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PhD Thesis

IDENTIFICATION OF NEW POTENTIALLY ACCESSIBLE BIOMARKERS SUITABLE FOR THE DEVELOPMENT OF THE TARGETED THERAPY OF GLIOBLASTOMA MULTIFORME

Candidate: Davide Musmeci

Tutor, University of Pisa: Prof. Generoso Bevilacqua

Tutor, University of Liège: Prof. Vincent Castronovo

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A Lucia e Giuseppe,

Summary

Background: Glioblastoma multiforme (GBM) is the most aggressive, highly invasive and neurologically destructive among malignant brain tumors. None of the treatments currently used is effective, making GBM lethal within 12 months from diagnosis. Despite major evolution in the understanding of the molecular mechanisms involved during GBM development and progression, patients are yet in need of a successful treatment. Nowadays, one of the most promising approaches aimed to cure cancer consists on the antibody-based therapy. The systemic delivery of specific antibodies, coupled with highly cytotoxic drugs, directed towards the tumor site is considered as a promising way of treatment. Unfortunately, the bottleneck of such approach consists in the identification of antigens accessible by the blood stream.

Aim of the work: This work aims to identify and validate *in-vivo* new biomarkers produced either by the tumor itself or by the surrounding stroma in response to tumoral demand, which are reachable by a systemic administered compound and could be used as therapeutic targets in human GBM.

Material and methods: Human GBM specimens, two different human glioblastoma cell lines (U373 and T98G) grafted in nude mice and U87-derived tumors grown in egg choriallantoic membrane (CAM) were biotinilated *ex-vivo*. Labeled proteins were successively isolated using affinity chromatography based on streptavidin beads. Proteins were eluted and digested with trypsin and the resulting peptides were analyzed using the 2D nano-LC MS/MS technique. Protein identification was carried out using the Mascot® search engine (Matrix Sciences, Boston, MA, USA) and the Swisprot® protein database (Swiss Institute for Bioinformatics, Basel, Switzerland). For the purpose of validation U87-derived tumors were grown on CAM. Intravenous administration of specific monoclonal antibodies into the CAM vessels was carried out in order to validate *in-vivo* the accessibility of the target.

Results and conclusion: expression profiles for each experimental model were determined using the Mud-PIT technique. About 30 to 35% of the total proteome were found to be accessible (membrane associated, extracellular and secreted proteins). Among the proteins identified, the study highlighted the hyaluronan receptor CD44 and tenascin-C (onco-fetal Tenascin) already known to be overexpressed in human GBM along with other new potential targets such as sparc-like 1, prosaposin and collagen 6 α1. The validation phase carried out using immunohistochemical analysis confirmed the overexpression of the proteins in high-grade gliomas. Additionally, *in-vivo*

experiment with the systemic administration of the monoclonal anti-human CD44 and COL6α1 antibodies into CAM vessels resulted in a site-specific tumor accumulation of the antibodies suggesting these proteins as readily accessible target for the treatment of GBM. Taken together, these results demonstrate the potential of the biotinilation technique in searching for potential accessible biomarkers. The study pointed at the usefulness of the CAM system as an alternative model of biomarker validation in comparison to the more cost and labor intense mouse model. Further investigations focusing on the development of antibody-based treatment of tumor bearing animals are the next step to envision before proposing these targets for clinical trials on humans.

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Preface

Thorough the centuries cancer became one of the major health problem of all developed country, only second to cardiovascular disease in terms of death rate. A comprehensive estimation of the incidence and mortality rates from cancer can be extracted from the GLOBOCAN project of International Agency for Cancer Research (http://globocan.iarc.fr/). Over a population of 6.7 milliards peoples, in 2008, more than 12 millions new cancer cases were estimated. That led to the death of more than 7 million peoples all over the world. In Europe, cancer caused approximately 1.7 millions death in 2008, which means 4.700 death per day. In other words, approximately 196 people are dying for cancer each hour, in all European territory. Moreover, as the incidence of cancer is higher in elderly people, probably due to the accumulation of mutation within lifetime, it is expected that the current demographic shift to higher age in developed countries will further increase this major health burden of 22% in 2015.

High-grade gliomas are the most common primary brain tumor in adults. Among them glioblastoma multiforme (GBM) is the most aggressive. The current therapy for these tumors is based on maximum safe and secure surgical resection of the neoplastic mass followed by concurrent local radiation and adjuvant chemotherapy with Temozolomide. However, the ability of GBM cells to migrate through the narrow brain parenchyma, sometimes for relatively long distances, makes them elusive target for effective surgical removal and give to patients a life expectancy that rarely exceeds 12 months, which drops down to 3-4 months in case of no treatment. These data clearly indicate that major efforts are needed in cancer research, and especially in the filed of GBM, in order to fight this lethal disease.

This thesis is focused on the development of a methodological and technological approach able to identify new biomarkers potentially useful as target for new molecular drugs. The research activity necessary to the generation of this work was carried out at the Metastasis Research Laboratory (MRL) of the University of Liege, Belgium, in collaboration with the Division of Surgical, Molecular and Ultrastructural Pathology of the University of Pisa, Italy.

Section I - Introduction

1. Cancer Disease

1.1 - Pathogenesis of cancer

Neoplasia or neoplasm or tumor literally means the process of "new growth". A tumor can be benign or malignant. Malignancy is the ability of a cancer cell population to invade the surrounding stroma and to give origin to metastasis. *Cancer* is the common term used for all malignant neoplasms. A neoplasm originates as the result of hereditary or somatic alteration in genes that control crucial biological process. These genetic changes allow excessive and unregulated cell proliferation that further becomes autonomous and independent of physiologic growth stimuli. In this way, the entire population of cells within a neoplastic mass usually arises from a single cell that has incurred genetic changes, hence tumors are said to be *clonal*. All neoplasms, benign or malignant, are made up of two basic components: i) proliferating neoplastic cells that constitute their *parenchyma* and ii) supportive *stroma,* made up of extracellular matrix tissue and a huge network of blood vessels. Although parenchymal cells represent the proliferating "cutting edge" of neoplasms, and so determine their behavior and pathologic consequences, the growth and the evolution of tumors are critically dependent on the stroma supply.

As previously said, nonlethal genetic alterations are the key of the carcinogenesis process. These genetic damages, more precisely called *mutations*, generally hit *tumor promoting* and/or *tumor suppressor genes*. The number of mutations needed to produce the initiation of a neoplastic event may vary from one to several. An example that could better elucidate this theory is represented by the chronic myeloid leukemia (CML) where a chromosomal translocation that involves a piece of chromosome 22 being lost is reported. This was first observed by Nowell and Hungerford, who named this small chromosome the *Philadelphia* chromosome. Only later it was shown that this alteration was resulting from the reciprocal translocation between chromosomes 9 and 22, which produces the chimeric protein called Bcr/Abl, a constitutively active tyrosine kinase promoting cell proliferation. Therefore, CML appears to be triggered by this one-hit event, and this is probably the reason why the drug *Gleevec*, which targets the Bcr/Abl kinase, is effective as a single agent in CML. However, progression to a full-blown invasive metastatic cancer requires almost always multiple hits. This is the case of most adult solid cancers such as colon, lung, breast and prostate. A model of cancer progression is well represented by colon cancer, for which at least five hits appear to be required to produce an invasive carcinoma (Figure 1).

Because of genetic instability, a characteristic of most solid cancers, many genetic alterations are frequently accumulated during cancer progression. Thus, in contrast to single genetic defect cancers such as CML, the prospect of finding effective single therapeutic agents is unlikely for most solid

tumors. Multiple aberrant cell signaling pathways will need to be inhibited to achieve effective chemotherapeutic regimens. However, if there are identifiable time intervals between the multiple hits that lead to cancer, perhaps detectable by early screening for surrogate markers of progression, there may be a window of opportunity for preventive agents [Raymond W.R. 2007].

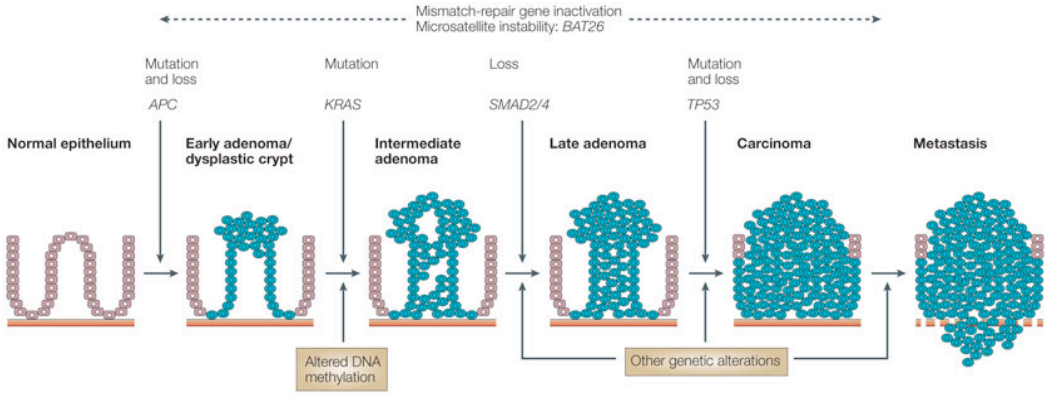


Figure 1: Progression from normal epithelium through adenoma to colorectal carcinoma is characterized by accumulated abnormalities of particular genes. [Davies RJ 2005]

1.2 - Molecular Basis of Cancer Progression

Heritable genetic mutations, passed down to the progeny of cancer cells, lie at the heart of carcinogenesis. Such mutations may be acquired by the action of environmental agents, such as chemicals, radiation, or viruses, or they may be inherited in the germ line. The term "environmental," used in this context, involves any acquired defect caused by exogenous agents or endogenous products of cell metabolism. Not all mutations, however, are "environmentally" induced. Some may be spontaneous and stochastic.

Two classes of regulatory genes are the principal targets of genetic damage: i) the growth-promoting *protooncogenes* and ii) the growth-inhibiting *tumor suppressor genes*. Mutations on *protooncogenes* are considered dominant because they transform cells despite a normal allele is preserved. *Protooncogenes* can be activated by increasing the synthesis of their corresponding protein, in normal form, or by alteration of the corresponding protein function, through gene mutation. In contrast, both normal alleles of the *tumor suppressor genes* must be damaged for transformation to occur. The loss of function of *tumor suppressor gene* caused by damage of a single allele is called *haploinsufficiency*. In addition to these two classes of genes, DNA repair genes affect indirectly cell proliferation or survival by influencing the ability of the organism to repair nonlethal damage in other genes, including *protooncogenes*, *tumor suppressor genes*, and genes that regulate apoptosis. Hence, disabilities in the DNA repair genes predispose to genome mutations accumulation and lead to neoplastic progression.

At the phenotypic level a malignant neoplasm has several attributes, such as uncontrolled and excessive growth, local invasiveness, and the ability to form distant metastases. These characteristics are acquired in a stepwise fashion during a phenomenon called tumor progression. For such reasons, the process of carcinogenesis is considered as a multistep process both at the phenotypic and the genetic levels.

Three are the main step characterizing tumor progression: initiation, promotion, and progression. Initiation can occur after a single, brief exposure to a potent initiating agent. The actual initiation events leading to transformation into a dormant tumor cell appear to occur within one mitotic cycle. Furthermore, initiation appears to be irreversible; the promoting agent can be given for up to a year later and a high percentage of tumors will still be obtained. Thus, the initiation phase only requires a small amount of time, it is irreversible, and it must be heritable because the initiated cell conveys the malignant alteration to its daughter cells. All these properties are consistent with the idea that the initiation event involves a genetic mutation, although other ‘‘epigenetic’’ explanations are possible. The promotion phase, by contrast, is a slow, gradual process and requires a more prolonged exposure to the promoting agent. Tumor promotion represents a cell proliferation phase

that propagates the initiated damage and leads to the growing of an altered clone of cells. Promotion occupies the greater part of the latent period of carcinogenesis; it is at least partially reversible and can be arrested by certain anti-carcinogenic agents. Finally, the progression stage of carcinogenesis is an extension of the tumor promotion stage and results from it in the sense that the cell proliferation caused by promoting agents allows the cellular damage inflicted by initiation, to be propagated, and the initiated cells to expand clonally. During the process of cancer progression loss of growth control and escape from host defense mechanisms become predominant phenotypic traits. At the molecular level, progression results from accumulation of genetic lesions that in some instances are favored by defects in DNA repair genes. Genetic instability, indeed, is the hallmark of the progression phase of carcinogenesis and leads to chromosomal translocations and *aneuploidy* that are frequently seen in cancer cells. Such alterations in the genome of the neoplastic cell during the progression phase lead to the increased growth rate, invasiveness, and metastatic capability of advanced cancer. Evidences for multistage induction of malignant tumors have been observed for mammary gland, thyroid, lung, and urinary bladder and in cell culture systems, thus it seems to be a general phenomenon.
[Robbins & Cotran 2005]

1.3 - Morphological basis of cancer progression

Malignant tumors range from well differentiated to undifferentiated. Malignant tumors composed of undifferentiated cells are said to be *anaplastic*. Lack of differentiation, or *anaplasia,* is considered a hallmark of malignant transformation. Anaplasia literally means "with no shape", the absence of any differentiative character. However, an increasing amount of literature suggests that most cancers do not represent "reverse differentiation" of mature normal cells but, in fact, arise from stem cells (known also as *cancer initiating cells*) that are present in all specialized tissues.
As said before, cancer progression is the consequence of an uncontrolled cell proliferation from one hand and of the accumulation of genetic alterations on the other hand. The increase of these two parameters causes a series of modifications in the shape of tumor cells and in the architecture of the tissue to which these cells belong, namely:

- atypia: nuclei become larger and darker, with abnormal shape, with a prominent nucleolus. These changes are respectively due to the fact that there is an increase in DNA quantity, modifications of the nuclear proteins, a higher protein synthesis.
- pleomorphism, polymetrism and polychromatism, due to the fact the atypical characters can be of different entity in the different tumor cell clones.

- presence and number of mitosis: sign of entity of the proliferation activity of the neoplastic cell population.

These characters, reflecting the degree of severity of the disease, are very useful for grading a malignant tumor in scales that can be different according to the type of neoplasm, that in any case represent an important prognostic parameter. For instance, as explained later, their morphology allows gliomas to be grouped in four classes, with an increase in their malignant behavior moving from low to high grades.
[Robbins & Cotran 2005].

1.4 - Cancer therapy and its limitations

Classical anti-cancer therapy, including chemotherapy and irradiation, often suffers from poor selectivity and, thus, from severe toxic side effects for healthy tissues. Many therapeutic protocols for solid cancer count on maximal surgical resection of the neoplastic lesion combined with successive irradiation and/or chemotherapy, aimed to slow down the fast proliferating cancer cells. However, cell proliferation occurs also in healthy organs such as the spinal cord, mucosa, hair follicles, bone marrow, and during pregnancy. Since the vast majority of tumor cells have to be killed to achieve the complete remission of the patient, the application of high doses of drugs is required, at the expenses of severe toxic side effects. Furthermore, most chemotherapeutic agents do not preferentially accumulate at the tumor site. The dose of drug that reaches the tumor may be as little as 5-10% of the dose that accumulates in normal organs. The high interstitial pressure and the irregular vasculature of the tumor account, in part, for this difficult uptake of drugs by tumor cells. On the top of that, the activity of multidrug resistance proteins, present on the plasma membrane of cancer cells, may further decrease drug uptake *[Baguley BC. 2010]*. In conclusion, the majority of pharmacological approaches for the treatment of solid tumor is not sufficiently selective, limiting the dose escalation and the possibility for the patient to be cured.
Fortunately, many molecular pathways and biological characteristics of different tumor entities have been unraveled during the last decades. This knowledge could now be used to generate specific tumor therapies, either by directly targeting of the proteins involved in the neoplastic process, or by delivering targeted drugs to the tumor site.

In the 19th century, Paul Ehrlich was the first to envision antibodies as the “magic bullets” that would specifically trace and kill tumor cells. However, the introduction of antibodies in basic cancer research was possible only after 1975, when Köhler and Milstein described the possibility to generate murine monoclonal antibodies (mAb) *[Köhler G. 1992]*. Their development as therapeutic

agents, however, was hampered by the immune response of the patients, which readily eliminated murine antibodies. To address this problem, the first chimeric antibodies were generated in late 1980 *[Riechmann L. 1988; Liu AY. 1987]* and already in the 1997 the first therapeutic antibody, *Rituximab*, was approved by the US Food and Drug Administration (FDA) for the treatment of B-cell non-Hodgkin's lymphoma *[Grillo-Lopez AJ. 2002].* Since that time, mAb-based therapies have become a key strategy in tumor therapy with the succeeding generation of several mAbs such as *Trastuzumab*, targeting the HER2/neu and used for metastatic breast cancer, *Bevacizumab* and *Cetuximab*, targeting respectively VEGF and EGFR, used in the treatment of colon cancer *[Scharma D. 2006].* Successively, cytotoxic drugs, cytokines, toxins or radionuclides have been conjugated to such mAbs and these constructs have been evaluated in preclinical and clinical trials. More detailed information on the mechanism of the targeted therapy will be given later on this thesis.

2. Malignant Brain Tumors

2.1 - Epidemiology

The term brain tumor includes all tumors occurring in the central nervous system (CNS). Brain tumors account for 1.4% of all new cancer cases and they are responsible for more than the 2% of all cancer-related deaths by year *[Ali-Osman F. 2005]*. Approximately 175,000 new cases of brain cancer are described in the world each year with an incidence going from 4 to 10/100.000 inhabitants, according to the histological type, patient's age, sex and country of residence. In developed country the incidence of brain tumor reaches 10/100,000 *[Béhin A. 2002; Stewart BW. 2005]*. Malignant brain tumors are currently classified on the basis of their histology and location. However, this classification is often complicated by the potential ability of any cell of the central nervous system to undergo malignant transformation, resulting in a mixture of cell types usually seen in many brain tumors. The main class of brain tumors is represented by malignant gliomas of astrocitic, oligodendroglial and ependymal origin, which account for more than 70% of all brain tumors. Other histological types are represented by: medulloblastomas, accounting for the 25% of child tumors; meningiomas and schwannomas, usually benign neoplasm; gangliomas, easily curable tumor using a combination of chemo- and radiotherapy; chordomas.

The age-adjusted incidence rates of brain tumor generally tend to be highest in developed rather than in developing countries. In Western Europe, North America and Australia there are 6 to 10 men per 100,000 inhabitants newly affected by brain tumor each year. Slightly lower is the situation for women, with 4 to 10 new case per 100,000 inhabitants (Figure 2). Even within Europe, incidence rates differ between countries (Figure 3). The lower incidence in developing countries may be partially due to a process of under-estimation, but different life-style and ethnic variances in susceptibility to develop brain tumors cannot be excluded. Generally, Caucasians are more affected than people of African or Asian descent, and this difference has been observed in children as well *[Farlay J. 2000]*. As the situation in Italy is concerned, brain tumors account for a yearly average of 10.5 new brain cancer per 100,000 males and 8.2 per 100,000 women. In 2002 a total of 2,414 and 1,873 new brain cancer cases were diagnosed in males and females respectively, leading to a total of 1,733 and 1,541 death, respectively.

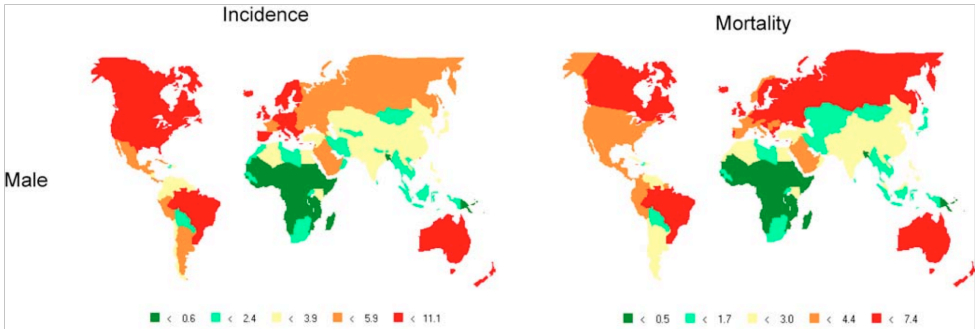


Figure 2: Global incidence and mortality rates of male individuals affected by nervous system tumor, adjusted to the World Standard Population (all ages; per 100,000 persons per year) *[Farlay J. 2000].*

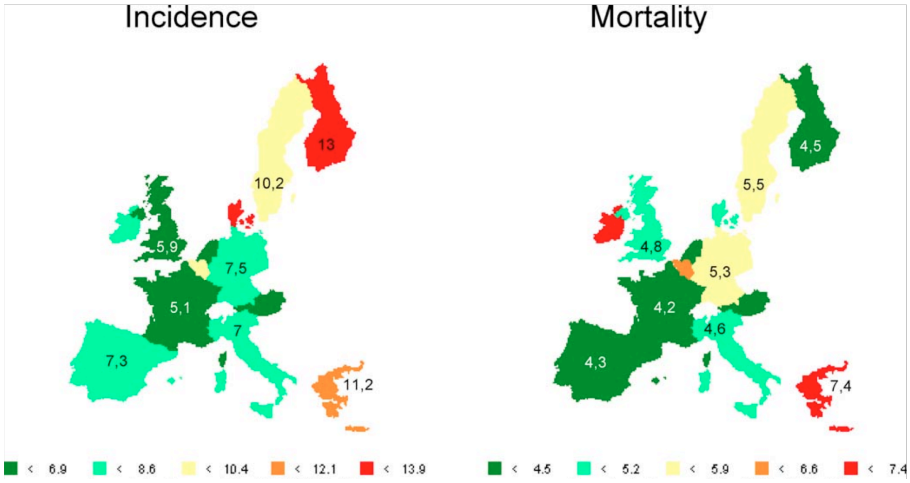
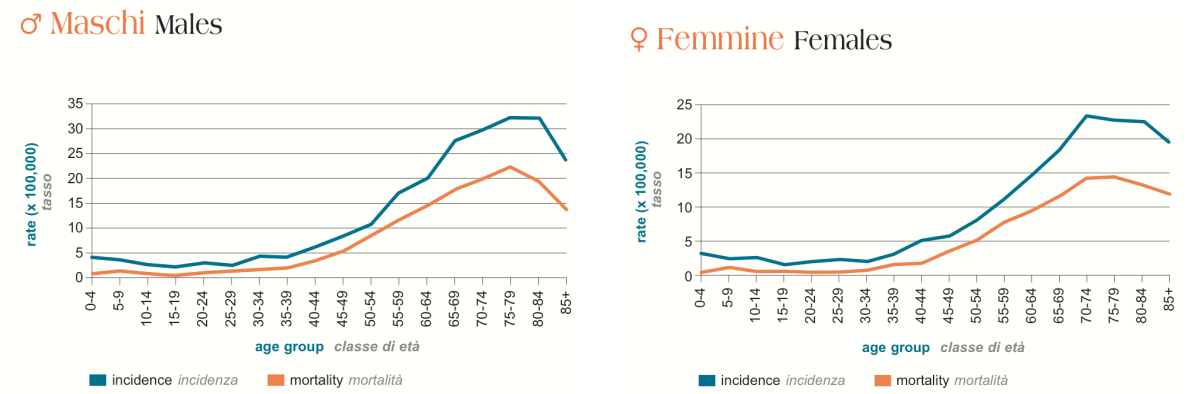


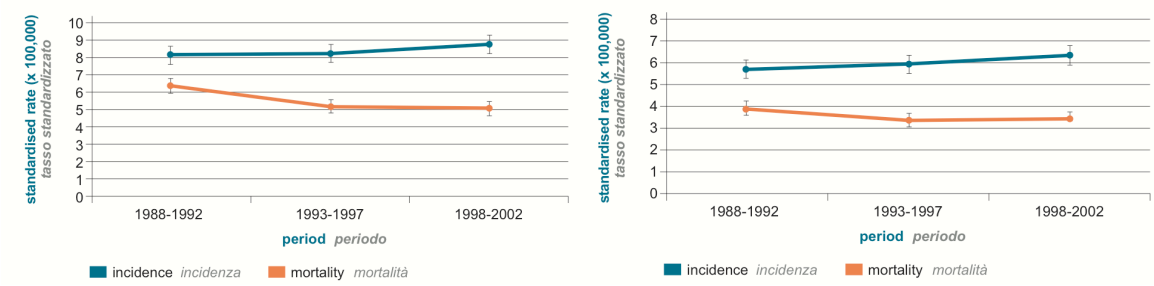
Figure 3: Incidence and mortality rates (per 100,000 persons per year) of nervous system tumors in Europe, adjusted to the European Standard Population *[Farlay J. 2000].*

However, in the all period spanning from 1998 to 2002 brain tumors represented only 1.3% of all neoplasms diagnosed among males and 1.4% among women. They were responsible of 1.9% and 2.1% of all cancer death among males and females, respectively. As shown in Graphs 1, brain cancer incidence and mortality picks lie approximately around 70 and 80 years old, respectively for men and woman. However, it is also relatively frequent already among young patients (0-44 years); in this age range it represents the 6th most frequent tumor among males (5.5% of all cancers) and the 9th among females (2.7% of all cancers). The cumulative risk (0-74 years) of developing a brain cancer was reported as 7.2‰ (1 case by 139 men) and 5.3‰ among females (1 case by 188 women), while the cumulative risk of dying from this cancer was 4.4‰ and 3.0‰ among males and females, respectively.



