Ministero dell'Università e della Ricerca Scientifica

Università degli Studi di Palermo

Centro di Oncobiologia Sperimentale

Dottorato di Ricerca in

Genomica e Proteomica della Ricerca Oncologica ed Endocrino Metabolica

Ciclo XXII: 2008-2010

Settore Scientifico Disciplinare: BIO/06

IDENTIFICATION OF PROTEOMIC CLUSTERS RELATED TO THE BREAST CANCER PROGRESSION

Tesi di Dottorato della:

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INTRODUCTION

TUMOR INITIATION

Experimental evidence suggests that the origin of neoplastic proliferation is predominantly monoclonal. The neoplastic transformation, then, is the appearance of a cell clone that, following an initial mutation, can prevail over the others because provided of increased replicative activity and / or because is refractory to the pro-apoptotic stimuli. Subsequently, successive genetic alterations and selective mechanisms provide new different subclones, many of which are able of circumvent the checkpoints of the cell cycle and to invade surrounding normal tissues.

In recent years, the cancer stem cell model of tumorigenesis has received increasing attention. This model postulates that tumours are driven and maintained by a minority subpopulation of cells that have the capacity to self-renew and to generate the more differentiated progeny which make up the bulk of a tumour (Clarke MF et al.2006). The former population has been termed cancer stem cells (CSCs), tumourigenic cancer cells, or tumour-initiating cells, by various investigators, to indicate that only these can give rise to new tumours when transplanted into immuno-deficient animals (Clarke MF et al.2006).

Evidence for the existence of CSCs initially came from studies of acute myelogenous leukemia (AML). Presence of CSCs have now been demonstrated in many solid tumours, including glioblastoma, medulloblastoma, breast cancer, melanoma, and prostate cancer (Takaishi S et al. 2008). The existence of CSCs has profound implications for cancer biology and therapy because it is likely that eradication of CSCs is the critical determinant in achieving cure. It has been proposed that CSCs may be particularly resistant to

chemotherapy and radiation therapy as has been shown in a study with glioblastoma (Bao S et al. 2006).

TUMOR PROGRESSION

The carcinogenetic process may be accelerated by repeated exposures to carcinogenic stimuli or by selection pressures favouring the autonomous clonal derivatives.

The stage of tumor progression is characterized by the expression of the invasive phenotype of tumor cells and is caused by additional morphological and behavioural alterations. The epithelial-mesenchymal transition (EMT) is an orchestrated series of events in which cell-cell and cell-extracellular matrix (ECM) interactions are altered to release epithelial cells from the surrounding tissue, the cytoskeleton is reorganized to confer the ability to move through a three-dimensional ECM, and a new transcriptional program is induced to maintain the mesenchymal phenotype (figure 1).

Moreover, during progression, epithelial cells lose acquire the ability to degrade the basal lamina and penetrate into the surrounding stroma, to secrete pro-angiogenic factors (resulting in vascularization of the tumor), to invade surrounding tissues and form metastases.

