples will allow us to assess the samples obtained per-operatively, based on the profile obtained and its comparison with the atlas.

Per-operative Testing. The final step will be to perform extemporaneous analysis of the tumor samples obtained during surgery to perform an

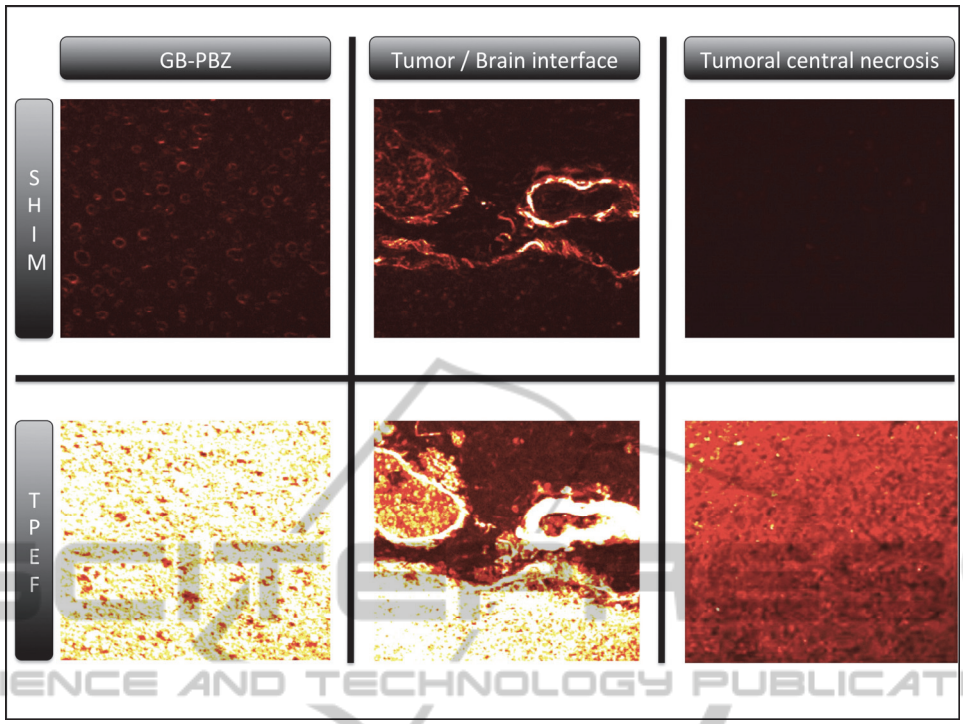

Figure 1: Illustration of the SHIM and TPEF acquisition of different GB area. The peritumoral brain zone from glioblastoma (GB-PBZ), the interface between florid tumor and peripheral brain and the central necrotic zone of the tumor.

immediate diagnosis of the tumor sample, which can take up to 10 days with classical pathological analysis, and also assess samples of the border of the resection's cavity to confirm the absence of tumoral cells and thus the complete resection of the tumor.

5.1.2 Other Potential Leads for Per-operative Study of the GB-PBZ

We are planning to try the optic coherence tomography (OCT) to study the 3D conformation and surface of the resection's cavity to ensure an optimal resection of the tumor. This opportunity comes from the commercialization of smaller, portable and usable in an operating theatre.

Also, one other possibility, depending on the identification of specific targets of GB tumoral cells will be a colorimetric staining of the resection's cavity walls with a reagent that will change colour when in contact with a specific biomarker present in GB cells.

5.2 Development of New Therapeutic Targets

5.2.1 Validation of the Proteins

We identified from our multimodal analysis of the GB-PBZ potential biomarkers suitable as therapeutic target.

First, we will perform a double validation of the presence of these proteins by immunochemistry (IHC) and western blot analysis on the samples from the patients used for multimodal analysis of the GB-PBZ, whose profiles are known.

5.2.2 Study of the Biomarkers in Tumor Samples

After the validation of the proteins, we will assess the expression of these proteins in a cohort of patients from the national clinico-biological collection of GB, coordinated by Pr Menei. Crossing the data obtained with the epidemiologic characteristics and the evolution of the patients will allow us to determine if the presence (or absence) of these biomarkers have an impact on the survival or the therapeutic response of patients with a GB.

A first cohort of 50 patients from the clinico-biological collection and tumors samples will be analysed using IHC.

If the results are concluding for this first cohort, the analysis will be extended to the whole population of the clinico-biological collection and be done routinely on new patients included in the national database.

5.2.3 Study of the Biomarkers in Blood Samples

The proteins identified in the PBZ are components of the brain cells exosomes, and thus can be assayed in blood samples from patients.

We look forward to this possibility because blood samples are easy to obtain and allow repetitive and reproductive measure of biomarkers blood level in GB patients during their post-surgical evolution and treatment.

We will assess the presence of these biomarkers in the peripheral blood of GB patients using an enzyme-linked immuno-assay (ELISA) technique.

As with the analysis of the tumor samples, a preliminary study will be performed on a small cohort of patients from the clinico-biological collection and will be extended to the whole cohort if the first results are positives.

5.2.4 Biomarkers as Therapeutic Targets

We will study the potential role of the identified biomarkers as therapeutic target in GB treatment. A step next will be to develop monoclonal antibodies targeted against these proteins to see if the specific neutralization of one of these proteins can improve the survival or the therapeutic response of a population of murine GB model.

If we identify a significant improvement in terms of survival after GB implantation in a murine model of the disease, we will begin a human clinical trial.

5.2.5 Biomarkers as Targets for Vectorized Therapies

One other axis of development will be the use of vectorized LNC aimed against the proteins identified in the GB-PBZ. Production of LNC, encapsulation of chemotherapeutic agents and targeted delivery of these nanocapsules are a field of expertise of my laboratory, which possess an international recognition in this domain.

We aim to produce LNC targeted against the identified biomarkers to use them as therapeutic agent against GB. In a first phase, we will produce LNC containing fluorochromes to assess the distribution of the LNC in a murine model of GB.

Then, if the results are positive, we will produce LNC containing a chemotherapeutic agent, and assess in the same murine model of GB the impact of the LNC injection in the functional prognosis and survival of the mice, with always as first aim the transfer of these researches to the clinical field in human.

6 EXPECTED OUTCOME

At short-term, during the next year, the results of the SHIM and TPEF imaging analysis of brain tumor samples will be the subject of a publication in a high impact review of Neuro-Oncology. Also the validation of the biomarkers in the brain tumor and the peripheral blood samples of the patients will be the subject of a publication in a high impact review in Neuro-Oncology.

The use of per-operative OCT or colorimetric staining of the tumor are planned for the end of my PhD thesis or will be the subject of my post-doctoral studies.

The use of the proteins of interest as therapeutic target or as target for chemotherapy-loaded LNC will be the subject of further studies in my laboratory, implicating Pharmacists and Galenists.

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