Graph 1: Incidence and mortality rates of brain cancer in men and woman, within different age group *[Brain cancer, tumors in Italy 2006].*

Incidence rates vary across Italy with a ratio between highest and lowest values of about 2.5 points. However, even thought incidence rates for brain cancer are increasing over the time, mortality shows a slightly decreasing tendency, probably as a results of better treatment modality used over the years *[Brain cancer, tumors in Italy 2006].* Graph 2 represents in fact the situation as from the 1988. A slight decrease of mortality is observable over a period of 22 years.



Graph 2: Incidence and mortality rates of brain cancer through different periods *[Brain cancer, tumors in Italy 2006]*

2.2 - Malignant gliomas

Originating from neuroepithelial cells, malignant glial tumors are the most common primary tumors of the human nervous system and include the most aggressive primary brain tumors in adults, glioblastoma multiforme (GBM). Yet, the etiology of this kind of tumors is not well known.

However, as for other human cancers, malignant gliomas take origin from genetic alterations occurring on *protooncogenes* and *tumor suppressor genes* and lead glial cells to transformation. In brain tumors, *protooncogenes* activation occurs almost entirely by gene amplification, resulting in an increased number of specific genes within a cell, and in a corresponding increased expression of the gene's encoded protein. 1985 marked the first report of a specific gene alteration in a CNS tumor: the epidermal growth factor receptor (EGFR) gene amplification in GBM. Since that time, neuro-oncology research has revealed many genetic abnormalities that indicate consistent genotype-phenotype associations. On the other side, tumor suppressor genes typically associated to malignant gliomas are TP53*,* NF2*, and* VHL*,* with their respective cancer syndromes: Li-Fraumeni (*TP53*), neurofibromatosis type 2 (*NF2*), and von Hippel-Lindau (*VHL*) disease. Moreover, several studies have also reported a wide variety of environmental risk factors, including diet, smoking, alcohol, occupation exposures, radiation, infections, allergies, head trauma, and family history that are being intensively investigated for their role in glioma development.

Malignant gliomas have a peak of incidence lying around age 60 years, and they represent one of the greatest challenges in oncology. Even thought major improvements in cancer research and treatment have been made during the last decades, malignant gliomas continue to have the reputation of highly lethal diseases with very austere prognosis. This is mainly due to the unchanged poor prognosis of GBM. Traditional treatments, which rely on nonspecific, cytotoxic approaches that generally act through DNA damage, have a marginal impact on patient survival and allow GBM to become lethal within approximately 12 months from onset *[Mirimamanoff RO. 2006; Stupp R. 2005; Polin RS 2005].* Only 3% of patients affected by GBM survive up to 2 years *[Krex D. 2007].* Moreover, the bony limited space and the very sensitive and non-regenerative neural tissue set critical limitation to surgical treatments in the head area. The blood-brain barrier and the blood-cerebrospinal fluid barrier make the delivery of anticancer drugs to the tumor site very challenging. Molecules that are able to cross these barriers must be electrically neutral, small and lipid-soluble. Unfortunately, this is not the case for most of the chemotherapeutic available nowadays.

On the histological basis malignant gliomas are classified as: astrocytomas, oligondedrogliomas, mixed oligoastrocytomas and ependymomas.

2.2.a - Astrocytomas

The majority of brain cancers are represented by astrocytomas, tumors arising from star-shaped glial cells called astrocytes. They can develop in patients of any age preferentially in the main part of the brain, the cerebrum, and rarely spreading outside, affecting other organs. The World Health Organization (WHO) classify these neoplasms in different subtypes according to their histological features (e.g. nuclear atypia, mitotic index, microvascular proliferation index, and necrosis) and on the basis of their invasiveness and/or malignancy.

In order of increasing *anaplasia*, astrocytomas are named as:

- *pilocytic astrocytoma* (WHO grade I): is the most common pediatric brain tumor (approximately 700 children per year are affected by this tumor) rarely undergoing malignant transformation. However, even though it is the most benign among astrocytomas, depending on its location, it can interfere with vital sensory functions and frequently recur after apparent complete resection. On histological basis, pilocytic astrocytomas appear as spindle shaped with numerous collections of reddish astrocytic fibers called Rosenthal fibers.
- *diffuse astrocytoma* (WHO grade II): it account for 25% of all gliomas and it is usually an infiltrative tumors. Despite its relative lack of aggressive histological features, the low-grade astrocytoma in adults is fatal in the great majority of patients. Grade II astrocytoma presents low cellularity with mainly oval pale nuclei, indistinct cytoplasmic borders and fibrillary processes with only mild pleomorphism.
- *anaplastic astrocytoma* (WHO grade III): is a highly malignant and infiltrative glioma with an increased tendency to dedifferentiate, giving rise to secondary glioblastoma. Histologically it is characterized by high cellularity, pleomorphism of the tumor cell with basophilic nuclei.
- *glioblastoma multiforme* (WHO grade IV): is the most lethal tumor of the central nervous system typically affecting adults. This type of glioma has a very poor prognosis, due in part to the rapid growth and infiltration of the brain parenchyma. GBM is a highly cellular tumor with pleomorphic, basophilic nuclei with indistinct cytoplasmic borders or plump pink cytoplasm and a delicate fibrillary background. Elevated mitotic index, necrosis, and capillary endothelial proliferation are common features of such tumors.

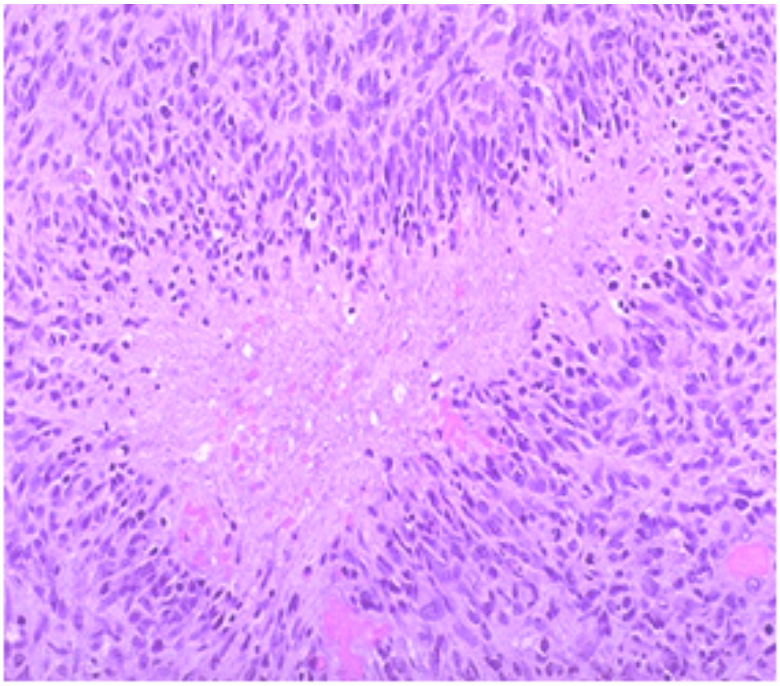


Figure 4: Histology of GBM. Pseudopalissade srurrounding necrotic zones along with hypercellularity, hyperchromatism and pleomorfism are typical features of these neoplasms.

2.2.b - Oligodendrogliomas and mixed oligoastrocytomas

These tumors represent a minor proportion of all primary brain tumors and are usually rare neoplasm arising from cells producing the lipid coat of the nerve cells axons, the oligodendrocytes. In the case of oligoastrocytomas, as known as mixed gliomas, the tumor shares characteristics of oligodendrocytes and astrocytes. They tend to develop preferentially in the temporal and frontal lobe of the brain and are most common in middle-aged adults. Although clinically less aggressive than astrocytomas, and with a slower growth rate, these tumors are able to cross into the cerebral spinal fluid (CSF) giving extra cranial metastasis and making surgical removal very challenging. They are both classified as low (WHO grade II) and high grade (WHO grade III).

2.2.c - Ependymomas

These tumors develop from ependymal cells, which line the ventricles of the brain and the central canal of the spinal cord. Ependymomas may spread from the brain to the spinal cord via the cerebrospinal fluid causing notable swelling of the ventricle or hydrocephalus. Ependymomas account for 4–6% of all brain tumors and occur mainly up to the age of 20 years old. In children, 30% of ependymomas appear before the age of 3 yr and

are more aggressive than in adults. Nearly 90% of pediatric ependymomas are intracranial: they occur in supratentorial or posterior fossa locations, and only 10% are intraspinal. In contrast to the earlier mentioned astrocytomas, low-grade ependymomas (grade I/II) develop metastases along the neuroaxis.

[Ali-Osman F. 2005]

2.3 - Clinical aspect

Clinical features of malignant gliomas are represented by headache, focal or generalized seizures with progressive neurological deficits, which are more or less important depending on the tumor location. Severe nausea and changes in mental status are usually present as well. All these symptoms typically occur as result of the rapid proliferation of tumor cells that generate a compression of the normal brain parenchyma. Edema and/or hemorrhage are also consequence of the process of infiltration, growth and expansion of the tumor mass within the brain, which is typical of this neoplasms.

2.4 - Molecular pathology of malignant astrocytoma progression

WHO grade II astrocytomas are best characterized by inactivating mutations of the *TP53* tumor suppressor gene on chromosome 17p, as well as overexpression of the platelet-derived growth factor (PDGF) α chain, and the PDGFα-receptor. Interestingly, loss of chromosome 17p in the region of the *TP53* gene is closely correlated with PDGFα-receptor overexpression, suggesting that *TP53* mutations may have an oncogenic effect only in the presence of PDGFα-receptor overexpression.

Allelic loss of chromosome 17p and *TP53* mutations have been observed in at least one-third of adult astrocytomas, regardless of tumor grade. An integral role for p53 in the early stages of astrocytoma tumorigenesis is further evidenced by so-called secondary glioblastomas; it has been demonstrated that grade IV lesions with homogeneous *TP53* mutations evolve clonally from subpopulations of similarly mutated cells present in the initial, grade II astrocytic tumors. Functional studies have recapitulated a role for p53 inactivation in the early stages of astrocytoma formation. For example, cortical astrocytes from mice that lack a functional p53 become immortalized when grown in vitro and rapidly acquire a transformed phenotype. In addition, although cortical astrocytes from mice with one copy of a functional p53 behave in a manner comparable to wild type astrocytes, subsequent loss of the one functional copy makes these cells

immortal and transformation can follow. Interestingly, those cells without functional p53 become markedly aneuploid, confirming prior reports that p53 loss results in genomic instability and that human astrocytomas with mutant *TP53* are often aneuploid. Thus, the abrogation of astrocytic p53 function appears to facilitate conditions leading to neoplastic transformation, setting the stage for subsequent malignant progression. The transition from WHO grade II astrocytoma to WHO grade III anaplastic astrocytoma is accompanied by several molecular abnormalities. Studies suggest that most of these alterations converge on one critical cell-cycle regulatory complex that includes the p16, retinoblastoma (Rb), cyclin-dependent kinase 4 (cdk4), cdk6, and cyclin D1 proteins. Individual members of this pathway are altered in up to 50% of anaplastic astrocytomas and in the vast majority, if not all, GBM. Loss of chromosome 9p primarily affects the region of the cyclin-dependent kinase inhibitor 2A (*CDKN2A)* gene and occurs in approx 50% of anaplastic astrocytomas and glioblastomas. The *CDKN2A* gene encodes for the p16 and $p14^{\text{alternate open reading frame (ARF)}}$ proteins, and expression of these proteins is most commonly altered by homozygous deletion of the *CDKN2A* gene, although point mutations and hypermethylation of *CDKN2A* have also been found to alter p16 and $p14^{ARF}$ expression. Chromosome 13q loss occurs in one-third to one-half of high-grade astrocytomas, with the *RB* gene preferentially targeted by losses and inactivating mutations. Analyses of the loss of chromosome 13q, *RB* gene mutations, and Rb protein expression suggest that the *RB* gene is inactivated in approximately 20% of anaplastic astrocytomas and 35% of GBMs. Interestingly, *RB* and *CDKN2A* aberrations are inversely correlated in gliomas, rarely occurring together in the same tumor.

Located on chromosome 12q13-14, *CDK4* is amplified in approx 15% of anaplastic astrocytomas and GBMs. This amplification frequency may be higher among gliomas without *CDKN2A* loss, perhaps reaching 50% of GBMs with intact p16 expression. CDK4 amplification and cyclin D1 overexpression appear to represent alternative events to *CDKN2A* deletions in GBMs because these genetic changes only rarely occur in the same tumor. Within this pathway, CDK6 amplification also occurs, although not as commonly as CDK4 amplification.

Further progression to GBM is characterized by the loss of chromosome 10; although occurring in anaplastic astrocytomas, this alteration can be found in 60–95% of GBMs. At least two tumor suppressor loci are implicated on the long arm of chromosome 10, as well as one potential locus on the short arm. The phosphatase and tensin homolog (*PTEN*)/ mutated in multiple advanced cancers 1 (*MMAC1*)/ TGFβ-regulated and epithelial cell-enriched phosphatase 1 (*TEP-1*) gene at 10q23.3 is one example of a tumor suppressor gene that has been studied, with *PTEN* mutations identified in approx 20% of GBMs. Moreover, introduction of wild-type *PTEN* into glioma cells with mutant *PTEN* leads to growth suppression. Nonetheless, given the remarkably high frequency of

chromosome 10 loss in GBMs, glioma tumor suppressor genes other than *PTEN* likely reside on this chromosome.

The epidermal growth factor receptor (*EGFR*) gene is the most frequently amplified oncogene in astrocytic tumors and is characteristic of so-called *de novo* GBMs. Although *EGFR* is amplified in few anaplastic astrocytomas, approx 40% of GBMs display this amplification. *EGFR* amplification in GBMs is almost always accompanied by loss of genetic material on chromosome 10 and these tumors often exhibit *CDKN2A* deletions. GBMs with *EGFR* gene amplification display overexpression of *EGFR* at both the messenger ribonucleic acid (mRNA) and protein levels, stressing the importance of this growth signal pathway to GBMs. Approximately two-thirds of the tumors having EGFR amplification undergo intra-gene deletion rearrangements that result in the overexpression of mutant EGF receptors. The most common EGFR mutant, EGFR-vIII, is known to have constitutive, ligand-independent tyrosine kinase activity, as well as an extended half-life that stimulates cell proliferation and enhances the tumorigenicity of human glioma cells in nude mice. Furthermore, the activity of this mutant has been shown to promote tumor angiogenesis, as well as to confer tumor resistance to apoptosis by increasing Bcl-XL expression. *EGFR* amplification and *TP53* mutations appear to be mutually exclusive genetic aberrations in GBMs. One-third of GBMs have *TP53*/chromosome 17p alterations, one-third display EGFR gene amplification, and one-third have neither change. Experimental data supports this distinction by showing that cells lacking functional p53 are not transformed when cultured in the presence of epidermal growth factor (EGF) but are transformed in the presence of other growth factors; GBMs with *TP53* mutations may therefore not be expected to acquire *EGFR* gene amplification if activation of the EGF-EGFR pathway does not produce a increased growth advantage in such cells. A number of additional molecular alterations occur in astrocytic gliomas for which little functional information is known. Less common genomic alterations associated with low-grade astrocytomas include loss of chromosome 22q, suggesting the presence of a chromosome 22q glioma tumor suppressor gene, and gains of chromosome 7q. In anaplastic astrocytomas and GBMs, allelic loss on 19q is quite common, being observed in up to 40% of these tumors and suggestive of a putative tumor suppressor gene.

[Ali-Osman F. 2005]

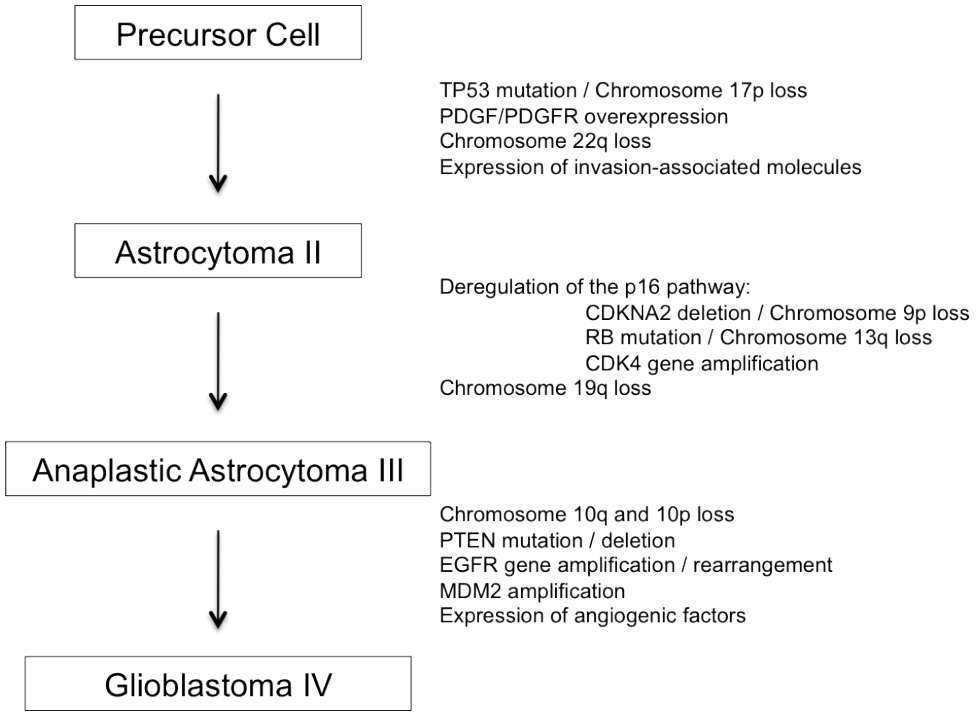


Figure 5: Molecular genetic alterations characteristic of different grade of astrocytomas

2.5 - Pathological markers of glioma

According to the first theory attempting to explain glioma formation, it was thought that central nervous system cancer cells arise from glial cells and thus resemble their precursors in varying degrees. Thus, as envisaged, mature neurons do not give rise to tumors, while glial cells surrounding them, such as astrocytes and oligodendrocytes, that remain proliferative, would be subject to tumorigenic stimulus. This resemblance helps histopatological diagnosis and could serve to predict the potential behavior of the neoplasm.

Another theory, which is newly coming into light, is based on recent findings that suggest that there may actually exist cancer stem cells (CSC) that are responsible for several tumor types, including brain tumors. These stem cells are portrayed as very primitive cells, resembling embryonic cells and neural stem cells that are still capable of potent mitotic activity and self-renewal. This theory gives a good explanation for the recurrence of tumors and why some tumors seem to be out of reach for even advanced therapies *[Singh SK. 2004].*

Until recently, treatment decisions regarding malignant gliomas began with establishing diagnosis by ordinary pathology only, which during the last 100 years provided knowledge about the biology and the clinical behavior of different neoplasm. During the last 10 years, however, data from histopathological examination of tumors tissue have been supplemented by an increased use of

molecular markers for tissue diagnosis. Knowledge about the molecular biology of cancer continues to increase and currently some molecular signatures, used to classify tumors rather than to predict response to therapy, are available to clinicians. According to the molecular biology of this tumors, the principal markers used in the case of malignant gliomas are 1p/19q co-deletion, methylation of the O-6 methyl-guanine-DNA methyltransferase (MGMT) gene promoter, and alteration in the epidermal growth factor receptor (EGFR) pathway.