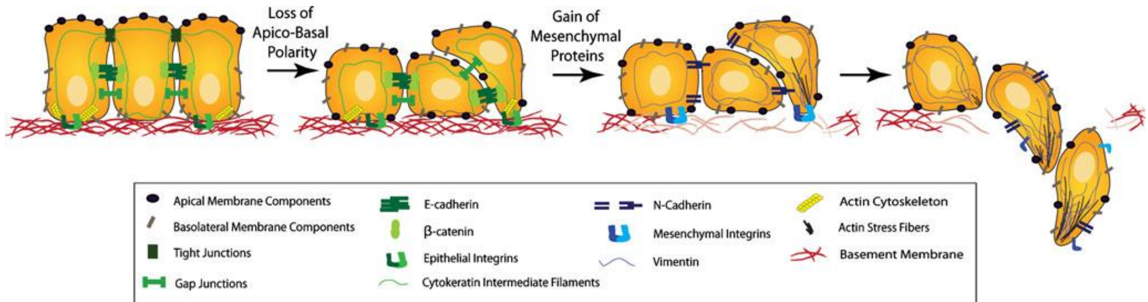


Figure 1: Epithelial-Mesenchymal Transition (EMT) and Epithelial Plasticity. Tight junctions which typically maintain apico-basal polarity dissolve allowing the mixing of apical and basolateral membrane proteins, adherens and gap junctions are disassembled and cell surface proteins such as E-cadherin and epithelial-specific integrins (green) are replaced by N-cadherin and integrins specific to extracellular components (blue). The actin cytoskeleton is remodeled into stress fibers which accumulate at areas of cell protrusions. The epithelial intermediate filaments, cytokeratins, are replaced by vimentin. Meanwhile, the underlying basement membrane is degraded and the cell invades and moves into the surrounding stroma, devoid of cell-cell contacts.

In this new context, tumor cells alter the characteristics of the adjacent stroma to create a supportive microenvironment. This is strongly supported by evidence that over 80% of the fibroblasts demonstrate an activated phenotype in breast cancer and are named cancer associated fibroblasts, CAFs, (Liotta LA et al. 2001). Also our previously work demonstrated that fibroblasts induce considerable proteomic modulations on 8701-BC cell line when cocultured, mainly in the cytoskeleton proteins and glycolytic enzymes. Additionally, fibroblast-conditioned medium increased neoplastic cell proliferation and invasion; suggesting that fibroblast stimulation may enhance the malignant potential or breast cancer cells in vitro (Cancemi P. et al 2010).

It has been shown that fibroblasts play major roles in tumor invasion by secreting various matrix-degrading proteases as well as their activators such as uPA (Camps JL et al. 1990). uPA can cleave MMPs to activate these proteins, and up-regulation of MMPs activity results in significant ECM degradation, which contribute to angiogenesis and metastasis.

The tissue inhibitors of metalloproteinases (TIMPs) have been shown to down-regulate MMPs activity. TIMPs can also regulate other growth factors indicating that TIMPs may also be involved in some important oncogenic signal pathways (Flavell SJ et al. 2008).

WARBURG EFFECT

It is now emerging that the tumor microenvironment influences the rate of cell proliferation and may have a profound effect on tumor progression and resistance to therapy (Hoogsteen IJ et al. 2007).

In contrast to normal differentiated cells, which rely primarily on mitochondrial oxidative phosphorylation to generate the energy needed for cellular processes, most cancer cells instead rely on aerobic glycolysis, a phenomenon termed “Warburg effect”, because described by Otto Warburg.

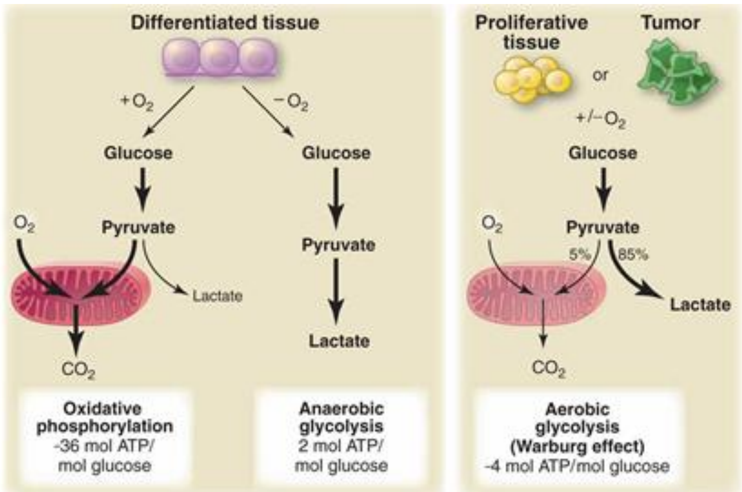


Figure 2: Schematic representation of the differences between oxidative phosphorylation, anaerobic glycolysis, and aerobic glycolysis (Warburg effect).

It is only under anaerobic conditions that differentiated cells produce large amounts of lactate (figure 2). In contrast, most cancer cells produce large amounts of lactate regardless of the availability of oxygen and hence their metabolism is often referred to as “aerobic glycolysis”. Warburg originally hypothesized that cancer cells develop a defect in mitochondria that leads to impaired aerobic respiration and a subsequent reliance on glycolytic metabolism. However, subsequent work showed that mitochondrial function is not impaired in most cancer cells, suggesting an alternative explanation for aerobic glycolysis in cancer cells.