2.5.a - 1p/19q loss

After the empirical discovery of favorable responses to chemotherapy in a high portion of recurrent anaplastic oligodendrogliomas (WHO grade III), Cairncross et al reported that such tumors carried a loss of the short arm of chromosome 1 (1p) *[Cairncross JG. 1998]*. Moreover, tumors carrying co-deletions of 1p and the long arm or chromosome 19 (19q) had substantially improved survival time. This finding have been investigated and then confirmed several times over the past 10 years and the correlation have been extended to new current therapy regimen such as temozolomide and radiotherapy *[Brandes AA. 2006; Kouwenhoven MC. 2006; Bauman GS. 2000]*. These data suggest this marker as a useful indicator of tumor vulnerability to a broad range of therapeutic options. The frequency of 1p/19q co-deletion has been estimated to 80-90% in grade II and 50-70% in grade III oligodendrogliomas. Even though no specific tumorigenic genes have been identified, 1p and 19q loss inversely correlates with TP53 mutations, 10q deletions, and amplification of EGFR. Deletions involving 1p and 19q are uncommon in glioblastomas, but, in those cases where it has been reported, these deletions seem to predict shortened survival, possibly indicating true genomic instability *[Esteller M. 2000]*.

2.5.b - MGMT methylation status

The orally administered alkylating drug Temozolomide, the current agent used as standard care drug for GBM since 2005, acts methylating the O6 position of the guanine nucleotide, resulting in cell death. The constitutively expressed DNA repair enzyme MGMT will transfer the methyl group from the O6 position of the modified guanine to a cysteine residue of the enzyme itself, mitigating the cytotoxic effects of the drug in normal cell. Approximately 50% of glioblastomas have decreased concentration of MGMT, making these tumors more susceptible to the effects of temozolomide *[Esteller M. 2000]*. Hegi and

colleagues studied 106 patients (46 with methylated tumors, 60 with un-methylated tumor) treated with temozolomide and reported a 2 years survival of 46% of patient carrying *MGMT* gene methylation versus only 23% of those patients with un-methylated tumor. The primary mechanism by which MGMT expression is reduced in glioblastoma, in fact, seems to be methylation of the *MGMT* gene promoter, a common way of gene silencing *[Hegi ME. 2005; Martinez R. 2007]*. Several studies suggested this correlation true also in pediatric glioblastomas and other low-grade gliomas *[Pollack IF. 2006; Everhard S. 2006]*.

2.5.c - EGFR pathway alterations

Molecular targeted therapies have been recently successful in chronic myeloid leukemia by the use of *Imatinib* to inhibit the BCR-ABL tyrosin-kynase fusion protein, and the use of *Trastuzumab* in the treatment of HER-2/neu positive breast carcinoma *[Druker BJ. 2006; Schnadig ID. 2006; Lohrisch C. 2001]*. Approximately 60% of glioblastomas present an up-regulation of EGFR mediated signaling, driven generally by an *EGFR* gene amplification. Additionally, in about half of the glioblastomas over-expressing EGFR, the expression of the mutant form EGFR variant III also is found. This variant lacks of the ligand-binding domain and therefore constitutively activates the downstream cascade.

The prognostic use of EGFR overexpression and its mutations is still not clear, but it might be useful for the identification of a subgroup of tumor with more aggressive behavior *[Jeuken J. 2009]*. The presence of such alterations might raise the possibility to use EGFR-targeted drugs in patients affected by glioblastoma; a similar situation to the non-small-cell lung cancer with activating EGFR mutations that showed remarkable response to *Erlotinib* and *Gefitinib*. However, two studies published in 2005 sought to clarify whether assessment of EGFR status was useful in aiding the decision of using such small-molecule kinase inhibitors. Results from both studies reported that tumor responding to these drugs had an intact PTEN-AKT pathway, which is unfortunately not the case of GBM *[Mellinghoff IK. 2005; Haas-Kogan DA. 2005]*. Subsequent studied, in fact, reported no major responses or benefit in patients with GBM being treated with these drugs *[Rich JN. 2004a; Kesari S. 2005]*.

2.6 - Glioblastoma: a cancer without hope

As already said GBM is the worst among brain cancer occurring in U.S. mainly in 60 years old men, with an incidence rate of 13.000 cases by year *[Schwartzbaum JA. 2006].* Typical symptoms of GBM depend on which part of the brain is affected. Headaches, nausea and vomiting, cranial nerve disorders and seizures are typical results of increased intracranial pressure. GBM does not metastasize via blood stream but has a great tendency to invade within the host brain parenchyma. Indeed, the aggressive and invasive nature of GBM has been described as the principal reason of the severe prognosis of GBM. Even though, chemotherapy with Temozolomide resulted as safe and feasible treatment of GBM, greatly accountable for the 1-year patient survival there are no effective treatments targeting tumor-infiltrating cells, migrating throughout brain parenchyma *[Hoelzinger DB. 2007].* Despite the usage of the most modern tools of the operating theatre, as online magnetic resonance imaging or computer-assisted navigation systems, neurosurgeons can only control the rapidly proliferating tumor mass by repetitive resections, reducing in this way the increasing intracranial pressure, caused by tumor growth, and thereby delaying imminent death of the patient. Additionally, malignant brain tumors are often located adjacent to or within functionally important areas of the brain where the use of aggressive surgery would turn into severe neurological damages for the patient. Accordingly, it appears obvious that the general principle of tumor surgery, which requires safety margin resection around the tumor mass of 1–2 cm, cannot be respected in neurosurgery. Furthermore, the blood-brain barrier and blood-cerebrospinal fluid barrier make the delivery of anticancer treatment to the tumor site a very challenging task. The first is formed of tight junctions between endothelial cells of capillaries, and the second has tightly bound choroid epithelial cells accompanied with an active organic acid transport system which impede crossing of molecules. Interstitial pressure in the brain slows down the molecular transfer to cells. Most molecules are not capable of penetrating these boundaries. Generally, molecules that are able to pass these barriers must be electrically neutral, lipid-soluble and small; unfortunately many, otherwise potent, chemotherapeutic drugs do not fit in this category. Even if the cytotoxic agent meets these criteria, it is extremely hard to reach therapeutic drug concentration and to maintain it within the proximity of the tumor site.

Moreover, neural tissue is very sensitive to radiation therapy resulting often in normal neural tissue death as well as a higher risk for radiation-induced mutations. These particular limitations demand the use of only carefully planned treatments only for those tumors that are known to respond. Considering all of this, it appears obvious that efficient treatments are based on comprehensive knowledge on tumor biology.

It has been already emphasized that histopathological examination may not be accurate enough and that genetic and molecular pathologic analysis of tumors are being used to complete diagnosis. These analyses constitute also the basis for targeted treatments, attempting to affect only those cells that express certain cancer-cell-specific features. This would minimize the damages to surrounding cells and reach a greater proportion of cancerous cells. However, it is still difficult to find such targets because GBM pathogenesis, like in the rest of malignant brain tumors, has been shown to be dependant on several tumorigenic pathways.

Many gene therapies and targeted molecular therapies have major limitations because their target products are often subject to mutation, making the treatment unstable. In addition, even specific features in certain cancer cells can be resulting from changes in very different sites along the molecular pathways. So far, it seems that future attempts to develop more effective treatments rely on accurate diagnosis, tools to estimate prognosis and development of combined therapies; an equation that is more complicated than its several factors taken alone.

2.7 – *de-novo* and secondary glioblastoma

Primary brain tumor present a set of characteristics common to all cancer: cell proliferation in absence of growth stimuli, avoidance of apoptosis and no limits to replication, escape from external growth-suppressive forces and immune response, new blood vessel formation, and extreme ability to invade normal brain tissue. These features are the results of a series of mutation occurring in the cells during their dedifferentiation, leading to tumor progression. At least two specific molecular pathways, by which high-grade gliomas develop, can be distinguished. Most GBMs are diagnosed without any antecedent lower-grade tumor being detected; they consist of the so called *de-novo* or *primary* GBMs. However, a smaller group of high-grade astrocytomas is known as *secondary* GBMs, arising as the result of the natural recurrence and progression of previously occurred lower-grade tumor. As showed by figure 6A, these two variants of GBM are determined by different sets of molecular changes. It has been reported that cancers with dysfunction in p53 pathway show resistance to radiotherapy. Indeed, high-grade gliomas are characterized by an elevated genomic instability, which is tolerated, without any induction of apoptosis, because of the disruption of the normal p53 pathway.

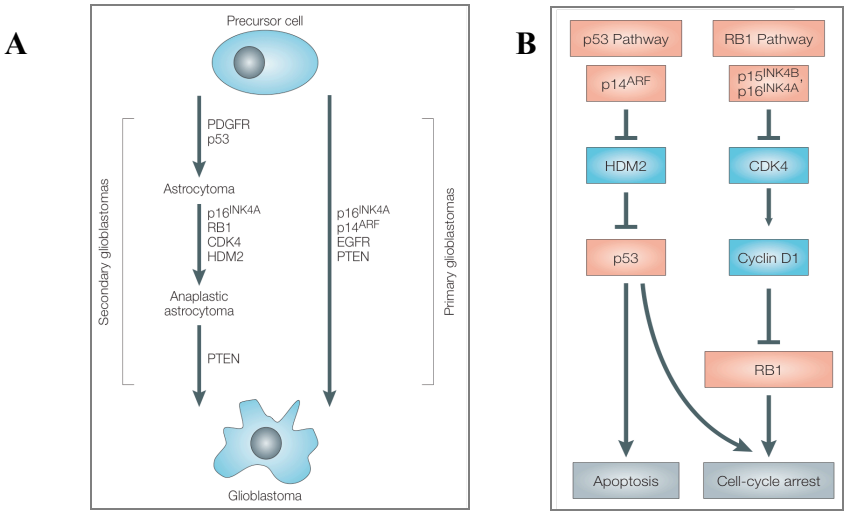


Figure 6: A) Molecular pathways to Glioblastoma formation - GBM can arise from the malignant progression of lower grade astrocytomas (grade II or grade III), or more commonly *de-novo* as primary tumor. B) Different genetic pathways are implicated in the formation of primary or secondary GBM, each of them has a crucial role in cellular transformation *[Rich JN. 2004b]*.

Low-grade astrocytomas and secondary GBMs often show mutation or loss of p53 protein, while in *primary* GBMs the protein is present but mutations and amplifications are commonly seen in its are the modulating factors. For instance, HDM2 (human double-minute 2) inhibits p53 function through inhibitory binding of p53 itself or facilitating its ubiquitinilation and successive degradation, via E3-ligase. Yet, HDM2 is amplified in a minority of astrocytomas and GBMs. $p14^{ARF}$ negatively regulates the HDM2 activity, thus p53 degradation. However, $p14^{ARF}$ gene is located on the chromosome 9p21 that indeed is frequently deleted or methylated in most of GBMs. Mutations in the pRB pathway are responsible for the unrestricted cellular proliferation of these tumors. Defects in the cell-cycle components are uncommon in low-grade gliomas, but as they progress in grade of maligannncy, tumors rapidly acquire molecular alterations that disrupt the pRB cascade. Specifically, loss of RB1 or amplification of CDK4 occurs in *secondary* gliomas, whereas deletions, or suppression by promotor methylation, of CDK inhibitors (CDKIs), $p16^{INK4A}$ and $p15^{INK4B}$ occur in *primary* GBMs. This kind of alterations, interesting the pRB pathway are found in parallel to the above discussed mutations of the p53 pathway in the majority of malignant gliomas (Figure 6B). Several growth factor pathways are also involved in the phenotype of malignant gliomas. These include epidermal growth factor (EGF), platelet-derived growth factor (PDGF),

insulin-like growth factor-1 (IGF1), hepatocyte growth factor/scatter factor (HGF/SF), vascular endothelial growth factor (VEGF) and transforming growth factor-β (TGF-β) *[Rich JN. 2004b].*

The mechanisms by which these pathway get activated are very important, because many interventions have been proposed and designed to target receptor activity at certain points (Table 1).

Target	Agent	Drug Class	Development stage in glioma
EGFR	Geftinib	TKI	II
	Erlotinib	TKI	II
	AEE788	TKI	I
	TP-38	Ligand-toxin conjugate	II
PDGFR	Imatinib mesylate	TKI	I/II
	SU6668	TKI	I
	MLN518/608	TKI	Preclinical
VGFR	PTK787/ZK222584	TKI	I
	SU5416	TKI	I

Table 1: Several clinical trials have been undertaken for adult and pediatric patients with recurrent malignant gliomas with target agents. Some of these agents are listed in the table above with their target and the stage of development *[Rich JN. 2004b].*

2.8 - Therapeutic strategies available: benefits and pitfalls

Generally, the first step in the treatment of brain tumors is surgery. With today's modern techniques, surgery is generally safe for most of the patients. The goals of surgery are: i) to remove as much tumor as possible; ii) to reduce the symptoms caused by the pressure that the tumor mass put on the normal brain parenchyma; and iii) to obtain tumor tissue for diagnosis and treatment planning. In some circumstances, such as certain medical conditions or concerns about the location of the tumor, a biopsy may be done in place of surgery. The tissue obtained during the biopsy is then used to make the diagnosis.

Surgical intervention to remove a brain tumor is carried out by making an opening in the skull over the tumor, what is known as a craniotomy. Brain mapping and functional MRIs help the neurosurgeon to determine vital region of the brain so as to avoid damages in these area during surgery. The surgeon can use stereotactic computerized equipment and image-guided techniques as

navigational tools, like a GPS (global positioning system) system. Those tools help to guide the neurosurgeon's access into some of the difficult or deep areas of the brain. Lasers may be also used during surgery to vaporize tumor cells. Ultrasonic aspirators are tools that break up and suction the tumor out of brain tissue. High-powered microscopes help the neurosurgeon to better see the tumor. Because the tentacle-like cells of astrocytomas grow into the surrounding tissue, these tumors cannot be totally removed.

Radiation and chemotherapy are then used to treat the remaining tumor. In adults, radiation therapy usually follows surgery. There are different types of radiation that may be given using various doses and schedules. Conventional fractionated external beam radiation is the "standard" radiation given 5 days a week for 5 or 6 weeks. A "fractionated" radiation could also be given in small doses at a time over several weeks. External beam radiation is actually the same radiation a patient is subject during a simple chest x-ray. Most forms of local radiation treat the tumor and its surrounding area. However, a sort of "local radiation" may be used to boost conventional radiation. Monoclonal antibodies may be capable of carrying radiation or drugs to the tumor site. Many of these radiation techniques are under investigation and are offered in organized testing plans called clinical trials.

Two main classes of chemotherapy drugs used in the therapy of brain tumors can be described: cytotoxic and cytostatic drugs. Cytotoxic drugs are designed to destroy tumor cells. They work by making tumor cells unable to reproduce themselves. Bis-chloronitrosoure, lomustine, procarbazine, cisplatin, temozolomide, and irinotecan are examples of cytotoxic drugs. Of this group, temozolomide is commonly used, along with radiation therapy, as a primary treatment for newly diagnosed glioblastomas. Cytostatic drugs are used to alter tumor behavior. These drugs work by changing the tissue in and around the tumor. There are several different types of cytostatic drugs. For example, angiogenesis inhibitors are cytostatic drugs that stop the growth of new blood vessels around a tumor. An example of angiogenesis inhibitors is well represented by the anti-VEFG-A bevacizumab. Differentiating agents, such as phenylacetate, are cytostatic drugs that make malignant cells look and act like normal cells. Sometimes, cytotoxic and cytostatic drugs can be combined in the attempt to obtain a synergic action. Researchers are also developing new ways of delivering drugs to the tumor. The convection-enhanced delivery (CED), for example, uses a pump to slowly "flow" chemotherapy drug or biologic substances into the tumor site. Another method uses a biodegradable wafer that is positioned into the tumor cavity after surgery that slowly releases a chemotherapy drug within the remaining tumor tissue. Other approach uses micro particles, which release drugs into the tumor at a pre-determined rate. Chemotherapy may be used in infants and very young children to delay radiation therapy until the age of three or four. Clinical trials are underway to evaluate the most effective ways of treating these tumors in infants and children.

Several drugs are also used to alleviate symptoms of brain tumors. Among them steroids are used to decrease swelling (edema) around the tumor. Anti-epileptic drugs control seizures. Anti-emetics prevent vomiting and help control nausea. Drugs to help treating fatigue or depression may be helpful as well.

Yet, the scientific community continues to look for new drugs to treat glioblastoma and other malignant brain tumor, and many drugs are under investigation. Some of them are new drugs, some are drugs proven useful in treating other types of tumors in the body, and still others are standard brain tumor drugs administered differently. However, because they belong to the chemotherapy drug class, they can affect normal cells and patients can expect side-effects from treatments such as hair loss or lack of appetite. For this reason the investigation towards more specific targeting drugs, able to accumulate in the tumor area only and to spare normal cells from their toxic effects, are desperately needed.

3. Targeted Tumor Therapy

3.1- General aspect

As previously discussed, a promising avenue towards more selective, better anti-cancer drugs relies on the targeted delivery of bioactive molecules (drugs, cytokines, pro-coagulant factors, photo/radio-sensitizers, radionuclides, etc.) to the tumor area by means of binding molecules (e.g., monoclonal antibodies) specific to tumor-associated antigens. In this way, the selective accumulation of drugs at the tumor site would spare normal tissues and lead to an increased therapeutic drug dose at the tumor site. This will bring to a higher treatment efficacy and less side effects for the patient. The same strategy, using ligands coupled to imaging agents (e.g., fluorophores), can also be useful for the diagnostic localization of tumor lesions by imaging analysis.

Even though the Paul Ehrlich's "magic bullet" concept was envisioned at the end of the 19th century, several technologies had to be developed before the selective delivery of therapeutic drugs to the tumor site could become a reality. Recent advances in protein engineering have made possible the generation of high-affinity human mAbs against virtually any biomolecular target. Furthermore, new technologies are becoming available for the generation of high-affinity binding peptides *[Collins J. 2001]*, aptameres *[Brody EN. 2000]* and synthetic organic molecules *[Erlanson DA. 2003; Melkko S. 2004]* that may be used as ligands for the development of targeted anti-cancer strategies. To note, there is a fundamental difference between therapeutic strategies aiming to the inhibition of the biological function associated to a molecular target and those strategies meant to delivery anti-cancer agents to the tumor lesion. Indeed, the term "target therapy" encompasses a wide variety of different approach, which can be mainly divided into *direct* and *indirect* approaches.

Direct approaches target tumor-associated or tumor-specific proteins to alter their signaling pathway either by mAbs binding to the relevant antigens or by small-molecule drugs that interfere with these proteins (approach also known as molecular targeting). These techniques are being already widely used in cancer treatment, especially in the case of vascular tumor targeting. The existing anatomical and physiological differences in the endothelium and surrounding stroma of tumors, compared to normal tissue, provides the basis for the development of vascular tumor targeting strategies. Tumor vasculature is often disorganized and tortuous, and presents different shunts (vascular tract in which blood passes directly from an arteriole to a venule) that make blood flow inconstant. The blood lacks of oxygen and nutrient resulting in a highly hypoxic and acidic microenvironment, which is under oxidative stress *[Brown NS. 2001]*. Endothelial cells respond

trascriptionally to all these stimuli and contributes to active angiogenesis in the tumor mass. The first mAbs approved in clinic was bevacizumab (Avastin, Genetech) that aim to inhibit the function of VEGF in colon cancer. Indirect approaches, on the other hand, rely on the identification of tumor-associated proteins expressed on the cells surface that serve just as a target device for binding of fusion proteins (ex. mAbs) chemically linked to different kinds of effector molecules. Ligand based tumor targeting approaches allowed to achieve excellent ligand localization in the tumor site, with tumor/organ ratios of >10:1 already a few hours after intravenous injection *[Carnemolla B. 2002]*. In this case, knowledge about the biological function of the target is not necessary and the therapeutic activity is left to the action of the drug itself. In this context the choice of the target results to be very important because the right choice can allow internalization of the construct or binding to the cell surface. However, one of the major challenges for these biomedical approaches consists of the identification of suitable antigens, which are specifically and/or abundantly expressed in the tumor environment, and which are accessible to intravenously injected drugs.

In this thesis I will refer to targeting only as the selective delivery of a molecular agent to a site of disease (indirect approach).

The research for novel targets and ligands is just as important as the development of strategies, which convert a ligand (capable of selective localization in the tumor site) into a therapeutic agent that retains the selectivity for the tumor. Several characteristics have to be considered in the selection of an ideal therapeutic target. First the target has to be present in the cancer cell and not in the surrounding normal tissues, then it must be a satisfactory pharmacological target - secreted molecules and cell surface receptors are accessible and often have well characterized sites of interactions with other molecules, activation states and mechanism of degradation.
Our laboratory was one of the first groups developing a novel methodology for biomarker identification, based on the high throughput proteomic analysis of tumor tissue that underwent a process of isolation of potentially accessible biomarker *[Castronovo V. 2006; Castronovo V. 2007]*. This methodology counts on the treatment of perfusable cancer-bearing organ or human surgical specimens, obtained after surgery from cancer patients, with a reactive ester derivative of biotin, which enables the covalent modification of proteins easily accessible from the bloodstream. This procedure eliminates from circulation by washing both blood cells and proteins, which could compete with the biotinylation reaction. As a result, accessible proteins carrying primary amino groups (e.g., unprotected exposed N-termini or lysine side-chains) are covalently modified with biotin. Due to the high affinity of streptavidin for biotin, biotinylated proteins can be efficiently

purified from total tissue extracts on streptavidin resin in the presence of strong detergents. Unbiotinylated proteins can be washed away, while the biotinylated proteins retained on the resin can be submitted to identification by mass spectrometric techniques. Without such a reduction of proteome complexity, the identification of these accessible antigens out of the total tissue extracts by proteomic methodologies might be extremely difficult due to the large amounts of intracellular proteins that would hide the protein of interest.

3.2 - Ligands for targeting applications

Different molecular species (such as pepties, small organic molecules and aptamers) are being considered for targeting applications. At present, however, monoclonal antibodies are the only clinically proven class of high–affinity binding molecules which can be generated against virtually any marker of disease. However, while the production of good-quality human monoclonal antibodies is by now an established experimental methodology in laboratories, which have large antibody libraries and are conversant with affinity-maturation procedures, the identification of suitable antigens whose inhibition provides a therapeutic benefit for the patient remains essential the important component of developing the antibody-based targeted therapy.

3.2.a - Antibodies

At present, monoclonal antibodies are the only general class of specific binding molecules that can be generated against virtually any antigen. Because of their binding affinity and long circulation time in blood, they are ideally suited to bind to membrane and extracellular proteins, some of which can be relevant for tumor progression. Monoclonal antibodies can potentially initiate immune responses, including complement-mediated or antibody-dependent cell-mediated cytotoxicity, and might also block crucial protein-protein interactions, including growth-factor receptor and integrin-ligand binding. Antibodies undergoing internalization have been armed with radioisotopes, including α-β-γ-emitters or toxins to initiate cell killing *[Rich JN. 2004b]*. The US Food and Drug Administration (FDA) approved 20 different monoclonal antibodies for therapeutic application of different cancers, among these: Herceptin *[Goldenberg MM. 1999]*, Mylotarg *[Sorokin P. 2000]*, and Avastin *[Ferrara N. 2004]*. The invention of hybridoma technology for the generation of monoclonal antibodies *[Köler G. 2005]*, followed by the identification of several disease–associated antigens, has stimulated plenty of pre-clinical and clinical studies for the development of

imaging and/or therapy molecules.