One proposed explanation for Warburg’s observation is that tumor hypoxia selects for cells dependent on anaerobic metabolism (Gatenby RA et al. 2004). Tumors are characterized by gradients of O_2 levels, based on the distance of tumor cells from a functional blood vessel. Tumor cells surrounding the blood vessel are well oxygenated, whereas the tumor cells more distant from the vessel are poorly oxygenated and express high levels HIF-1. Several evidences suggested the existence of a “metabolic symbiosis” between hypoxic and aerobic cancer cells, in which lactate produced by hypoxic cells is taken up by aerobic cells, which use it as their principal substrate for oxidative phosphorylation (figure 3).

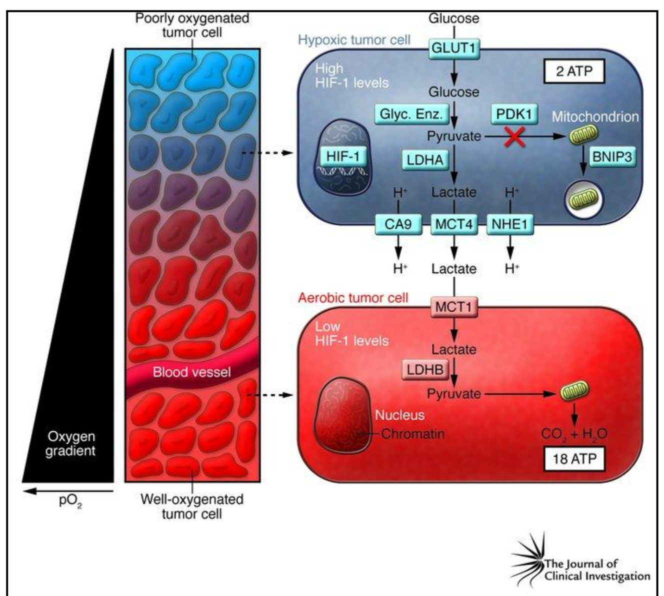


Figure 3: Intratumoral hypoxia and metabolic symbiosis. Hypoxic cells express high levels of which induces the expression of proteins that increase: uptake of glucose (e.g., glucose transporter 1 [GLUT1]); conversion of glucose to pyruvate (e.g., glycolytic enzymes [Glyc. Enz.]); generation of lactate and H+ (e.g., LDHA); and efflux of these molecules out of the cell (e.g., carbonic anhydrase IX [CA9], sodium-hydrogen exchanger 1 [NHE1], MCT4). The lactate produced by hypoxic cells is taken up by aerobic cells, which use it as their principal substrate for oxidative phosphorylation.

Although the altered metabolism in cancer has been well documented, the exact mechanisms leading to increased glycolysis and abnormal tumor cell growth under hypoxic conditions are not completely understood. A possible link could be the "early response" gene c-myc, because of its frequent overexpression in transformed cells, its stimulatory effect on cell growth (Hurlin PJ et al. 2004), and ability to upregulate the transcription of several glycolytic enzymes (Osthus RC et al. 2000) Myc is overexpressed in approximately 70% of all human tumors (Gordan JD et al. 2007), and activate α-enolase (48 KDa), a glycolytic enzyme which catalyzes the conversion of 2-phosphoenolpyruvate from 2-phosphoglycerate (Kim JW et al. 2005). Moreover, α-Enolase is also a hypoxic stress protein, which may contribute to hypoxic tolerance of tumors by increasing anaerobic metabolism (Pancholi V, 2001).

HYPOXIA AND ANGIOGENESIS

As a result of variable blood flow (oxygen supply) and rapid utilization of glucose within solid tumors (oxygen utilization), most tumor cells are subjected to a microenvironment that is hypoxic. Hypoxia is quite common in breast cancer where it has been related to poor prognosis (Vleugel MM et al. 2005) with increased risk for tumor recurrence and metastasis.