Different antibody formats show different pharmacokinetics and different tumor-targeting properties. Full IgG exhibit slow elimination from the blood and accumulate mostly in the liver. Rapidly clearing antibody fragments, like single-chain variable fragments (scFvs) are typically preferred for imaging application in nuclear medicine. However, intact immunoglobulins are preferred for many therapeutic applications, which rely on the antibody's ability to interfere with signaling events and to activate antibody-dependent cellular cytotoxicity mechanism or complement. The main limitation to the use of antibodies in brain cancer is their delivery; as large, adhesive proteins they cannot be delivered to intracranial tumors without either modulation of blood-brain barrier integrity, or local delivery through intracystic delivery or by the recently introduced convection-enanched delivery (CED), technique that deliveries compounds in a large area of the brain with the use of small-bore intracranial catheters.

3.2.b - Peptides

A large number of peptides have been suggested to be able of selective localization on tumor neovasculature. The somatostatin analogue - Sandostan® LAR® (octreotide, Novartis) - has been approved in Europe and USA for the imaging of neuroendocrine tumors *[Kowalski J. 2003]*. Several other agents are under development such as integrin-binding peptides with argnine-glycine-aspartate (RGD) motifs or bombesin* peptide analogs *[Chen X. 2006; Okarvi SM. 2003]*. However, RGD-containing peptides have so far yielded disappointing tumor/organ ratio. Novel technologies for the isolation of high-affinity binding peptides are currently available, but *in-vivo* stability of linear peptides remains a cause of concern *[Ahlskog J. 2006]*. On the other hand, regulatory peptides, binding to internalizing receptors, have been proven to be valuable for the imaging of neuroendocrine tumors and their metastases *[Behr TM. 2001; Breeman WA. 2001]*.

* Bombesin is a 14-aminoacid peptide originally identified from the frog skin. It has two homologs in mammals called Neuromedin B and gastrin releasing peptide. It has been reported as a tumor marker of lun, gastric cancer, and neuroblastoma.

3.2.c - Small organic molecules

Another class of compounds used as therapeutic agents in cancer treament consists of the small organic molecules. This constitutes the vastest class of drugs on the market. They have several advantages over biopharmaceuticals characteristics, namely oral bioavailability, easy manufacture, lack of immunogenicity and favorable tissue biodistribution properties. However, the isolation of high affinity small organic binding to protein antigens remains a difficult task *[Arkin MR. 2004]*, which often fails when the epitopes to be recognized do not contain hydrophobic pockets. An increasing amount of experimental evidence suggests that linking two or more organic compound together can increase affinity and specificity of the drug. In this way, exploiting the chelate effect, the molecule may recognize adjacent surfaces on the target protein *[Melkko S. 2006]*. Methods for identification of such drugs include *SAR by NMR* (structure activity relationship by nuclear magnetic resonance) and the recently developed *EDAC* (encoded self-assembly chemical libraries) *[Dumelin CE. 2006]*.

3.2.d - Aptamers

Besides mAbs and small organic compounds, aptamers (single-stranded nucleic acids capable of adopting a complex three-dimensional structure) are possible the only class of molecules from which specific binding molecules against a variety of target proteins can be isolated *[Nimjee SM. 2005]*. At 8-15 kDa, aptamers are intermediate in size between mAbs (150 kDa) and small peptides (1-5 kDa) and are slightly smaller that scFvs (25 kDa). Pegaptanib (MacugenTM, Pfizer and Eyetech) was the first aptamer to be approved by U.S. FDA for the treatment of age-related macula degeneration *[Doggrell SA. 2005]*. Aptamer technology allows the generation of large libraries of single-stranded DNA or RNA molecules, which can be panned for targeting antigen binding. The nucleic acids selected can be then amplified by PCR- based techniques.

3.3 - Effectors for antibody-based tumor therapy

As described before antibodies are the most investigated and used ligands for targeted tumor therapy. Indeed, they are very useful as ligands for targeting, while it is the effectors molecules physically linked to the antibody that perform the real therapeutic role. This section reviews briefly the different strategy that could be used for tumor-targeting.

3.3.a - Immunoglobulins

Antibodies in the intact IgG format can have a direct therapeutic function as a consequence of their antigen binding and neutralization (Avastin binds and neutralize the vascular endothelial growth factor (VEGF)). However, more often, part of the antibody's action relies on the activation of macrophages, neutrofils and natural killer cells (antibody-dependent cellular cytotoxicity ADCC) or the complement system (complement-dependent cytotoxicity), leading to the direct killing of the target cell by the immune system.

3.3.b - Chemotherapeutic drugs

The conjugation of the chemotherapeutic agent deoxorubicin to an internalizing antibody targeting adhesion molecules for leucocytes showed very good anti-tumor activity in mice but only poor efficacy in humans. Better results were obtained with the 100-1000 times more toxic maytansinoids or calicheamicins *[Lambert JM. 2005]*. Furthermore, it remains to understand whether antibody-drug conjugated to non-internalizing antigens could be used therapeutically, thanks to the use of cleavable linkers.

3.3.c - Toxins

Antibodies have been fused to different toxins that derive from plants or microorganisms, like ricin A, Saporin, Pseudomonas exotoxin or Anthrax toxin. However, clinical investigations revealed severe toxicity problems, such as vascular leak syndrome and immunogenicity, related to a high degree of humoral response in human. When the target antigen is expressed to some extent on normal cells in vital organs even normal cells will be killed, leading to complication. In order to overcome this problem, various strategies have been tried, such as the administration of immunosuppressive agents (cyclophosphamide) or site-directed mutagenesis to generate less immunogenic variants of the toxins. With the advent of human protein toxins, (ex. the human ribonuclease angiogenin) researchers may be able to circumvent the immunogenicity problem but this approach is still likely to rely on the use of internalizing antibodies *[Mathew M. 2009]*.

3.3.d - Radionuclides

Radionuclides have been extensively tested as toxic moieties for conjugation to antibodies. A therapeutic radionuclide is preferably a β-emitter with a half-life of several days. The path length of β-particles is several millimeters and therefore permits irradiation of several layer of cells, including poorly accessible cells or antigen-negative cells. The approval of (90Y)-ibritumomab (Zevalin, Biogen Idec) and ^{131}I-tositumomab (Bexxar, GlaxoSmithKline) two radiolabeled anti-CD20 antibodies for the radioimmunotherapy (RIT) of non-Hodgking's lymphoma, has recently revived the interest in this approach as an avenue for the selective eradiation of disseminated tumors *[Stern M. 2005]*. However, the problem of radioprotection put high difficulties in the development of RIT protocols. Börjesson and collegues described anti-tumor effects in incurable patients with bulky head and neck squamous cell carcinoma treated with a ^{186}Re-labeled humanized mAb to CD44v6 *[Börjesson PK. 2003]*.

3.3.e - Cytokines

Proinflammatory citokines, such as interleukin-2 (IL2) or tumor necrosis factor (TNF) have been used in the therapy of certain tumor for years. Due to their very small therapeutic window and their high toxicity, they are solely applied in tumors, which cannot be treated otherwise *[Fishman M. 2002]* or by isolated limb perfusion. Another member of this group, interleukin-12 (IL12) never found its way to routine clinical application due to its extremely high toxicity and even several death during phase II clinical trial *[Colombo MP. 2002]*. Anibody-cytokine fusion compound would allow pursuing a logical avenue for improving the therapeutic index of anti-cancer cytokines, whose clinical use is hampered by excessive toxicity *[Davis CB. 2003]*. The work reported by Carnemolla, and collegues represents a good example of the efficacy of these fusion proteins. Using a human recombinant single-chain Fv (scFv - L19) to the ED-B domain of fibronectine coupled with IL2, they demonstrated in vivo the possibility of targeting newly forming tumoral blood vessels in different mouse model of cancer such as teratocarcinoma, myeloma, small cell lung cancer, and colon cancer *[Carnemolla B. 2002]*.

3.3.f - Photosensitizers

Photodynamic therapy (PDT) is a promising approach for the treatment of superficially localized tumors. A limitation, however, is the lack of selectivity of photosensitizes that often results in severe toxicity. Photoimmunotherapy (PIT) exploits the conjugation of such compounds to mAbs. Recently published data report an encouraging tumor response in animal model of cancer *[van Dongen GA. 2004; Fabbrini M. 2006].*

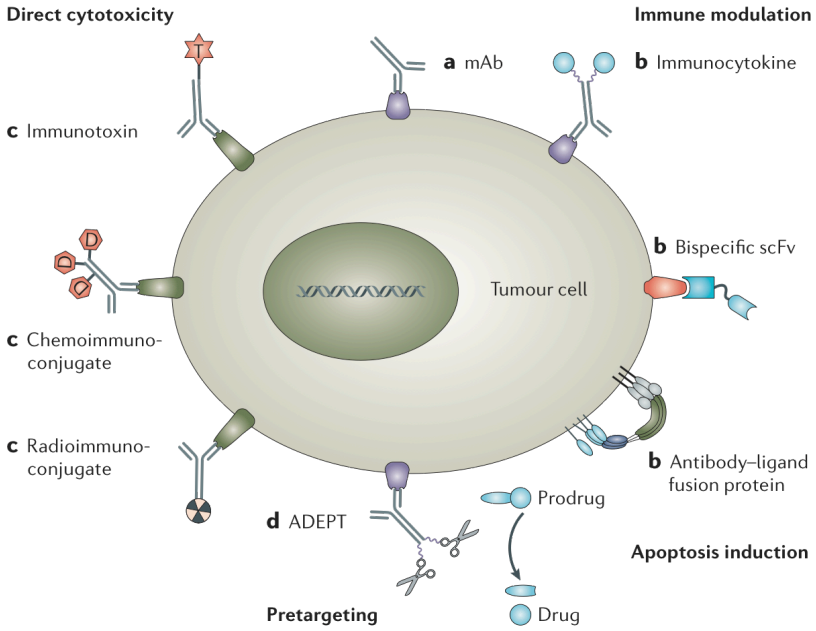


Figure 7: ***Concept of therapeutic Antibodies*** **– a)** Targeting monoclonal antibodies to the tumour can result in the destruction of the tumor cells by antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity. **b)** Similarly, targeting cytokines or immunomodulatory molecules either by bispecific scFv or antibody–ligand fusion proteins to the tumour modulates the immune response against the tumour. In addition, antibody–ligand fusion proteins can induce apoptosis to targeted cells as well as bystander cells by, for example, presenting FasL. **c)** A more direct approach to kill the targeted cell is the conjugation of cytotoxic drugs (D), toxins (T) or radionuclides to the monoclonal antibodies *[Wu AM. 2005].* The direct targeting approach requires the homogenous expression of antigen in the tumor cell population; depending on the radionuclides used, however, radio-immunoconjugates can exert bystander effects and kill surrounding cells which do not express the antigen. **d)** The antibody-directed enzyme prodrug therapy (ADEPT) approach specifically aims at causing bystander effects by targeting enzymes to the tumor cell and delivering a prodrug that is converted to a chemotherapeutic by the targeted enzyme *[Schrama D. 2006].*

4. Proteomics approaches for cancer biomarker discovery

4.1 – General aspect

The conclusion of the human genome project and concurrent explosion in expression array technologies led to unprecedented enthusiasm about the potential to understand and to treat cancer disease. Yet, genomic and transcriptomic studies yielded very precise and sensitive information about genes and proteins expressed in different cancer. However, as protein expression is also regulated at the translational level, the amount of a given transcript does not necessarily correlate with the corresponding protein level. For that reason, even thought studying gene expression levels is an important piece of the puzzle, the essential component in cancer research will be to investigate the real protein pattern expressed by cancer cells. This is a field known as proteomics *[Chuthapisith S. 2007].*

It was Mark Wilkins who first coined the term "Proteomics", between 1994 and 1997, during his permanence in Genève (Switzerland) as post-doc *[Wilkins M. 2009].* This term refers to the characterization of the complete set of proteins encoded by the genome of a given organism (proteome). Yet, the filed of proteomics involves not only the acquisition of data about the expression of proteins in cells and tissues, but also the analysis and the integration of these data with already existing knowledge, both in related and disparate fields.

The identification of new biological markers (biomarkers) represents an essential application of proteomic technologies in cancer research. New biomarkers can be helpful to predict patient prognosis, to monitor treatment response as well as be used as targets for the delivery of specific bioactive drugs to the neoplastic lesion, in the process of developing new, more specific, therapeutic protocols.

Prognostic biomarkers usually provide information about malignant potential of tumor and patient survival time, facilitating further treatment choice. Estrogen and Progesterone receptors (ER and PR), proliferation markers such as Ki67, markers of angiogenesis such as VEGF and growth factor receptors as HER-2/neu and p53 are currently used in clinic for this purpose *[Alaiya A. B. 2005].* Diagnostic markers as CEA *[LaBaer J. 2005]*, CA19-9 *[Steinberg W. 1990]*, cytokeratin 7 and 20 for gastrointestinal malignancy *[Rekhi B. 2008]*, and CA-125 for ovarian cancer *[Riedinger JM. 2006]* PSA for prostate cancer *[Hernàndez J. 2004]*, and thyroid transcription factor-1 for lung cancer *[Ordonez NG. 2000]* are commonly used to aid histopatological tumor diagnosis and classification, allowing for an optimal treatment choice. Additionally, screening biomarkers are used to detect early stage disease with high sensitivity and specificity. The marker that has probably

been most widely used for cancer screening is prostate-specific antigen (PSA) as a screen for prostate cancer.

However, early detection is only helpful if a therapeutic intervention can be undertaken when early stage disease has been diagnosed, resulting into an improved survival and better outcome for patients. As discussed previously, the most recent and promising therapeutic strategy to cure cancer consists on the systemic administration of drugs coupled to specific ligands (e.g. monoclonal antibodies "mAb") able to reach proteins exclusively present in the tumor cell or in its surrounding stroma. Yet, the bottleneck for developing such treatments consists on the identification of specific targets that are easily reachable by a systemically administered mAb-linked drug. In this field the use of proteomic technologies plays a key role. Investigation for cancer biomarkers using high throughput proteomic approaches have become possible due to the wealth of data gained from the human genome project and to the technological advances made in mass spectrometry and separation techniques. One of the first high throughput proteomic approach consisted on the use of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Introduced thirty years ago, and still used in many research laboratories, 2D-PAGE (Figure 8) put the basis of modern proteomics. Generally, the whole protein extract coming from cell culture, tissue sample or biological fluid is separated in a 2-dimensional gel (2-D PAGE), where the first dimension is dependent on the protein isoelectric point (the pH at which a molecule carries no net electrical charge) and the second on the molecular mass of the protein. Spots of interest, for instance proteins whose expression is significantly increased or decreased between normal and cancer condition, are then excised from the gel and identified using mass spectrometry (MS) analysis. The main limitation of this approach lays on the resolution power of the electrophoretic separation, which reflects in a lack of sensitivity. Indeed, many proteins (specially cancer biomarkers) are expressed at such low levels that they might be hidden by abundant protein (serum proteins) thus escape MS detection *[Veenstra TD. 2006]*.

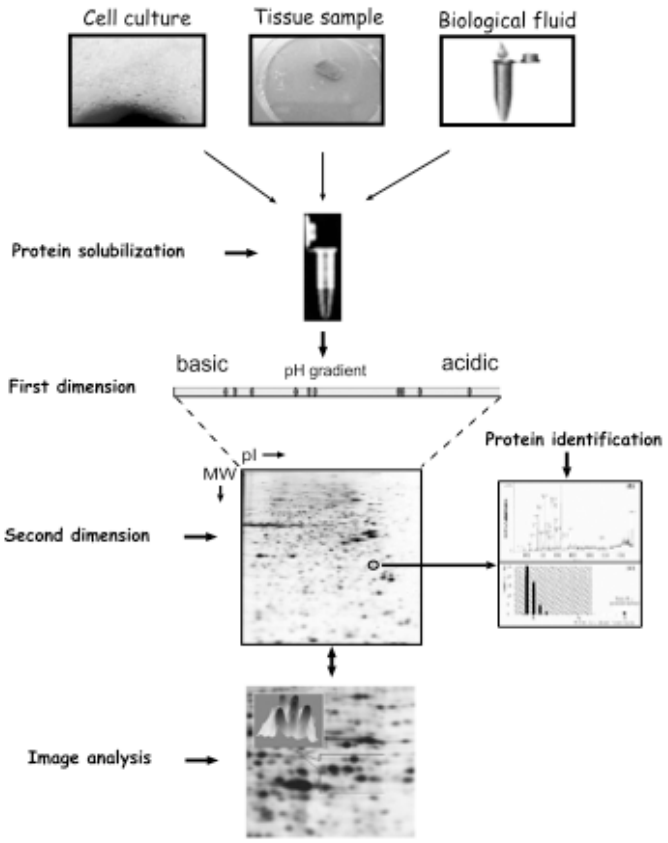


Figure 8: Schematic representation of a classic 2-D PAGE experiement *[Veenstra TD. 2006].*

4.2 - Mass Spectrometry (MS)

The development of the first mass spectrometer is attributed to J.J. Thomson, who, at the beginning of the 20th century, measured the m/z ratios of several atoms and small molecules *[Thomson JJ. 1913].* In the first half of the 20th century, improvements in ionization methods and analyzers occurred with the parallel application of mass spectrometry in the field of organic chemistry for the elucidation of chemical structures. However, it was not until the beginning of the 1990's that the field of biological mass spectrometry became significant. This was due to the introduction of soft ionization methods, such as Matrix Assisted Laser Desorption Ionization (MALDI) by Tanaka, and Karas and Hillenkamp, and ElectroSpray Ionization (ESI) by Fenn that allowed the analysis of macromolecules as proteins and peptides *[Zhang H. 2004].* Over the past 20 years, MS has undergone significant improvement with the development of more sensitive, accurate and high-resolution techniques. Moreover, the introduction of more advanced separation techniques such as high performance liquid chromatography (or High-Pressure Liquid Chromatography - HPLC) and the possibility to interface the latter directly with the ESI ionization source allowed to

increase the purity of the compounds that are introduced into the MS, allowing for a better protein identification.

The continuous advances in the field of biological mass spectrometry has now made it possible to routinely identify proteins from the corresponding endoproteolitic peptides, at total protein amounts within the femtomole range. In the majority of proteomics studies, the complex protein mixture is digested into small peptides, normally using enzyme such as trypsin, prior to MS analysis *[Rappsilber J. 2002].* The advantage of this digestion step is not only due to the higher solubility of peptides in solution, with respect to intact proteins, but also because of the accuracy of current mass spectrometers that, even though is very high, is still not sufficient to confidently identify intact proteins *de-novo* based exclusively on their molecular weight. Consequently, proteins are typically identified through peptides acting as surrogates for their parent protein. The resulting spectrum obtained from such a sample is reported as a *peptide map* or a *peptide fingerprint* (Figure 9). Protein identification is achieved by comparing the collection of measured masses to an *in-silico* peptide maps derived from a protein or genomic database. This process is known as *bottom-up* approach.

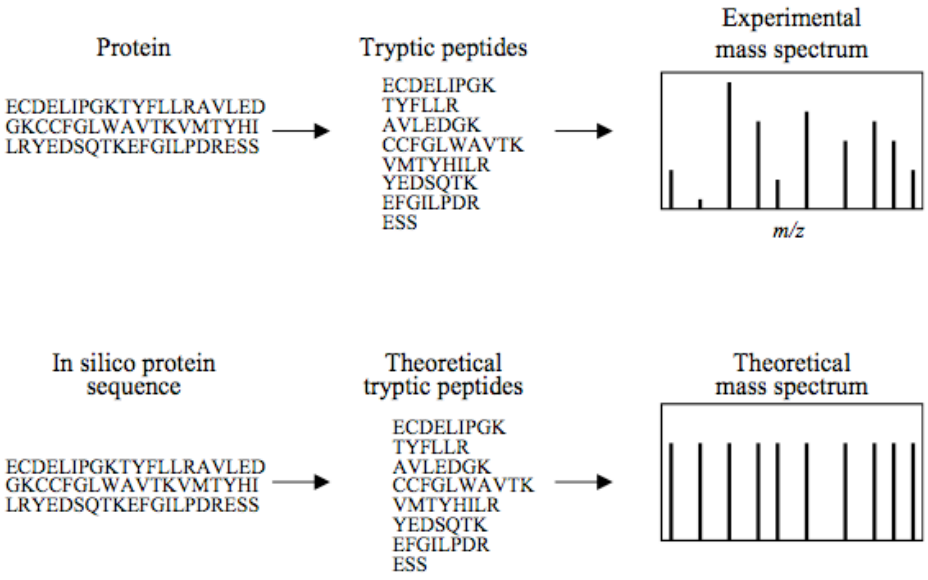


Figure 9: Example of protein identification by MS analysis. Prior to injection in the mass spectrometer, proteins are digested into small peptides using trypsin. The measures of the masses of the different preptides results in a spectrum, which is usually called *peptide fingerprint*. Definitive protein identification is achieved by comparing the collection of measured masses to an *in-silico* peptide fingerprint derived from a protein database.

Furthermore, the technological evolution in the proteomic field led to the introduction of the tandem mass spectrometry or MS/MS (Figure 10). Instead of relying on the real mass of a specific peptide, individual peptide ions can be isolated and fragmented by collision-induced dissociation (CID). After fragmentation of the peptide, the masses of the fragmented ions are recorded and used to obtain partial or complete sequence information. When peptides are subjected to MS/MS, they are not completely sequenced into their constituent amino acids, but rather a collection of various lengths fragments of the same peptide is obtained. This information provides *sequence ladders* that enable partial primary sequence information of the peptide to be deduced. The raw data obtained are then analyzed using bioinformatic tools that can compare the experimental data to in-silico MS/MS mass spectra calculated from the protein sequences in the database *[Veenstra TD. 2006].*

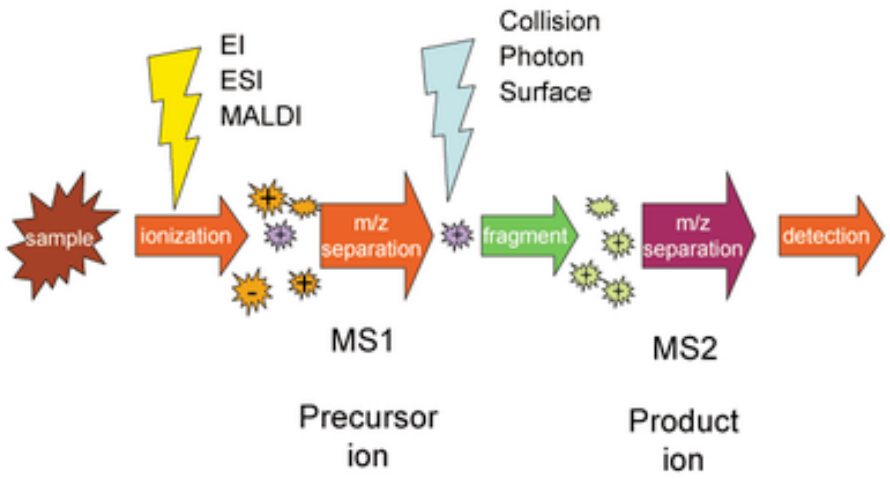


Figure 10: Schematic representation of tandem mass spectrometry (MS/MS). The sample undergoes a primary MS analysis after having be charged by the ionization source. Then one or several masses (usually the three most intense) are chosen, isolated and fragmented again to undergo a second MS analysis. This approach provides the *sequence ladders* that are then compared with *in-silico* database to achieve protein identification. (Image from the web)

4.3 - How does mass spectrometers work?

A mass spectrometer is defined as an instrument capable of measuring the mass-to-charge (m/z) ratios of molecules. It is generally made up of three devices, namely i) an ionization device, ii) a mass analyzer and iii) a detector. It is within the ionization source that the sample of interest is ionized and then desorbed into the gas phase. The mass analyzer acts to guide the gas-phase ions through the instrument to the detector. At the detector, the ions m/z ratios are measured. Sample

inlet and data output recorders are needed, in addition, but they are not part of the mass spectrometer as such.

The functioning of a mass spectrometer can be explained as such: the electromagnetic field is able to deflect the trajectory of charged particles, and since the kinetic energy imparted by motion through an electromagnetic field gives to the particles an inertia dependent on the particle's mass, the mass analyzer can use this features to steer certain ions to the detector, based on their m/z ratio, by varying the electromagnetic field. The magnetic field can be also used to narrow the mass range (i.e. to select peptides of interest for tandem mass spectrometry [MS/MS]) or to scan through a range of masses to catalogue the ions present (survey scan). There are many different kinds of mass spectrometers, which are described generally by the types of ionization sources, mass analyzers, and detectors that are used. The most commonly used ionization sources for biological molecules are Matrix-Assisted Laser Desorption/Ionization (MALDI) and Electro-Spray Ionization (ESI) *[Merchant M. 2000; Mann M. 2001]*. Other ionization methods, which, however, will be not discussed in this thesis, include Fast Atom Bombardment (FAB), Chemical Ionization (CI), Thermal Ionization (TIMS), Secondary Ionization (SIMS) and Plasma Desorption (PD). All of these ionization methods can detect within the picomole to femtomole range, key feature required for the analysis of biological samples. Examples of mass analyzers are quadrupole, time-of-flight (TOF), ion trap (IT), ion cyclotron resonance (ICR), orbitrap and magnetic sector instruments. The first three are the most commonly used analyzers in biological mass spectrometry, but there numerous combinations of mass analyzers exist in the so-called hybrid instruments. There are several ways to detect ions. Routinely information are recorded when an ion hits the detector plate, such as Multi Channel Plates, or MCP. As ions hit the plate a cascade of electrons is released, amplifying the single ion detection. This flow is called *image current* and can be detected and amplified. When a scan is conducted in the mass analyzer, the charge induced in the detector during the course of the scan will produce a mass spectrum, which represents a record of the m/z values at which ions are present and their relative intensities (Figure 11). These spectra are then analyzed to produce the most probable list of proteins that could have been the source of the particular set of peptides. One of the difficulties inherent to high-throughput proteomics is filtering the peptide assignments to derive a list of highly likely correct identifications. In small experiments, an expert in MS can examine the spectra and determine which peaks are likely to lead to robust identifications. Because of time and personnel constraints, this method is infeasible for larger experiments *[Veenstra TD. 2006]*.

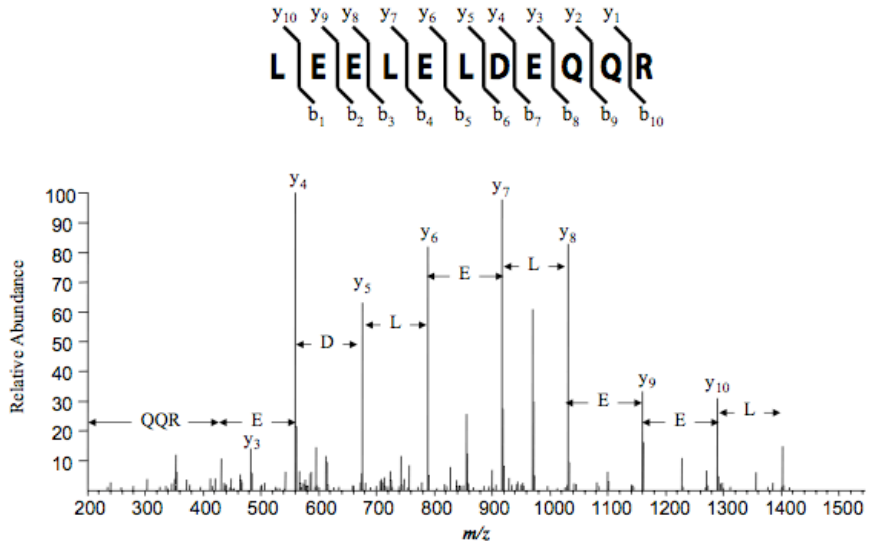


Figure 11: Example of a peptide fingerprint obtained after the analysis of a complex peptide mixture using an HPLC system coupled to an ESI-MS/MS spectrometer. The *m/z* values and the signal intensity produced by each ion are represented respectively in the X and Y axis. The analysis of the distance between the different peaks allows the determination of the amminoacidic sequence *[Veenstra TD. 2006]*.

4.4 - Building up a proteomic experiment

In order to ensure consistent sample collection, preparation and analysis between and among the study and the control samples, any proteomic experiments requires a careful planning of its three major components: sample preparation, protein separation and protein identification.

4.4.a - Sample preparation

Before proteins can be separated, the sample must be first collected and prepared. Although not commonly discussed the sample isolation and preparation is a critical factor in a proteomic experiment. The central principle of most experiments is to keep constant all variables between the different conditions to investigate, except for the particular compound being examined. As the high sensitivity of the mass spectrometer, bias is a significant risk to validity in all proteomic studies, and it can often be explained by errors or inconsistencies during sample preparation. If samples are handled differently, this introduces noise into the signal for one of the compared conditions making very difficult, if not impossible, post-processing or post-experimental data manipulation to validate the results. Some important steps in preparation include types of collection tubes, the time from collection to spinning and/or freezing, difference in temperature, number of freeze/thaw cycles and/or other factor involved in the analysis on the mass spectrometry instrument.

4.4.b - Protein separation

Separation of proteins is a key step in any proteomic experiment. The greater separation is achieved prior to protein or peptide identification, the greater is the resolving power of the analysis. Common methods of protein separation include one- and two-dimensional electrophoresis and liquid chromatography. While one-dimensional electrophoresis separates proteins only based on their molecular weight, two-dimensional electrophoresis separates the sample first based on the isoelectric point (pI – the pH at which a molecule carries no electrical charge) followed by a separation based on the protein size. Gel-based separation methods are rapidly becoming replaced by methods that involve peptide separation by liquid chromatography (LC) techniques linked to a mass spectrometer. The most basic form of LC involves separation of digested peptides through a C18 resin. A more complex and accurate separations **(used to perform the current study)** can be achieved through the use of other *on-line* methods, such as a cation exchange resin coupled with a C18 column (multi-dimensional protein identification technology, MudPIT). These separations can also be run ”off-line”, that means not in series with the mass spectrometer.

4.4.c - Peptide and Protein Identification

Protein identifications are almost always achieved through mass spectrometry. A mass spectrometer consists of an ion source, a gas analyzer that can measure the mass-to-charge ratio (m/z), and a detector that can measure the number of ions at each m/z value. The two techniques commonly used to volatilize and ionize the proteins and peptide source are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). Because ESI ionizes the peptides right out of a solution, it is easily coupled to liquid-based separation techniques, such as HPLC. MALDI, on the other hand, sublimates and then ionizes the samples out of a solid crystalline matrix and is therefore suited to more relatively simple peptide mixtures.

The mass analyzer measures the mass-to-charge ratio of the ionized compounds. There are four main types of mass analyzers: the ion trap, time-of-flight, quadrupole, and Fourier transform ion cyclotron (FT-MS). While ion traps are relatively inexpensive and sensitive, a disadvantage is their low mass accuracy. On the other hand, the FT-MS captures ions under high vacuum in a high magnetic field and has high resolution, mass accuracy, sensitivity and dynamic range. Nevertheless, the expense and operational complexity of the FT-MS has slowed its acceptance.

4.5 - Electrospray Ionization

In contrast to MALDI, ESI ionizes molecules from a liquid phase. The compound is dissolved in an organic solvent mixture, typically methanol or acetonitrile, containing a small concentration of acid (e.g. formic acid 0.1-1%). The introduction of the sample into the mass spectrometer can be carried out by a number of methods. In the simplest case, sample is directly infused through a syringe and a narrow transfer capillary. Another example is the nanospray sample delivery method. In this system, a small amount of sample is placed into a needle, which has a very small tapered opening on one side. Sample is forced out because of capillary forces and high voltage is applied to the needle. The most commonly used method is the coupling of the electrospray directly with reverse phase chromatography. In this setup the capillary end of the chromatographic system is connected to the needle to which the voltage is applied. Typical flow rates of 200 to 500 nL min-1 (nanospray) are used for the chromatography. A fine spray of charged droplets emerges from the capillary and is directed into the vacuum chamber of the mass spectrometer through a small orifice. An electrostatic field is formed between the capillary and the walls of the mass spectrometer, and as the droplets travel they evaporate resulting in the formation of gas-phase ions. The magnitude of the charge-repulsion effect becomes more significant, and at a certain charge/solvent composition (termed the Rayleigh limit), Coulomb explosion of the analyte-solvent clusters occurs. The clusters become smaller and more highly charged within the skimmer region until single molecular ions are formed either by further explosion of clusters or by desorption of molecular ions from the clusters. ESI instruments are usually coupled to ion traps.

The charged ions are accelerated through the analyzer towards the detector. These ions can then be analyzed according to their *m/z* ratio.

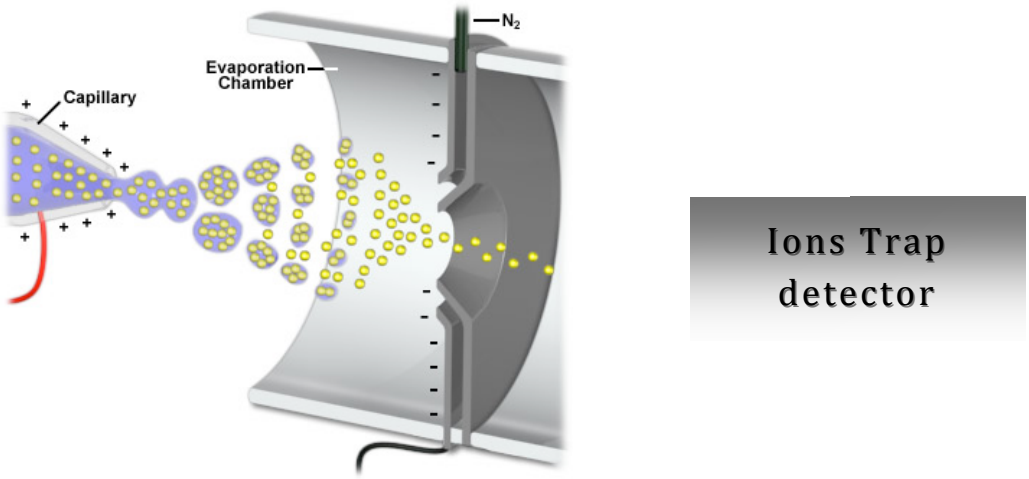


Figure 12: Schematic representation of electrospray ionization. The sample is delivered through a capillary and a tapered needle. Through a high voltage, droplets are extracted. Due to the evaporation of solvent, the charge repulsion reaches a critical value (The Rayleigh limit), when droplets explode (Coulomb explosion), creating multiply charged ions. These enter the mass spectrometer under the applied electrical field.

Section II - Aim of the work

Glioblastoma Multiforme (GBM) is the most aggressive, highly invasive and neurologically destructive among malignant brain tumors. Any of the actual treatments currently used is effective, making GBM lethal within 12 months from diagnosis. The identification of new biomarkers suitable for developing an antibody-based therapy provides fertile ground for novel therapy and new hope for patients. The rationale for the use of antibody-linked drugs is based on the presence, in or on the surface of cancer cells, of antigens that are not detectable on normal cells. Indeed, the eligibility criteria for a good biomarker are: i) to be expressed on the membrane of tumors cells or in their surrounding stroma; ii) to be accessible to the specific monoclonal antibody systemically administered *via* blood stream; iii) to be **not** expressed (or **not** accessible) in normal cells of different tissues.

Consequently, aim of the current work was to identify new potentially accessible biomarkers of human GBM with special emphasis on the following:

1. Accessible stroma-associated biomarkers expressed by the host tissue undergoing alteration as a result of cancer development.

2. Accessible cancer-associated biomarkers.

Additionally, we aimed to develop a method of intravenous administration of specific mAbs to a chicken model of GBM that could be useful for an initial *in-vivo* validation of the biomarkers identified in the discovery phase of the study.

Section III - Material & Methods

5. Material and Methods

Unless otherwise specified all the reagents used in the present study were obtained from Sigma Aldrich Co. (St. Louis, MO, USA). The *in vivo* experiments were performed on the basis of Authorization LA1230509 of the Animal Ethics Committee of the Federal Department of Health, Nutritional Safety and the Environment (Belgium).

5.1 - Tissue harvesting

5.1.a - Human specimens

Surgical removal of 5 human glioblastomas was performed according to the standard care protocol at the neurosurgery department of Pisa hospital. After the surgery the tissue was sent to the pathology department and a small fresh part (not strictly necessary for diagnosis) was kept for our experiments. Diagnostic procedures were never influenced during tissue harvesting.

5.1.b - Cell culture and mouse model of GBM

The human U373 (ATCC code HTB-17) and T98G (ATCC code CRL1690) GBM cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained as detailed previously *[Belot N. 2001; Branle F. 2002]*. Briefly, cells were cultured at 37°C in Falcon plastic dishes (Gibco, Nunc, Belgium) containing Eagle minimal essential medium (MEM; Gibco, Nunc, Belgium) supplemented with 10% fetal calf serum (FCS), a mixture of glutamine (0.6 mg/ml final concentration (FC); Gibco, Nunc, Belgium), penicillin (200 IU/ml final concentration; Gibco, Nunc, Belgium), streptomycin (200 IU/ml FC; Gibco, Nunc, Belgium) and 0,1 mg/ml gentamycin (Gibco, Nunc, Belgium). The FCS was heat inactivated for 1h at 56°C. Nude mice xenografts were obtained by grafting 1 million/8µl of either T98G or U373MG cells into the left temporal lobes (0,5 mm posterior, 2 mm left to the Bregma, 3mm depth) of 8-weeks old female nu/nu mice (collaboration with Pr. R. Kiss, Bruxelles University). A set of 6 mice were injected with each of the 2 cell lines while 6 more were injected with PBS and used as normal control. All mice in a given experiment had cells implanted into their brains left temporal lobe on the same day. Mice were then sacrificed 5 weeks after the graft and left hemispheres were collected for biotinilation.

5.1.c - Chorioallantoic membrane (CAM) assay

U87 CAM-derived tumors were grown up according to the protocol described by Hagedorn ad colleagues *[Hagedorn M. 2005]*. Briefly, at the embryonic developing day (EDD) 4 a small window was opened in the shell of white chicken eggs (gallus gallus) to expose the CAM. The window was successively sealed with clear tape and eggs were immediately replaced into the incubator set at 37° C - 80% humidity for 7 more days to allow CAM development. At EDD 11 the window was reopened a plastic ring was placed on the CAM, and a suspension of 5 million U87 glioma cells in 15µl volume was gently deposed on the membrane after a soft scratch of the surface. Embryos were scarified at EDD day 18 and tumors were collected for further analysis.

5.2 - Tissue biotinilation and protein extraction

The biotinilation procedure was performed as detailed previously *[Castronovo V. 2007]*. Briefly, tissue samples were sliced and soaked into freshly prepared EZ-link Sulfo-NHS-SS biotin (1 mg/mL, Pierce, Thermo Scientific, USA) in PBS (pH 7.4) at 37°C for 20 minutes. This was followed by a 5 min quenching step using 50 mM Tris-HCl (pH 7.4) at 37°C. The samples were then rinsed with PBS (PH 7.4) and snap-frozen in liquid nitrogen. Following this, 100 mg of tissue were grinded using a mikro-dismembrator (B. Braun Biotech, Melsungen, Germany) and dissolved in 1 mL of lysis buffer (PBS 50mM, NaCl 0,5M, NP40 1%, DOC 0,5%, SDS 1%) containing a protease inhibitor cocktail (Protease Inhibitor Cocktail 100x, Thermo Fisher Scientific, Waltham, USA) and not oxidized glutathione to preserve the disulfide bound of the Sulfo-NHS-SS biotin reagent. Subsequently, the samples were sonicated (2×30'') using a 2 mm microprobe (Vibra-Cell 75022, Bioblock Scientific, Illkirch, France). Finally, protein concentration was determined using the BCA protein assay reagent kit (Pierce, Thermo Fisher Scientific, Waltham, USA). In order to isolate the biotinylated proteins, the necessary amount of streptavidin–sepharose resin (Amersham Biosciences, 150 µL/mg of total protein extract) was equilibrated by five washes in buffer A (1 % NP40 and 0.1 % SDS in PBS 50 mM). Following this, the total protein lysate and streptavidin resin were mixed together and incubated for 2h at RT under constant rotation. After the incubation, the unspecific binding was reduced by washing the resin four times with buffer A, four times with buffer B (0.1% NP40, 1 M NaCl in PBS), twice with buffer C (0.1 M sodium carbonate in PBS, pH 11) and once with a solution of NaCl (0.5 M) in PBS (50 mM). The biotinylated proteins were eluted using 100 mM DTT solution (2×30' at 60°C), 150 mM alkylated with iodoacetamide (30' at RT in the dark) and finally precipitated with 20 % trichloroacetic acid (over night at 4°C). The protein pellet was washed twice with pure pre-chilled (-20 °C) acetone and allowed to dry for 5 min

at RT. Finally the pellet was dissolved in 200 mM ammonium bicarbonate buffer and the pH was adjusted at 8.0. Protein concentration was determined using the BCA protein assay. For the mass spectrometry investigation, the dissolved proteins were digested with trypsin (1/50, protease/ protein) over night at 37°C followed by second digestion using fresh trypsin (1/100) for additional 4 h. Prior to injection in the nanoHPLC system, a quantity corresponding to 5 µg of peptides were desalted using ZipTip® (Millipore, Billerca, MA, USA) according to the manufacturer's instructions. Subsequently, the peptides were lyophilized and dissolved in 20 µL water containing 0.1 % formic acid.

5.3 - Mass Spectrometry analysis

The peptide containing samples were analyzed on the 2D-nano-HPLC system Ultimate 3000® (Dionex, Sunnyvale, CA, USA). The HPLC system was connected on-line to the electrospray ion-trap mass spectrometer Esquire HCT ultra® (Bruker Daltonics, Bremen, Germany). For the MS analysis, approximately 5 µg of digested protein sample was loaded onto the Bio-X-SCX column (500 µm i.d. x 15 mm; Dionex, p/n: 161395). Subsequently, 4 different concentrations of salt injection were performed (45, 75, 150 and 500 mM ammonium acetate). After each salt injection, the eluted peptides from the SCX column were trapped on a C18 pre-column (Acclaim PepMap©, 300 µm i.d. x 5 mm; Dionex, p/n: 160454) and desalted for 5 minutes at a flow rate of 30 µL/min using solvent A (97.9 % water, 2 % acetonitrile and 0.1% formic acid). Following this, the peptides were separated on the C18 analytical column (Acclaim® 75 µm x 150 mm; Dionex, p/n: 162224) using a 140-minute solvent gradient (t = 0 minutes, 0 % B [B: 80 % acetonitrile, 19.9 % water and 0.1 % formic acid]; t = 140 minutes, 40 % B) at a flow rate of 0.3 µL/minute. The MS scanned the mass range from 200 to 1600 m/z. The 4 most intensive peptides found in this mass range (bearing +1, +2 and +3 charges) were selected automatically and fragmented in the MS/MS modus (m/z range 100-2500).

5.4 - Spectra deconvolution and data processing

The acquired spectra from all the samples were deconvoluted with the Data Analysis® software version 3.4 (Bruker Daltonics, Bremen, Germany). The MS/MS database search was conducted using the Mascot® search engine version 2.2.2 (Matrix Sciences, Boston, MA, USA). The human non-redundant and non-identical protein database Swissprot® (Swiss Institute for Bioinformatics, Basel, Switzerland) version 57.7, filtered for the taxonomy *Homo Sapiens* or *Mus*

Musculus, was used. The mass tolerances of precursor and fragmented ions were set at 0.6 and 0.3, respectively; fixed modifications were carbamidomethyl; variable modifications were oxidization of methionines. Trypsin cuts before prolines and one missed cleavage were allowed. A cut-off score value of 30 was used to eliminate poor MS/MS spectra. This cut-off value assured that all the high-intensity MS/MS fragments were assigned to a specific amino acid in the peptide sequence. In order to ensure this, the MS/MS spectra bearing a score below 30 were per default manually checked (conducted only for the proteins of interest).

In the study with the animal model a semi-quantitative analysis was performed for the proteins that were identified in both tumoral and normal samples and in more than one sample. Accordingly, the peak-list files from all the fractions were combined into one file for each individual and disease state. These combined files were submitted to the Swissprot® database using the Mascot® search engine. The results yielded protein identifications along with the exponentially modified protein abundance index (emPAI). The emPAI is calculated using the following equation:

$$emPAI = 10^{(Nobsd/Nobsbl)} - 1$$

where *Nobsd* is the number of observed and *Nobsbl* the number of observable peptides per protein. Ishihama and colleagues showed that the emPAI value is directly proportional to the protein quantity *[Ishima Y. 2005].* Abundance ratios (protein emPAI tumoral vs. normal) were calculated for each individual animal separately. Only proteins displaying scores above 100 were considered for the emPAI quantification. Following this, a mean value was calculated including the standard deviation of means. The normalization was conducted assuming the Gaussian distribution of the data, where the maximum number of the proteins has an emPAI abundance ratio of 1.0. Specifically, a median value was calculated and all the ratios in a given sample were divided with this value. Proteins not eligible for emPAI quantification (scores lower than 100 and/or not present in at least two animals) were screened for the frequency of presence or absence in all the samples. Those proteins that were identified more frequently in the tumoral conditions rather than in the normal counterpart were also considered as potentially modulated.