The hypoxic response is significantly controlled in most cells by HIF-1, a heterodimeric transcription factor composed of the nearly ubiquitous HIF-1α and its dimerization partner HIF-1β. HIF-1 activates approximately 200 genes encoding proteins that regulate cellular metabolism, proliferation, motility, haematopoiesis, and angiogenesis (Semenza GL, 2000). Upon initiation of the hypoxic signal, HIF1-α translocates to the nucleus, dimerizes with HIF1-β to form the HIF-1 complex and induces the expression of its transcriptional targets via binding to hypoxia-responsive elements (HREs) (Chilov D et al. 1999).

In response to various alterations in the microenvironment, and in particular, to intratumoral hypoxia, angiogenesis-stimulating factors produced by the cells themselves induce the formation of new blood vessels from pre-existing vasculature, to increase delivery of oxygen and nutrients to tissues (figure 4). Many angiogenic genes, such as VEGF, angiopoietin-2, VEGF receptors (Flt1 and KDR), and neuropilin-1 (Hickey MM et al. 2006; Simons M, 2005) are induced by hypoxia by several mechanisms, including direct transcriptional activation by

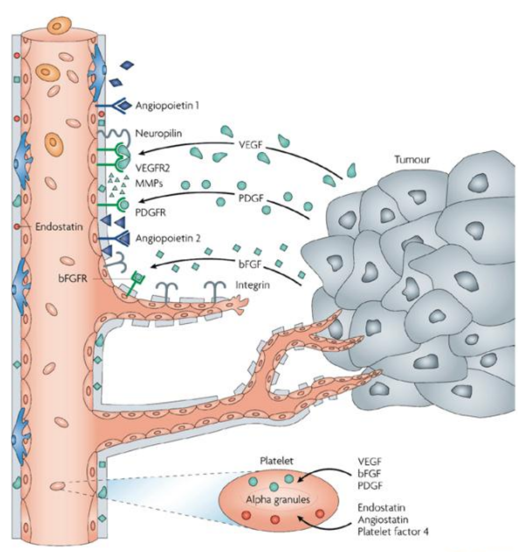


Figure 4: schematic representation of formation of new blood vessels from pre-existing vasculature.

HIFs or indirect up-regulation by HIF-induced molecules. In addition, other transcription factors induced by hypoxia, such as Related Transcription Enhancer Factor-1 (RTEF-1) and early growth response 1 (EGR-1), can both target VEGF to enhance angiogenesis (Shie JL et al. 2004; Yan SF et al. 2000). Additional angiogenic growth factors such as IGF are also induced by hypoxia, but can signal through a HIF-1-independent pathway (Slomiany MG et al. 2006).

However, unlike normal tissues, the newly formed vascular network has a number of "defects", both structural and functional (expansion, basement membranes and endothelial surfaces incomplete or absent, irregular and tortuous architecture, arterio-venous shunts, blind ends and lack of contractile components of the wall) (Vaupel et al., 1989) resulting in an irregular blood flow sometimes reducing the transport of oxygen and nutrients to cancer cells.

BREAST CANCER

Breast cancer is the leading cause of cancer-related death in women world-wide.

About 90% of solid tumors is part of the category named carcinoma that is cancer of epithelial-glandular origin with high degree of malignancy. In breast cancer we can distinguish:

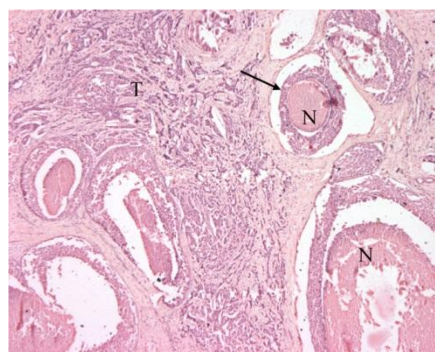


Figure 5: Malignant tumor infiltrating [T], characterized by initial intraductal proliferation of cancer cells with typical area of central necrosis [N].

carcinoma in situ (non-infiltranting) characterized by uncontrolled proliferation of malignant epithelial cells that do not exceed the basement membrane; this proliferation can occur within the lobule (lobular carcinoma in situ, LCIS) or into the duct (ductal carcinoma in situ, DCIS).