5.5 - Determination of protein origin

For the reasons of genetic proximity between the two species (Homo Sapiens and Mus Musculus) it was not always possible to discriminate mouse from human proteins. Using a mammalian database search it was however possible to distinguish three protein groups: i)

identified uniquely as mouse, ii) identified as human and those which iii) were reported as both human and mouse identification. The latter group was the point of major interest, as it was important to determine if some of those proteins had peptides that were unique to the mouse. The analysis therefore consisted of examining the sequence coverage with respect to the mouse or human canonical protein sequence. Those proteins displaying i) more sequence coverage in the mouse sequence and had also ii) unique mouse peptides were considered as host produced (mouse) proteins. Differences in the amino acid sequence were confirmed by *de-novo* sequencing of the MS/MS spectra.

5.6 - Immunohistochemistry

The polyclonal anti-SAP (dilution 1/200 – *ProteinTech Group, Inc – Chicago, USA*) and anti-SPRL1 (dilution 1/50 - *R&D systems, Minneapolis, USA*) antibodies were used to assess protein expression by immunohistochemistry analysis in tumor bearing mouse brain tissues (both U373 and T98G) and human glioma (all grades) as well as normal brain tissues. Monoclonal anti-CD44H *(dilution 1/100 - R&D systems, Minneapolis, USA)* and anti-COL6α1 *(dilution 1/200 - LifeSpan BioScience)* antibodies were used to assess protein expression in human glioma TMA and for intravenous Abs injection of chicken embryo (following paragraph)**.** In total, 4 animals per tumoral/normal condition as well as 14 glioblastoma multiforme, 8 G3, 8 G2 as well as 7 G1 astrocytomas including 5 normal adjacent tissues were evaluated.

Five μm sections were sliced from paraffin blocks, unparaffined 2 times in xylene for 5 min and hydrated in methanol gradient (100%, 95%, 70%, and 50%). Blocking unspecific peroxidase was performed for 30 minutes with 3% H_2O_2 and 90% methanol. Following all antibody incubations, slides were washed with PBS for 10 minutes. The incubation with the primary antibody was performed overnight at 4°C. Following 30 minutes incubation Slides with the biotinylated secondary antibody, slides were incubated with the avidin biotin complex kit (ABC kit) for an additional 30 minutes. 3,3'-diaminobenzidine tetrachlorhydrate dihydrate (DAB) with 5% H_2O_2 was used for colorization. The slides were counter-stained with hematoxylin.

5.7 - Intravenous injection of chicken embryo

Intravenous injection was used to introduce a compound into the chicken's circulatory system. This approach results technically very challenging, however, the small time-window

needed to grow up a tumor and the lack of excretion in the chick embryo system provide an advantage over other in vivo systems.

In order to locate suitable blood vessels for the injection, eggs were candled at EDD 17 by directing a light ray down their major axis. Traces of the vessel-branches were drawn on the shell using a pencil, and an electric drill was used to allow the shell to be cut out. Few mineral oil drops were used to remove the pre-cut shell and to prevent the rupture of the underlying membrane. If so, in fact, a huge hemorrhage can impair embryo viability, impeding the experiment. Improved resolution of the blood vessel was achieved by top-lighting the egg during the injection through the previously done upper window. This was very important, since there is very little tactile resistance when injecting a CAM and the injection relies almost completely upon visual cues. Once the vessel was free of the shell the egg was placed onto an appropriate support in order to maintain a stable position. Successful injection was achieved using a 29-gauge bevel needle on a 1ml syringe, which was placed on a micromanipulator (Cat. Number: MD4L - World Precision Instruments – Sarasota, USA). The micromanipulation system was used to minimize any movement of the needle during the injection, hence minimizing the risk of hemorrhage. The injection was generally done "with flow", resulting in a transient clearance of the vessel. After the injection the small bleeding was blocked using a cotton bud placed on the scar for few seconds, and the egg was replaced into the incubator.

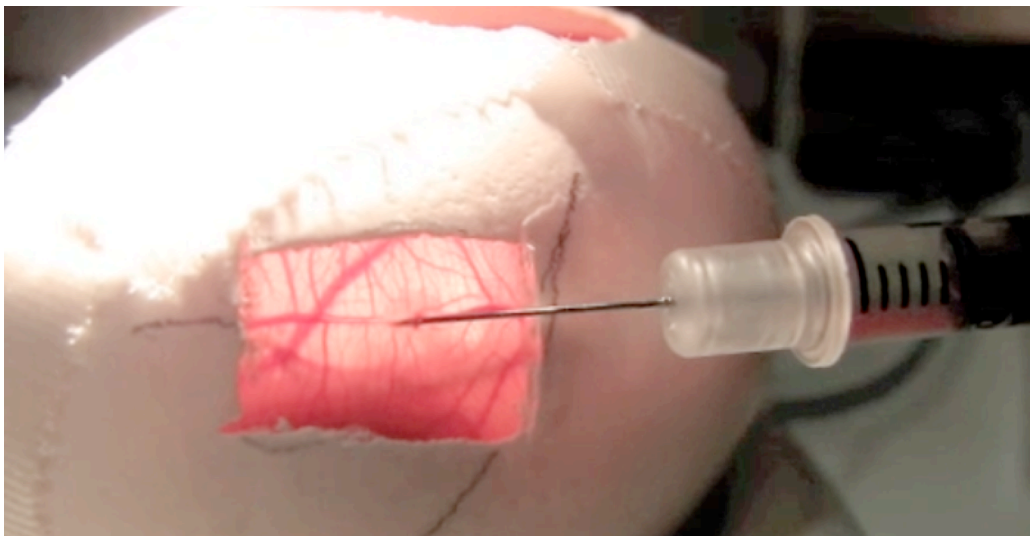


Figure 13: Representative picture taken during the *i.v.* injection of a compound into the chicken's circulatory system. As visible in the immage the "with flow" injection produced a transient clearance of the vessel.

5.8 - Immunofluorescence

Direct immunofluorescence analyses were performed on U87-derived tumor whose embryo, that allowed tumor development, had received an intravenous injection of a specific antibody. At EDD 18 embryo were sacrificed and the U87-derived tumor was fixed with PAF 4% for 30' at R.T., rinsed with PBS, included in tissue TEK and then frozen at -20°C. Five μm thickness frozen sections were cut and further fixed in MetOH 80% Acetone 20% solution for 10' and unspecific reaction was blocked by incubating the slide with BSA IgG free 1% in PBS for 1h. Afterwards slides were incubated with 1/500 Alexa-488 conjugated antibody dissolved in PBS-BSA IgG free 1% at 4°C over night. After extensive washing with PBS and a wash with Millipore® H_2O, slides were mounted using Vectashield mounting medium with DAPI (Vector Labs H-1200, Burlingame, USA) and observed with a fluorescence microscope.

Section IV - Results

6. Results originating from the mouse model of glioblastoma

6.1 - Sample processing, mass spectrometry and data analysis

Using the procedure illustrated in figure 14 we have investigated the expression of potentially accessible host-produced protein using mouse model of high grade glioma. A total of six animals for each condition were involved in the proteomic analysis. The solubilization of 100 mg of tissue resulted in approximately 5 mg of whole protein extract. A total of 20 to 50μg of proteins were obtained from each sample following the streptavidin-based purification. A total of 751 and 785 proteins were identified from the analysis of the T98G and U373 derived tumor respectively. Because of the genetic proximity between the mouse and human species, three different groups of proteins could be distinguished. The first set included proteins identified uniquely as mouse, the second composed of uniquely human and third group consisted of proteins where the identified peptides were conserved between the species and no specific assignment to either species could be performed. Figure 15A illustrates the results obtained and as expected most of the proteins had mouse origin (approx. 50% for both models). This is due to the fact that the human tumor mass was considerably smaller in comparison to the mouse brain hemisphere. Therefore only 4-7% of identified proteins were assigned as human and the remaining (~40%) were of unknown origin.

The next step consisted of evaluating the differential expression of the possibly host produced proteins in each tumor condition with respect to the normal mouse brain. A protein was considered as differentially expressed when it was detected in the tumor samples only or found with a fold change emPAI ratio greater than 1.5. The emPAI value represents the logarithm of the ratio of observed and the theoretically observable peptides in the MS analysis. Given these parameters, a total of 68 and 89 mouse proteins were found as differentially expressed in the T98G and U373 condition respectively (Figure 15B). According to the aim of the present study the attention was subsequently focused on all proteins that were differentially modulated and potentially accessible. Therefore the differentially expressed mouse proteins were further assessed for their subcellular localization using the information from the Swissprot® database. A protein was considered potentially accessible when it was described as localized in cell membrane or as secreted in the extracellular matrix (17 and 25 proteins in T98G and U373 model respectively; Figure 15C).

In conclusion a total of 13 unique accessible proteins (some in common for both experimental conditions) were identified as over expressed in the host tissue are displayed in the Table 2.

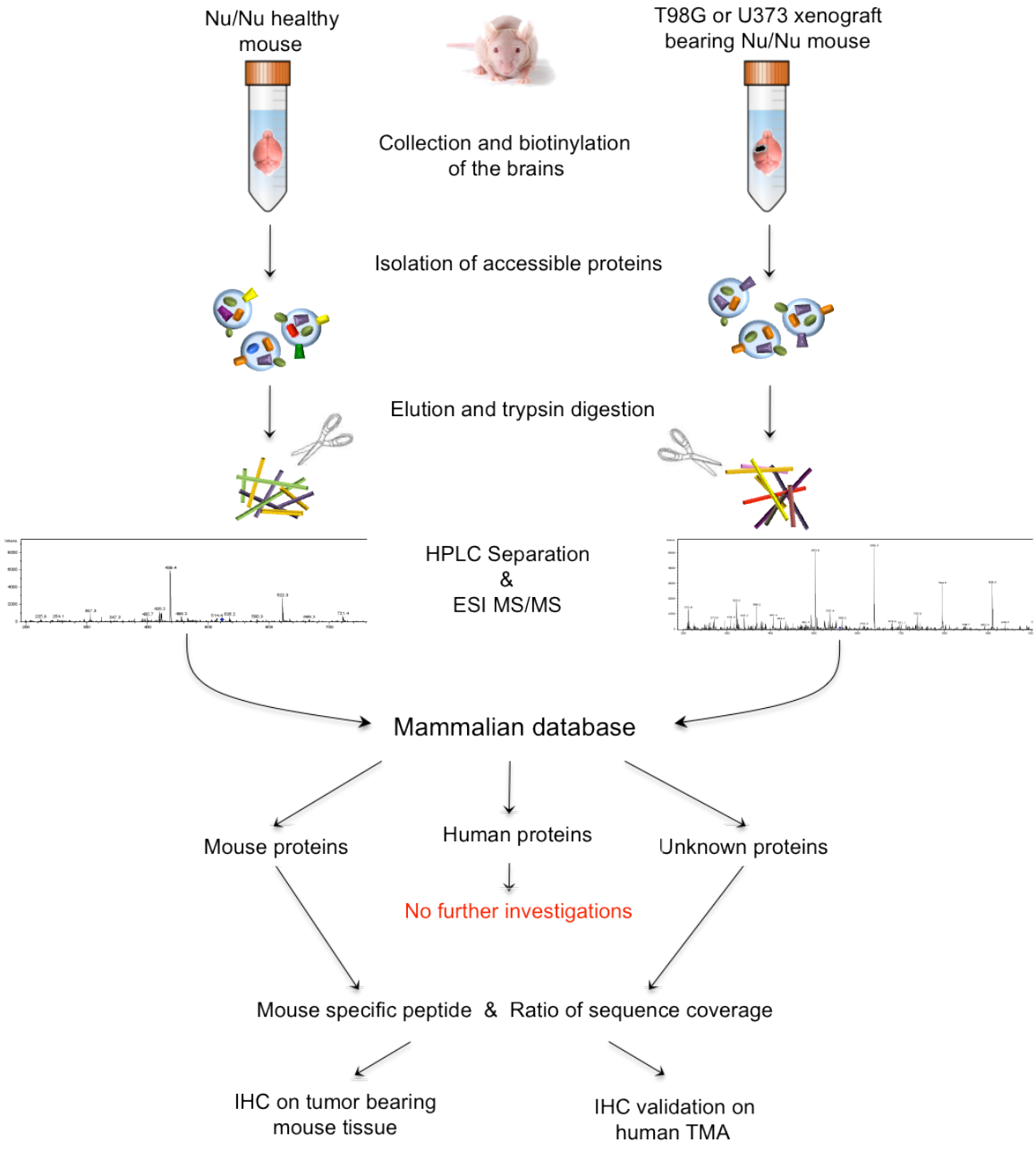


Figure 14: Workflow of the differential proteomic analysis. The experiment begins with the tumor generation and is followed by labeling of the accessible proteins using modified biotin reagent. Biotinylated proteins are purified using streptavidin beads and digested with trypsin into peptides. The peptides are then sequenced using 2D-nano-HPLC-ESI-MS/MS. Subsequently, the peptide sequences (from differentially expressed proteins) are compared with existing mammalian databases. Selected proteins are than validated using IHC firstly in tumor bearing mice and then on different grade glioma human specimens.

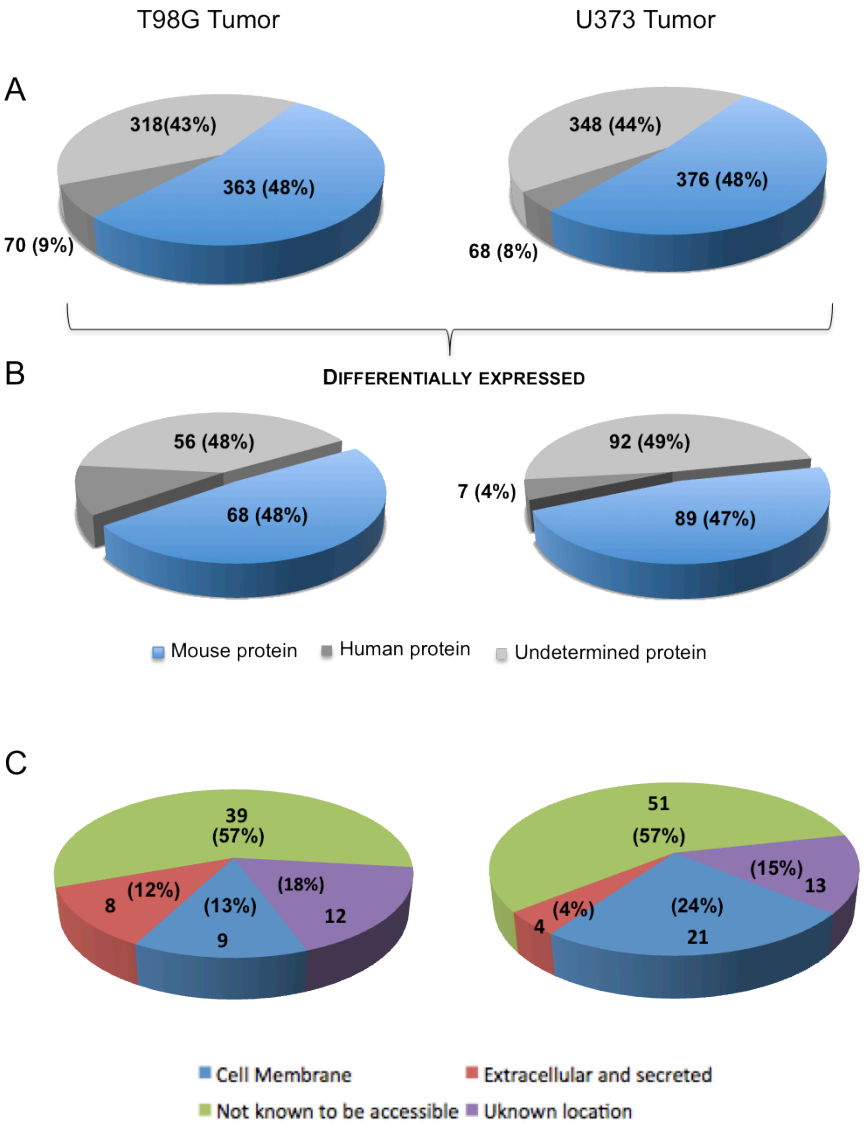


Figure 15: Overview of the identified proteins in the two tumor models at different steps of analysis. The charts on the top (A) indicate the numbers of mouse, human and undetermined proteins following the database search of MS/MS data. Below, the chart B displays the number of modulated proteins following the comparison of each tumoral sample with the normal brain. Finally, the chart C shows the localization of the differentially expressed mouse proteins (Uniprot® database). The abbreviation n.d. indicates a group of proteins for which the origin could not be unambiguously determined (any specific mouse peptide)

				Presence (N. of Rep.)			MASCOT Score			Unique Peptides			Seq. Coverage (%)			Ratio	
SPID	Entry	Protein	SCL	CTRL	T98G	U373	CTRL	T98G	U373	CTRL	T98G	U373	CTRL	T98G	U373	T98G	U373
Q3UHJ0	AAK1_MOUSE	AP2-assoc. protein kinase 1	M	1	3	n.d.	112	185	n.d.	2	5	n.d.	1.3	2.8	n.d.	2.0	n.d.
Q60771	CLD11_MOUSE	Claudin-11	M	2	1	3	68	51	211	2	1	6	6.8	6.8	6.8	1.0	2.2
Q68FD5	CLH_MOUSE	Clathrin heavy chain 1	M	5	3	6	1196	592	1493	34	18	48	12.1	6.7	18.3	0.6	1.6
Q6PFD5	DLGP3_MOUSE	Disks large-assoc. protein 3	M	n.d.	n.d.	2	n.d.	n.d.	166	n.d.	n.d.	2	n.d.	n.d.	1.8	n.d.	n.d.
Q9D164	FXYD6_MOUSE	FXYD domain-containing ion transport regulator 6	M	n.d.	2	n.d.	n.d.	81	n.d.	n.d.	3	n.d.	n.d.	23.4	n.d.	n.d.	n.d.
Q61885	MOG_MOUSE	Myelin-oligodendrocyte glycoprotein	M	2	1	3	68	78	97	3	2	4	7.7	8.9	12.6	1.0	1.6
Q99PJ0	NTRI_MOUSE	Neurotrimin	M	4	5	3	600	688	120	16	20	4	23.3	24.7	12.5	2.1	0.6
O55022	PGRC1_MOUSE	Membrane-associated progesterone receptor component 1	M; ER	3	4	3	173	208	364	4	5	8	7.2	17.4	17.4	2.1	3.3

P70663	SPRL1_MOUSE	SPARC-like protein 1	ECM	3	2	3	119	67	106	5	4	6	4.5	6.2	6.2	1.5	1.5
Q61207	SAP_MOUSE	Sulfated glycoprotein 1	SCR	1	1	n.d.	121	111	n.d.	3	5	n.d.	2.7	5.6	n.d.	2.8	n.d.
Q7M6Y3	PICA_MOUSE	Phosphatidylinositol-binding clathrin assembly protein	M	n.d.	n.d.	2	n.d.	n.d.	501	n.d.	n.d.	14	n.d.	n.d.	5.3	n.d.	n.d.
Q6RHR9	MAGI1_MOUSE	Membrane-associated guanylate kinase, WW and PDZ domain-containing protein 1	M	n.d.	n.d.	2	n.d.	n.d.	59	n.d.	n.d.	4	n.d.	n.d.	1.3	n.d.	n.d.
Q9D3A9	TTYH1_MOUSE	Protein tweety homolog 1	M	4	5	4	196	199	246	8	5	11	8.7	2.9	12	0.3	1.7

Table 2: Differentially expressed mouse proteins identified is T98G and U373 human tumor xenografts. The table displays the Swissprot database identifier (SPID) along with entry abbreviation, protein name and sub-cellular localization of the protein (SCL [ECM: extracellular matrix, M: cell membrane, ER: endoplasmic reticulum and N: nucleus]). The corresponding protein data include the number of biological replicates where the respective protein was observed, the Mascot score of the protein, number of unique (protein specific) peptides identified during MS/MS analysis, protein sequence coverage and abundance ratio of tumoral vs. normal sample (if applicable).

6.2 - Determination of mouse origin

In order to elucidate the possible murine origin of the identified modulated accessible proteins, MS/MS spectra of the specific mouse sequence were examined. First the peptides in question were blasted against the human protein database. The resulting analysis revealed the specific amino acid residues that are distinct between the mouse and the human species. Subsequently, the MS/MS spectra were checked for the typical collision induced fragments corresponding to the *y* and *b* peptide ions. Figure 16 provides MS technical evidences for the mouse origin of sulfated glycoprotein 1 (SAP) and sparc like-1 (SPRL1) where a specific fragment in the MS/MS spectrum was assigned to the mouse peptide and not to the human one. The differences in the amminoacidic sequence are indicated in bold red.

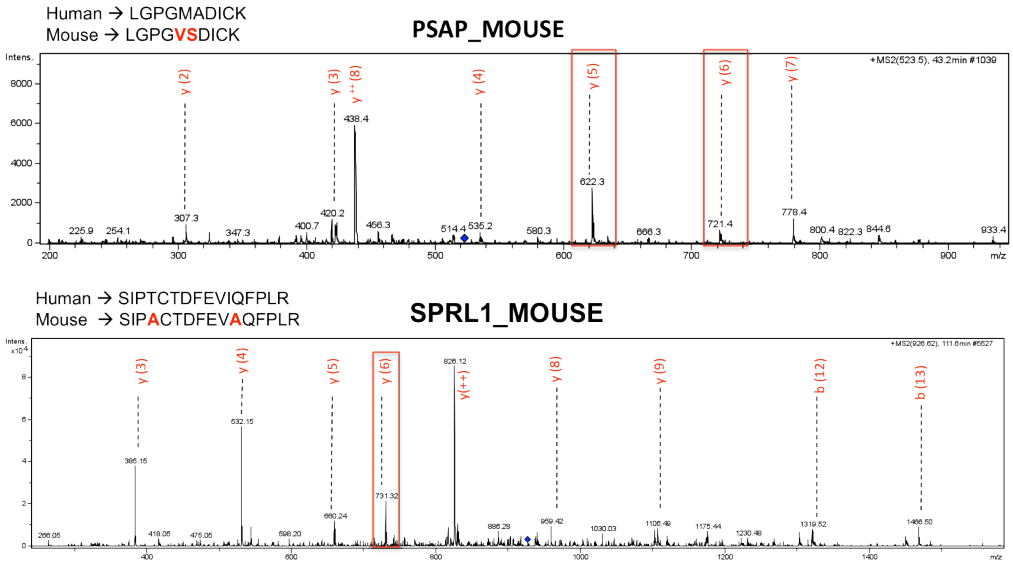


Figure 16: Examples of the MS/MS data corresponding to the mouse specific peptide of PSAP and SPARC-like 1. The ions assigned to a specific fragment are indicated in the spectrum along with the observed mass. The red boxes highlight the ions that allowed the identification of the specific mouse-sequence related aminoacids. In the case of Sparc-like 1 the *alanine* residue next to the carboxi-terminal group is not visible in the spectrum.

6.3 - Immunohistochemistry

With the exception of the two secreted proteins SPRL1 and PSAP, the remaining modulated proteins (identified as of mouse origin) were membrane based. Therefore we have decided to direct our attention towards these two proteins and characterize their expression

pattern in mouse bearing tumors (n = 4), human glioblastoma (n = 14) and several grades (G) of astrocytoma (G1 [n = 7], G2 [n = 8] and G3 [n = 8]).

In tumor bearing mouse brains a diffuse and intense expression of SAP was detectable in the cytoplasm of human cancer cells. Any sign of positivity was detectable in the neoplastic area. Normal brain tissue from control animals showed no positivity. On human specimens, SAP expression was always detectable in the cytoplasm of tumor cells, in all grade grades of malignancy, with an increasing percentage of positive cells moving from grade II to grade IV. More precisely all GBM (grade IV) samples and 62% (5/8) of grade III specimens showed protein expression. A lighter expression was detectable in 50% (4/8) of grade II astrocytomas. Grade I gliomas and normal brain tissue (consisting of healthy tissue adjacent to the tumor) showed no SAP expression (Figure 17).

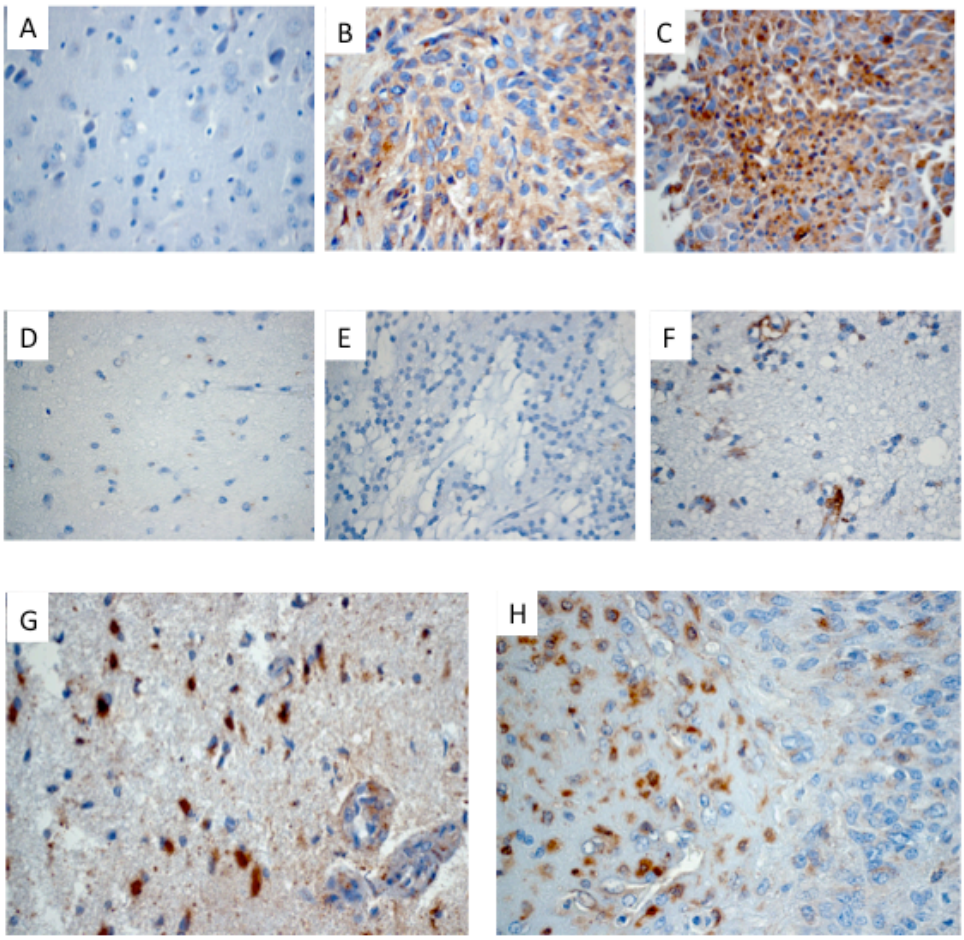


Figure 17: Immunohistochemical anamysis of prosaposin (SAP) expression. No protein expression was detectable in the normal mouse brain tissue (A). A strong diffuse citoplasmic expression was obsevable in T98 (B) and U373 (C) tumor-bearing mouse. Normal human brain (D) as well as in grade I astrocytoma (E-F) resulted negative for SAP expression. On the contrary, a strong expression was detected in grade II to IV astrocytomas (G-H). The magnification used: 400X.

As in the case of PSAP, an intense expression of SPRL1, mainly localized in the cytoplasm of cancer cells, was detectable in both T98G and U373 tumor. A lighter immunostaining was also detectable in the neuropil of normal and neoplastic mouse brain. However, staining was limited to neurons and clearly located in the nucleus only. In human samples, a high cytoplasmic protein expression was detectable in the 50% of GBM samples. Immunostaining was mostly localized in cells around hyper-plastic vessels. In lower grade gliomas SPRL1 was generally less expressed. Only 25% grade III and 14% grade II astrocytoma showed a strong staining. Grade I tumors and normal adjacent brain tissue showed abundant protein expression in all cases analyzed. However, while in all grade astrocytomas immunostaining was generally located in the cytoplasm of tumor cells, in normal brain tissue SPRL1 was exclusively present in the nucleus of sparse neurons (Figure 18).

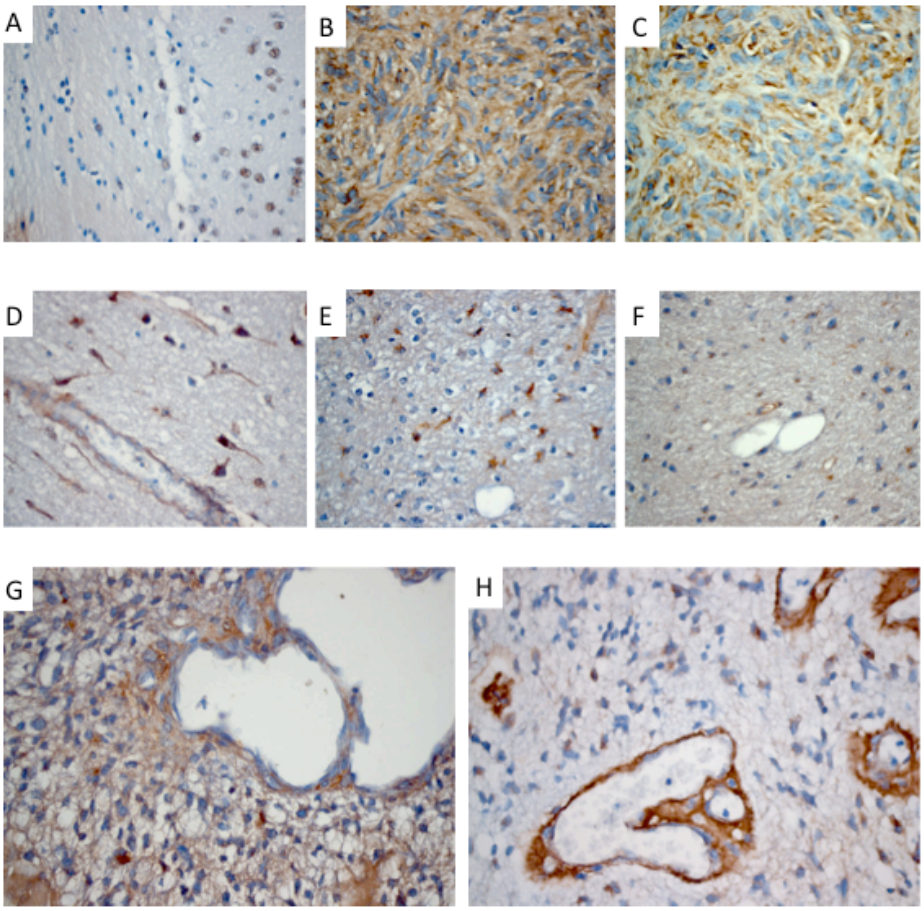


Figure 18: Immunohistochemical analysis of sparc like-1 (SPRL1). In normal mouse brain SPRL1 expression was exclusively localized in the nucleus of neuronal cell (A). Intense and diffuse cytoplasmic expression was detected in T98G and U373 tumor cells (B-C). As for normal mouse brain, a weak protein expression was detectable in the nucleus of some neurons in normal human brain (D) as well as in grade I and II astrocytomas (E-F). Diffuse expression of the protein was detected in grade III astrocytoma (H) and glioblastoma samples (I) where vessels (red arrow) were usually highly positive. Images displayed at 400x magnification.

7. Results originating from human glioblastoma samples and U87-derived tumors

7.1 - Tissue processing

Proteomic analyses of five human glioblastoma specimens and five U87-derived tumors grown on CAM led to the identification of 574 and 539 unique proteins, respectively. Pooled together, this resulted in the identification of 839 unique proteins. Among these, 274 proteins (32%) resulted to be expressed both by human and U87-derived tumors. According to our main focus of identifying, and successively investigate the *in vivo* distribution of new accessible biomarkers of glioblastoma these data were treated as following: i) all the proteins identified in human and U87-derived tumors were sorted according to their subcellular location, in order to generate a list of potentially accessible biomarkers (information on the protein subcellular location were obtained by the Swissprot® database and when necessary integrated with literature information). Proteins were considered potentially accessible when they were described as localized in cell membrane or as secreted in the extracellular milieu; ii) since the lack of proteomic data coming from normal human brain tissue, we decided to use the data obtained from the previous work, conducted on mouse brain tissue, to filter-out the lists previously generated from all the proteins found expressed in normal mouse brain. iii) a final list of biomarkers was compiled, containing all the potentially accessible proteins identified in human, in U87-derived or in both tumors.

In conclusion a total of 105 proteins (54 associated to the cell membrane and 49 secreted or extracellular) were identified as differentially modulated. However, all those proteins identified in less the 3 out of 5 samples (human or U87-derived tumors) were no considered for further investigations. Finally this procedure allowed editing a list of 30 potential biomarkers, which were the object of additional investigations.

SPID	Accession ID	Protein names	N of Identification		Subcellular locations
			Human tumors (n=5)	U87-derived tumor (n=5)	
P05556	ITB1_HUMAN	Integrin beta-1	4	5	CM
P04083	ANXA1_HUMAN	Annexin A1	3	5	CM
P16070	CD44_HUMAN	CD44 antigen	3	5	CM
P04264	K2C1_HUMAN	Keratin, type II cytoskeletal 1	3	5	CM
Q13698	CAC1S_HUMAN	Voltage-dependent L-type calcium channel subunit alpha-1S	3	5	CM
P15311	EZRI_HUMAN	Ezrin	3	4	CM
Q03113	GNA12_HUMAN	Guanine nucleotide-binding protein subunit alpha-12	3	1	CM
P06756	ITAV_HUMAN	Integrin alpha-V	2	4	CM
P18564	ITB6_HUMAN	Integrin beta-6	2	4	CM
O00299	CLIC1_HUMAN	Chloride intracellular channel protein 1	1	4	CM
Q9P2E9	RRBP1_HUMAN	Ribosome-binding protein 1	1	4	CM

Q92542	NICA_HUMAN	Nicastrin	1	3	CM
P01009	A1AT_HUMAN	Alpha-1-antitrypsin	5	0	Secr/Ex
P06396	GELS_HUMAN	Gelsolin	5	0	Secr/Ex
P02787	TRFE_HUMAN	Serotransferrin	5	0	Secr/Ex
P02671	FIBA_HUMAN	Fibrinogen alpha chain	4	0	Secr/Ex
P09486	SPRC_HUMAN	SPARC - Osteonectine	3	2	Secr/Ex
P01008	ANT3_HUMAN	Antithrombin-III	3	1	Secr/Ex
P02675	FIBB_HUMAN	Fibrinogen beta chain	3	1	Secr/Ex
P02763	A1AG1_HUMAN	Alpha-1-acid glycoprotein 1	3	0	Secr/Ex
P02749	APOH_HUMAN	Beta-2-glycoprotein 1	3	0	Secr/Ex
P00738	HPT_HUMAN	Haptoglobin	3	0	Secr/Ex
P01857	IGHG1_HUMAN	Ig gamma-1 chain C region	3	0	Secr/Ex
P07355	ANXA2_HUMAN	Annexin A2	2	5	Secr/Ex
P09382	LEG1_HUMAN	Galectin-1	2	5	Secr/Ex

P24821	TENA_HUMAN	Tenascin-C	2	5	Secr/Ex
P12111	CO6A3_HUMAN	Collagen alpha-3(VI) chain	2	3	Secr/Ex
P02751	FINC_HUMAN	Fibronectin	1	5	Secr/Ex
P12109	CO6A1_HUMAN	Collagen alpha-1(VI) chain	1	4	Secr/Ex
P08123	CO1A2_HUMAN	Collagen alpha-2(I) chain	1	3	Secr/Ex

Table 3: List of potential accessible tumor biomarker obtained by the proteomic analysis of human glioblastomas and U87-derived tumors. "Number of identifications" stands for the number of samples (human or U87-derived tumors) where the protein was found. **SPID**: Swissprot® ID. **CM**: cell membrane. **Secr**: secreted. **Ex**: extracellular.

7.2 - Validation phase

Few of the proteins showed in table 3 such as Tenascin-C, Galectin-1, SPARC as well as Integrin α5 and Fibronectin have been already discussed in the context of GBM pathogenesis. On the contrary some of them, even though already reported as related to other cancers, have never been described as associated to glioblastoma yet [PubMed research conducted with: (name of the protein) and (glioblastoma) and/or (brain tumor)]. Accordingly to the aim of our work we decided to focalize our investigations on CD44 and on Collagen type VI alpha-1 and characterize their expression by immunohistochemistry analysis using tissue micro arrays containing several human glioblastoma specimens (n = 14) and different grades (G) of astrocytoma (G1 [n = 7], G2 [n = 8] and G3 [n = 8]). Furthermore, in order to investigate the potential of these proteins as biomarker of glioblastoma we decided to perform an *in-vivo* injection of specific mAbs into the chick's vascular system and to check the distribution of the mAbs in the tumor mass.

7.3 - Immunohistochemistry of CD44 and Collagen VI alpha-1

Immunohistochemical analysis conducted on different grade gliomas and normal brain tissues resulted in a strong diffuse expression of CD44, mainly localized in the plasma membrane of cancer cells. However, a lighter diffuse citoplasmatic immunostaining was detectable also. A total of 73% GBM samples showed a strong to moderate expression of the protein in more than 90% of cancer cells. As far as lower grade gliomas are concerned, the 71% grade III tumors showed a moderate positivity, the 28% grade II tumors showed a moderate to strong positivity. Grade I gliomas resulted in a moderate CD44 expression present in the 85% of the sample analyzed. Under the experimental conditions used, normal brain tissue resulted positive for CD44 in the 20% of the samples (Figure 19).

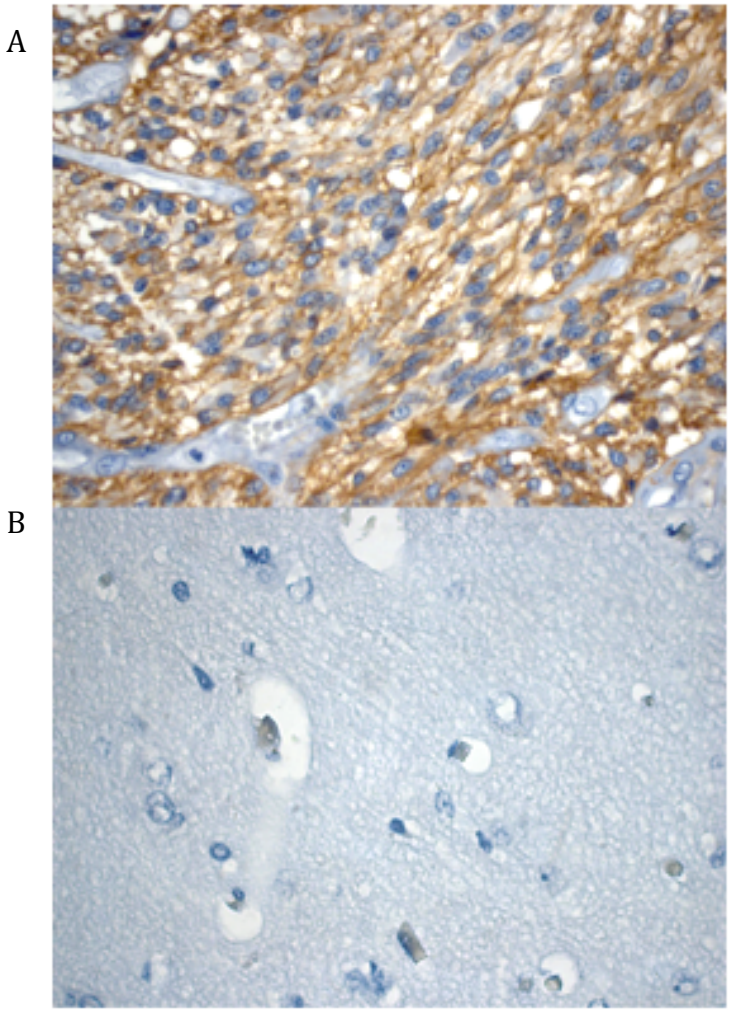


Figure 19: representative pictures of CD44H immunostaining in GBM (A) and normal brain tissue specimens (B). Strong to moderate protein expression was detected in the plasma membrane of cancer cells.

The expression of COL6α1 in different grade gliomas and normal brain tissues was also investigated in this study. The presence of a strong immunostaining revealed high extracellular protein expression exclusively located in the neuropil, and mainly around blood vessels. COL6α1 expression was detectable in more than 70% of human GBM samples analyzed. Lower grade gliomas showed a lighter protein expression in the 80% grade III and 12% grade II astrocytomas, while grade I astrocytomas resulted in a diffuse extracellular protein expression in the 57% of the samples. Normal brain tissue showed no immunostaining under the experimental conditions used in any the cases analyzed.

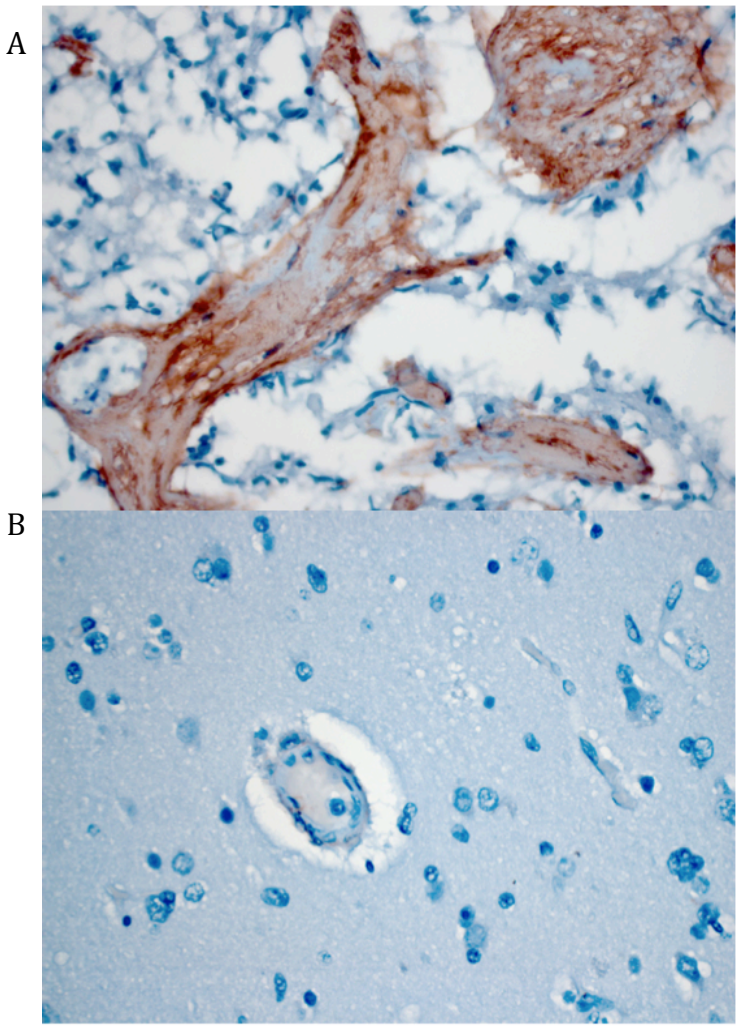


Figure 20: representative pictures of COL6α1 immunostaining in GBM (A) and normal brain tissue specimens (B). Strong protein expression was detected in the extracellular space surrounding cancer cells and especially around blood vessels, which are well visible in both pictures.

7.4 - In vivo validation of human CD44H and Collagen VI alpha-1

The last part of this thesis work was committed to demonstrate that the biomarkers identified by mass spectrometry as potentially accessible were truly reachable by their specific antibody systemically administered. In order to answer to this question we set up a method of intravenous injection using the chicken egg model that we used to grow up the U87-derived tumors. The process of setting up the technique was carried out using repetitive injections of Dextran-FITC solution until a staining of the vasculature system could be seen. As showed in figure 21, intravenous injection of 100 µl of Dextran-FITC (average mol wt 20,000) solution (final concentration = 5µg/µl) allowed the staining of both CAM (A) and tumor vascular bed (B). In this way, the CAM

model allowed not only the modeling of the glial tumor but also a way to test *in-vivo* the accessibility of the biomarkers found expressed both by human specimens and the U87-derived tumors.