Infiltrating breast cancer is a heterogeneous disease in its presentation, pathological classification and clinical course. It's characterized by the overcoming of the epithelial cells of the basal lamina and the ability to invade different anatomical regions from that of tumor formation.

MOLECULAR BASIS OF BREAST CANCER

Despite significant advances in diagnosing and treating breast cancer, several major unresolved clinical and scientific problems remain.

While the exact etiology of breast cancer is unknown, family history is one of the strongest determinants of risk, implying hereditary factors. Some observations suggest that 5-10% of

all cases of breast cancer may have a hereditary basis (Yoshida and Miki, 2004). The two genes that confer susceptibility to the onset of cancer breast are BRCA 1, BRCA 2 that belong to tumor suppressor family. In particular BRCA1 gene is located on the long arm (q) of chromosome 17 and BRCA2 gene is located on the arm (q) along chromosome 13. Although the structures of the BRCA1 and BRCA2 are very different, some functions of the two genes are related; indeed the correspondent proteins are essential for the repair of damaged DNA. Moreover women with a mutation at one of the two genes have, in fact, about 60-80% chance of developing breast cancer during their lifetime.

In the sporadic cases of breast cancer, alterations of protooncogenes are associated with the initiation, promotion, and/or maintenance of tumors. Oncogenes often found to be overexpressed in human breast cancer tissue include members of the myc and ras family (c-myc, Ha-ras-1), the members of the EGF receptor (EGFR, erbB) family, including erbB-2 (or HER-2 or neu), HER-3, and HER-4. Overexpression and mutation often leads to constitutive activation of these receptors (ie, signaling in the absence of their cognate ligands). Growth-promoting signals may be continuously transmitted into the cells, resulting in activation of multiple intracellular signal transduction pathways and unregulated cell growth. Genes normally involved in cell cycle control, especially members of the cyclin D family, may also function as oncogenes.

Several studies have shown that blockade of these growth factor receptors or pathways has therapeutic implications: for example, both in preclinical models and in human breast cancer, monoclonal antibodies to EGFR or HER-2 have dramatic antitumor effects. Furthermore, these antibodies have synergistic interactions with cytotoxic agents, such as the anthracyclines, the platinum analogs, and the taxanes (Baselga J et al. 1994).

In addition to BRCA1 and BRCA2, alterations in two known suppressor genes, the retinoblastoma gene (RB1) and the human p53 gene, have been identified in human breast

cancer cells, as well as in other solid tumors. Mutations in the p53 gene have been found in families with the Li-Fraumeni syndrome, who have a markedly increased incidence of breast cancer and other neoplasms. In addition, up to 50% of breast cancers have been shown to have mutations in the p53 gene.

Evaluation of mutational inactivation of suppressor genes, associated with breast cancer, could lead to early recognition of high-risk families, as well as to new treatment strategies to reverse the malignant phenotype by introducing normal gene copies through gene therapy or by treatment with the normal suppressor protein itself.

CLINICAL CLASSIFICATION OF BREAST CANCER

Breast cancer is a clinically heterogeneous disease, and existing histological classifications do not fully capture the varied clinical course of this disease. Immunohistochemistry (IHC) currently forms the cornerstone of molecular classification of breast cancer into ER-positive and ER-negative categories. Nucleic acid in situ hybridization is now also routinely used to classify breast cancer into HER-2 amplified or non-amplified categories. A large number of other single gene molecular markers were also assessed in the past 25 years using these technologies but failed to establish themselves in the clinic for various reasons.

Estrogen receptor: ER (α) expression is undoubtedly the most important biomarker in breast cancer, because it provides the index for sensitivity to endocrine treatment. ER-positive tumors (c. 80% of breast cancer) use the steroid hormone estradiol as their main growth stimulus; ER is therefore the direct target of endocrine therapies. While the absence

or presence of the ER is used to obtain treatment decisions, little attention has been paid on the value of the quantitative expression levels as a predictive indicator.

There have also been investigations concerning the amplification of the ER gene (ESR1). An initial report indicated that ESR1 gene amplification in breast cancer could be detected in 20% of all invasive tumors, and that there was a correlation between the gene amplification and ER expression levels (Holst et al. 2007). However, <3% of invasive breast cancer cases were reported as ESR1 amplified by other independent groups (Brown et al. 2008).