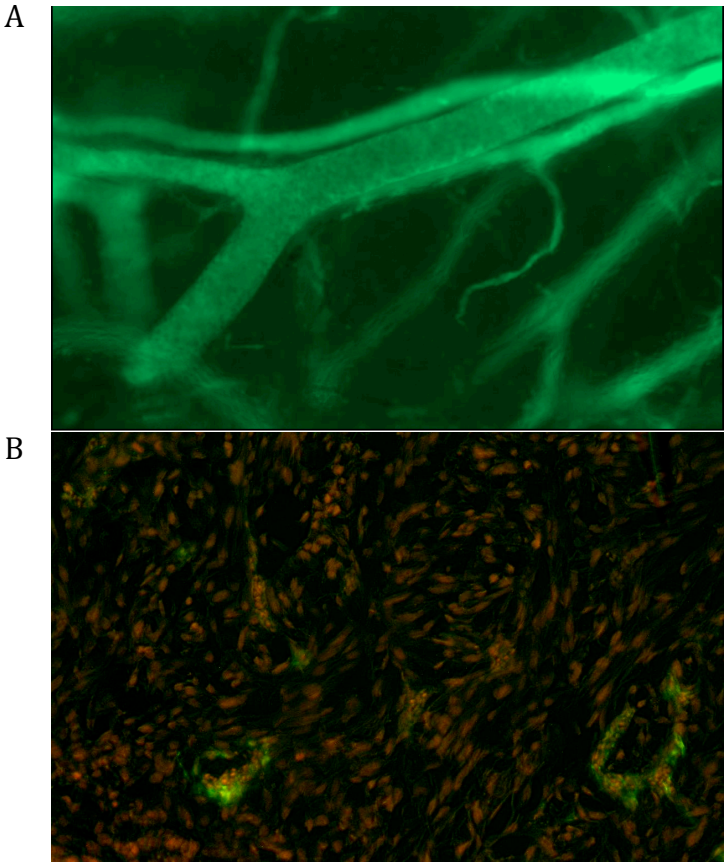


Figure 21: (A) CAM vasculature network after the intravenous injection of 100 µl of 5mg/ml Dextran-FITC solution. **(B)** U87-derived tumor vasculature bed stained in green after the *i.v.* injection of Dextran-FITC solution. Red nuclei results after the counterstaining done using To-Pro dye. Pictures taken using a 520nm filter, after fixation of the tissue with PAF 4% at 4°C.

Following, the *in-vivo* accessibility of CD44 and COL6α1 was evaluated by intravenous injection into the CAM vasculature of monoclonal specific antibodies. A total of 20µg of mouse anti-human CD44H *(R&D systems, Minneapolis, USA)* and 10µg of mouse anti human COL6α1 *(LifeSpan BioScience,* Seattle, USA*)* where injected *i.v.* as described in the material and methods section. 24 hours after the injection embryos were sacrificed and U87-derived tumors were excised and fixed with PAF 4%. Direct immunofluorence analyses were successively performed by probing frozen tissue sections with the secondary anti-mouse antibody.

Anti human CD44H selectively accumulated in the tumor area and no staining was detectable in the surrounding CAM tissue. As showed by the figure 23 a specific membrane (green) staining was evident in the U87 cell membrane. A similar result was achieved using the mouse anti human COL6α1. Immunofluorescence experiments showed that after *i.v.* administration the

antibody accumulated in the extracellular matrix of U87-derived tumor with no staining of the CAM tissue around (Figure 24). Injection of unspecific mouse IgG resulted in any staining neither of the U87-derived tumor nor of the surrounding CAM. Notably, immunostaining was stronger at the tumor base (closer to CAM vasculature) to become lighter at tumor periphery (far from CAM vasculature) where big vessels were less present, in both cases.

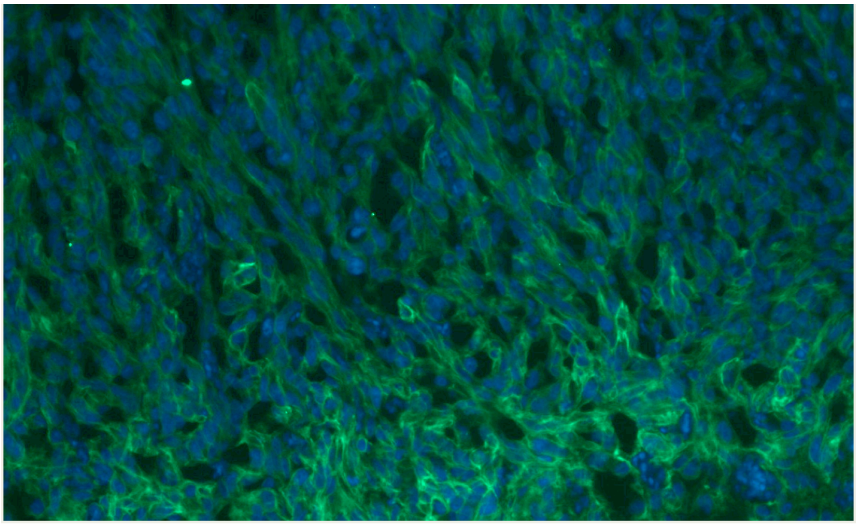


Figure 23: in vivo evaluation of CD44H accessibility by direct immunofluorescence anlaysis. U87-derived tumor slices were probed with the secondary antibody (Alexa 488 – green staining) allowing the revelation of the primary anti-human CD44H that was injected *in-vivo* through the CAM vasculature. Blue staining represents nuclei of cell stained with DAPI.

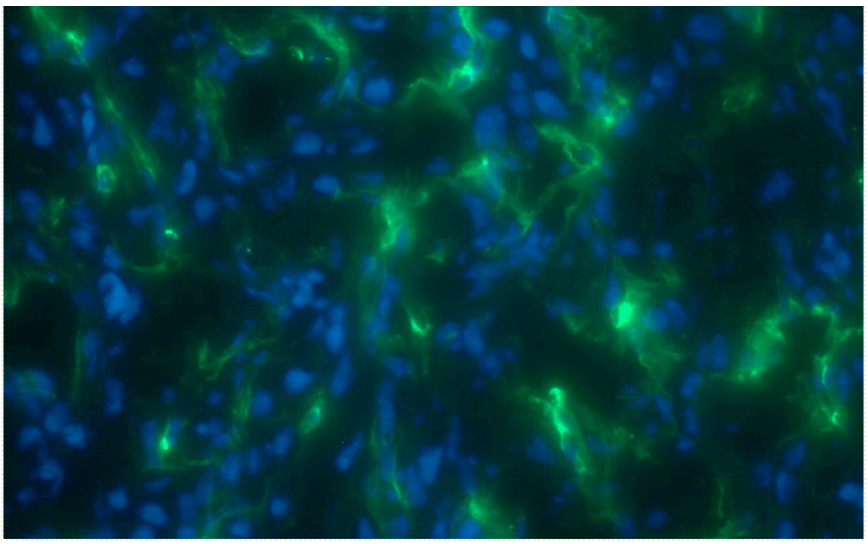


Figure 24: in vivo evaluation of COL6α1 accessibility by direct immunofluorescence anlaysis. U87-derived tumor slices were probed with the secondary antibody (Alexa 488 – green staining) allowing the revelation of the primary anti-human COL6α1 that was injected *in-vivo* through the CAM vasculature. Blue staining represents nuclei of cell stained with DAPI.

Section V - Discussion

Because of the general toxicity of current chemotherapeutic drugs, nowadays, clinician and researcher's expectations rely on the production of new specific targeting agents whose application can be easily translated to a major improvement in patients survival, through a less toxicity for normal organs and a higher cytotoxic effect for cancer cells. The delivery of toxic agents to the tumor area can be reached by targeting either proteins expressed on the membrane of cancer cells or proteins of the modified extracellular matrix, which constitute the tumor stroma. Indeed, antigens located in the proximity of tumor blood vessels are better reachable for compounds coming from blood stream. In addition, components of the modified tumor stroma are often more abundant and more genetically stable with respect to their cellular counterpart, offering an attractive alternative for biomarker investigation. However one of the major limiting factors for the development of such therapeutic modalities consists on the identification of specific tumor antigens. The evolution of high-throughput mass spectrometry technique applied to biological samples has made this task easier. It was along this perspective that, during my permanence at the Metastasis Research Laboratory, I carried out my researches. My scientific activity turned towards the identification of accessible biomarkers of GBM and was divided into two major parts. The first was principally focused on the discovery of new stroma-associated biomarkers produced by the host in response to cancer development. The second part was oriented towards the discovery of new cancer cell associated biomarker and to the use of a chicken egg model of GBM as a novel tool for the *in-vivo* validation of such biomarker. The biomarker discovery phase was carried out by applying the *ex-vivo* biotinilation method, developed in our laboratory *[Castronovo V. 2007]*, to human GBM specimens obtained after surgical interventions, as well as tumor samples obtained from the mouse and the chicken model of GBM. Tissues were immersed into the sulfo-NHS-SS-biotin reagent and through diffusion membrane associated, secreted and extracellular matrix proteins were labeled. The decision of focusing on this class of proteins (called here *accessible* proteins) takes place from the fact that they are considered as readily reachable for targeting drugs administered systemically to the patient via blood stream.

Part I - In order to investigate for stroma-associated biomarkers produced by the host tissue we used high throughput MS analysis of tumor samples obtained from human glial tumor cells (U373 and T98G) xenografted in nude mouse brain. Even thought transgenic mouse models could appear more appropriate for oncological studies aiming to investigate the host-tumor reaction, most of them often fail to exhibit all of the characteristics typical of human astrocytomas and furthermore, they would be not suitable for those applications trying to discern host from tumor produced proteins. On the other hand, xenograft model can only be performed on immunedeficient mice that, due to their particular features, lack major components of the immune mediated response

and hence could manifest only partially the host-tumor reaction *[Reilly KM 2001].* The MS analysis conducted on tumor bearing and normal mouse brain led to the identification of several modulated proteins (tumor Vs normal). However, along with our research line, we focused our interest on those proteins that, according to the MS data, appeared to originate by the mouse tissue, and more specifically on SPRL1 and SAP, described as extracellular matrix associated proteins. SPRL1 (also known as hevin) has been described as a matricellular glycoprotein belonging to the family of osteonectin, sharing over 50% of its amino acid sequence with SPARC (or osteonectin) *[Sullivan MM. 2004].* SPRL1 expression has been inversely correlated with tumor induced angiogenesis and tumor growth in a mouse model of hepatocellular carcinoma *[Lau CP. 2004]* and it has been described as anti-adhesive extracellular matrix protein downregulated in different cancer, such as prostate and non-small cell lung cancer *[Nelson PS .1998; Girard JP. 1999].* Also, SPRL1 has been reported to be capable to bind type I collagen *[Hambrock HO. 2003]* and to have a tumor suppressor role in pancreatic cancer *[Esposito I. 2007].* As far as Sulfated Glycoprotein-1 (SAP) is concerned, it is a homologue of human prosaposin, known to be processed into four 15 kDa saposins (A,B,C and D). Saposins are activators of various lysosomal lipid-degrading enzymes. Contradictory data are present in the literature regarding this protein in the context of human tumor growth and development. A study conducted on patients with recurrent breast cancer showed that gene expression level of prosaposin could predict the clinical outcome of the patients. High mRNA levels of the protein were significantly associated with a shorter progression-free-survival *[Meijer D. 2009].* Another study reports human prosaposin as involved in prostate cancer invasion, through the modulation of basal membrane adhesion proteins, and suggests this protein as a possible target for prostate cancer therapy *[Hu S. 2010].* On the other side, it has been demonstrated, using cell lines with different metastatic potential, that prosaposin inhibits prostate tumor metastasis acting via paracrine and endocrine stimulation of stromal p53 and thrombospondin 1 *[Kang SY. 2010]* suggesting that this protein could have a dual role in cancer development. In this work we used IHC analysis conducted on mouse and human brain tumor tissues in order to validate the MS data. The advantage of this technique over other methods, such as western blot or polymerase chain reaction, lays principally on the fact that formalin fixation preserve the tissue from any degradation, impeding in this way possible false results due for instance to the presence of necrosis. Moreover paraffin tissues are usually easy to obtain and immunostaining allows a visual localization of the protein expression, giving a initial information on its subcellular location.

On the basis of the immunohistochemical data it results difficult to confirm the MS data suggesting SPRL1 and SAP as proteins produced by the mouse tissue. Furthermore, it appears evident that, at least in these particular conditions, neither SPRL1 nor SAP can be classified as secreted protein.

Indeed, both proteins are detected in the human cell constituting the tumor mass within the mouse brain with an intense expression mainly localized in their cytoplasm. Only in the case of SPRL1 a nuclear immunostaining was detectable in normal mouse neurons within the tumor area, suggesting the possibility of an intracellular trafficking of the protein according to the different physiopathological conditions in cancer rather than in normal cells. On the other hand, in human tumors, even though the staining pattern observed was the same than in mouse tissue (cytoplasmic staining), an increasing percentage of cells expressing SPRL1 and SAP was observable moving from grade I-II to grade IV of malignancy, suggesting these proteins as probably associated to the pathological progression of human astrocytic brain tumors. Taken together, this data report for the first time SPRL1 and SAP as novel proteins highly expressed in high-grade malignant glioma, especially on GBM, and propose the same as biomarker related to the pathological progression of the disease. However, the proteomic data suggesting the murine origin of the modulated proteins identified, were not confirmed by a more empirical validation, suggesting once more IHC as the most important complementary validation technique of all proteomic approach aimed to biomarker discovery in human cancer specimens. Future studies are needed in order to establish the role of SPRL1 and SAP, in the pathogenesis of GBM.

Part II - Furthermore in this work, investigations aiming to the identification of cancer cell originating biomarkers were conducted using human GBM surgical specimens and U87-derived tumors growth on chicken egg's CAM. The biotinilation technique and the following MS analysis allowed, also in this case, the identification of several potentially accessible proteins whose role in glioma formation has never been reported. However, an important issue after the discovery of a biomarker consists of the validation step. Before going through the stage of pre-clinical validation the characteristics and tissue biodistribution of a biomarker has to be well investigated. The process of validating tumor proteins as targets of selective therapeutic drugs is much easier compared to the process required for biomarkers that are targets of inhibitory molecules. The latter, in fact, need an attentive development of selective inhibitor molecules wisely chosen in order to avoid that the protein, which is being inhibited, is functionally replaced by another one that can perform the same function. On the other hand, the complementary information provided by immunohistochemical studies conducted on a large series of human samples together with the *in-vivo* biodistribution experiments, can provide enough information about the suitability of a biomarker as target for the delivery of selective drug-conjugated antibodies. Along this perspective, we used IHC analysis conducted on a large collection of human all grade glioma samples and normal human brain. The chicken model of GBM was used as a tool for an initial *in-vivo* validation of the accessibility of the antigens, through an *i.v.* administration of specific mAbs in the chick vasculature. The rationale of

choosing the CAM model rather than a more comprehensive one, such as mouse model, come from some practical reasons that see the chick model as a technique which is i) well established and easily reproducible, ii) simple and fast to perform; a complete tumor develop within one week, iii) not restricted by ethical rules and iv) relatively inexpensive. Moreover, Hagedorn and colleagues have shown a major resemblance of the U87-derived tumor with human GBM at the transcriptomic and histological level.

Among the 30 proteins showed in the table 3, a total of 22 were identified both in human and in U87-derived tumors, while the remaining 8 proteins were only found in human samples. All of them had been reported as potentially accessible. The proteomic similarity between human GBM and U87-derived tumor confirmed the transcriptomic data presented by Hagedorn at al. indicating the chick model as a valid tools miming GBMs developing in human patients. Along with proteins, such as Tenascin-C, galectin-1 and CD44 antigen whose role in glioma pathogenesis has been extensively validated by other authors, we found novel proteins that have no previous record in relation to glial tumors, such as integrin-β6, COL6α1, COL1α2, glypican-6 and spactasin.

In particular, high levels of tenascin-C are abundantly expressed in glioma microenvironment and the protein was suggested as the main actor in the process of immune-suppression carried out by cancer cells *[Huang JY. 2010]*. Combined use of Temozolomide and a mAb targeting a specific domain of tenascin-C have been recently proposed as GBM treatment *[Pedretti M. 2010]*. High galectin-1 expression has been detected in high-grade gliomas. This protein appears to modulate glioma cells invasion, angiogenesis as well as resistance to chemo and radiotherapy *[Jung TY. 2008; Le Mercier M. 2009]*. CD44 antigen, a widely distributed cell-membrane adhesion protein, is able to bind to hyaluronic acid, collagens, fibronectin and osteopontin and it has different roles in normal and tumoral conditions, such as lymphocyte homing, matrix adhesion, wound healing, promotion of cancer cell growth, survival and migration. High protein expression was recently demonstrated by real-time PCR reaction, western blot and immunohistochemical analysis in a set of 73 GBM patients. However, despite its clear involvement in malignant gliomas pathogenesis, the utility of CD44 expression as diagnostic, prognostic or therapeutic biomarker remains unclear and needs further investigations *[Wei KC. 2010]*. The role of other proteins, such as SPARC, integrin αV, fibronectin and annexin A2 in glial tumors has been less discussed, however, data present in literature suggest their possible function in glioma pathogenesis. The essential role of SPARC, in the invasiveness of human glioma cells has been recently discussed by Seno and collegues *[Seno T. 2009]*. SPARC expression by cancer cells has been related with a decrease of tumor progression, while, contrarily to this observation, vascular expression of the protein seems to be associated with a worse patient prognosis *[Capper D. 2010]*. Additionally, our group has already reported high

SPARC levels in breast cancer *[Bellahcène A. 1995]*. As far as integrins molecules are concerned, they mediate cell-cell or cell-matrix attachment. They usually forms heterodimers composed of alpha and beta chain and they are described as major actor in the development and the progression of central nervous system neoplasms. Integrin αV-β3, for instance, is specifically expressed in endothelial cells of newly forming blood vessels. A recent study conducted on cell lines proposes integrin αV as the main player in the enhanced glioma cell migration and adhesion, through activation of JNK pathway after the binding of insulin-like growth factor binding protein 2 (IGFBP2) *[Mendes KN. 2010]*. Moreover, Schnell et al. propose the use of (18)F-labeled glycosylated Arg-Gly-Asp peptide PET as noninvasive imaging in αV-β3 expressing GBM *[Schnell O. 2009]*. Recenlty, short-harpin (sh)RNA mediated knocking-down of fibronectin resulted in a reduction of tumor growth in a syngeneic mouse model of GBM (GL261 cells) *[Sengupta S. 2010]*. On the other side, the down regulation of Annexin A2, using RNA interference technique, resulted in a significant inhibition of U87MG and U373MG migration *[Tatenhorst L. 2006]*. Besides the proteins discussed above, this work point out some antigens that, according to the review of literature, appear to be new in the context of glioma development. However few of them have been already described in other cancer types. It is the case of Integrin-β6, which have been proposed as molecular target of malignant colon and pancreatic cancer *[Bates RC. 2005; Hausner SH. 2009]* and collagen proteins, that have been described as promoter of pancreatic cancer metastasis and epithelial-to-mesenchymal transition in lung cancer *[Shintani Y. 2007]*.

In this study CD44 antigen, whose role as therapeutic target of GBM has not been reported yet, and COL6α1, which was never described in the context of GBM, were considered for further validation by IHC and *in-vivo* experiments. The identification of CD44 by MS technique and its successive detection by IHC analysis as a membrane bound antigens expressed by cancer cells in GBM tumor samples, along with the literature information discussed above, provide strong evidence that the technique used in this work is a valid method for biomarker investigation. Moreover, the discovery of the high expression of COL6α1 in more than the 70% of GBM analyzed highlights the potential role of this protein as novel accessible biomarker of GBM. In addition, our in vivo experiments support the hypothesis that both CD44 and COL6α1 are not only present in high quantity in GBM tumors, compared to normal brain tissue, but also readily accessible by vasculature system, as demonstrated by i.v. mAb injection, and put the basis to propose these proteins for further investigations aiming to the development of new targeted therapies of GBM.

Section VI - Conclusion and Perspectives

6.1 – Conclusions

Glioblastoma multiforme is the most common high lethal primary brain tumor occurring in adults. It represents the highest grade of malignancy of astrocytic tumors. Current treatment including surgery, radiotherapy and chemotherapy can only ensure a patient's survival that rarely exceed 1 year. The recently acquired knowledge about biological features and molecular pathways involved in GBM formation are being currently used to develop specific cancer therapies targeting directly those proteins involved in the neoplastic process. An example of that is represented by the several clinical trial involving different compounds, which are intended to inhibit proteins whose function is usually altered in GBM, such as EGFR and PDGFR *[Rich JN. 2004b].* However, direct therapeutic approaches, aiming to the inhibition of one rather than another molecular pathway, are often limited by i) the high genetic instability typical of cancer cell, which lead to protein mutations and consequently to the not recognition of the target by the ligand, and ii) by the fact that for each pathway which is being inhibited, an alternative ways, able to accomplish the same or a very similar function, may take place. On the other side, the use of indirect solutions, conceived as tools to deliver highly cytotoxic drugs specifically to the tumor site only, is not subjected to such limitations. Moreover**,** knowledge on the biological function of the target protein is not strictly necessary and the therapeutic activity is left to the action of the drugs itself that acts mainly in the tumor area. In this work, we used a procedure for the chemical modification, recovery and identification of proteins readily accessible from the blood stream. To this aim, we labeled accessible proteins by *ex-vivo* biotinilation of tumor specimens surgically resected from cancer patients as well as tumor samples derived from different animal models of GBM. We show that this procedure along with the use of the 2D-nano-HPLC MS/MS system allow the identification of a huge number of potential tumor antigens. Indeed, we report the identification of SPRL1 and SAP as novel biomarker possibly associated to the pathological progression of glial tumor. Along with markers, such as tenascin-C and CD44, which had already been shown overexpressed in glial tumor, we show a specific expression of COL6α1 in high-grade glioma tumors. Furthermore, we developed a valid and alternative way for the *in-vivo* validation of accessible antigens, using the CAM model of GBM. With this technique, which imply the *i.v.* injection of a specific mAb into the CAM vasculature system, COL6α1 and CD44 accessibility was validated. Taken together, our results suggest the efficacy of the biotinilation technique in the investigation for cancer accessible biomarkes and propose the chicken model of GBM as an easy and suitable tool for an initial *in-vivo* screening of tumor antigens.

6.2 – Perspective

The biotinilation technique, already used in our laboratory for the identification of new biomarker of GBM, kidney and breast cancer, is currently being used for the study of other types of cancer such as pancreas and colon cancer. Besides, further investigations and validations will be needed to confirm the physiological and pathological relevance of the markers identified in this work. The accessibility feature of such biomarkers will be validated *in-vivo* using the CAM model of GBM. Once the relevance of these antigens will be assessed we could foresee a preclinical evaluation of these biomarkers using more complex animal models of cancer. Imaging analysis performed by linking specific mAbs to fluorphores, could give information about the biodistribution of the target *in-vivo* without any risk of toxicity. Radiolabeled mAbs could be used in the following steps in order to conduct a pre-clinical evaluation of the new therapeutic agents. These two investigation phases conducted firstly on animals could be then applied to human patients who will be recruited in comprehensive and organized clinical trials.

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