Progesterone receptor: The expression of the PgR is strongly dependent on the presence of ER. Tumors expressing PgR but not the ER are uncommon and represent <1% of all breast cancer cases in some large series (Viale et al. 2007). There is evidence that in metastatic breast cancer the response to anti-estrogen treatment is better among patients with tumors expressing both ER and PgR versus those who only show ER positivity but lack the PgR expression (Elledge et al. 2000).

HER2: the oncogene HER2 was first identified to be an indicator of patient's prognosis. In cases of HER2 being overexpressed (HER2 positive), breast cancer patients are more likely to suffer from relapse and tend to have a shorter overall survival. Through the development of the monoclonal antibody trastuzumab, which is targeted at HER2, the amplification status of HER2 became also a highly predictive biomarker (Slamon et al. 1987, Mass et al. 2005). Overexpression and amplification of HER2 can be detected in about 15% of all primary breast cancer, and this group of patients benefit significantly from anti-HER2 therapies.

Within the last few years, many more promising agent targeting HER2 have been developed including monoclonal antibodies and tyrosine kinase inhibitors. From them, HER2 status is likely to be a predictive markers as well (Widakowich et al.2008). The

preliminary finding that patients expressing a truncated cytoplasmic HER2 receptor (p95HER2) show a poor response to trastuzumab (Anido et al.2006), but may benefit from the tyrosine kinase inhibitor lapatinib (Scaltriti et al. 2007), might have some implications for future HER2 testing.

Ki67: is a nuclear non histone protein. The characteristic that Ki67 was universally expressed among proliferating cells and absent in quiescent cells led to the further evaluation of Ki67 as a marker of proliferation. Although little is known about the exact function of the protein in cell division, Ki67 is expressed during G1, S, and G2 phases of cell cycle with a peak during mitosis and an absence in G0 phase (Lopez et al.1991)

The correlation of Ki67 and other biomarkers in invasive breast cancer has been studied intensively. Unsurprisingly, given that the Nottingham grading system defines mitotic rate as one of its three criteria (Trihia et al. 2003), there is a good correlation with tumor grade. The relationship with ER has been predominantly described as an inverse correlation with lower proliferative activity in ER-positive tumors (Haerslev et al. 1996). There are hints of a correlation with HER2 as well, but this is not completely defined (Nicholson et al. 1993, Rudolph et al. 1999).

Although determination of ER status is an essential part of the diagnostic workup of all breast cancer patients (useful to determine whether a patient is a candidate for endocrine therapy or not) these current clinical parameters, for breast cancer diagnosis, cure, and prognostic/ predictive properties, appear inadequate to discriminate between cancer subtypes and to support proper therapeutic decisions. Indeed, patients with the same type and stage of disease often display significantly different clinical outcome and responses to therapy. Moreover, patients with tumors that lack expression of estrogen receptor (ER), progesterone receptor (PR), and HER2 amplification (triple negative breast cancer) are not

candidate for currently available FDA-approved, target therapies and therefore treated with standard chemotherapy regimens.

MOLECULAR CLASSIFICATION OF BREAST CANCER

The advent of high throughput gene-expression profiling technologies allowed investigators to ask how many different molecular subtypes of breast cancer exist based on complex mRNA expression patterns. It might be useful to identify the various molecular classes because the different subsets may have different natural histories. Also, the molecular signatures that define particular groups may lead to the discovery of new therapeutic targets and treatments that are effective in particular molecular subsets. The first study to examine comprehensive gene-expression patterns of breast cancer have revealed five major molecular subtypes of breast cancer: basal-like, luminal A, luminal B, HER2+/ER–, and normal breast–like. The molecular differences result in distinct clinical outcomes and responses to treatment; in general, the basal-like tumors have the worst, and luminal A-type tumors the best, prognosis (Sorlie T et al. 2001).

The luminal subtypes of breast cancers express high amounts of luminal cytokeratins and express genetic markers of luminal epithelial cells and normal breast cells (Rakha EA et al.2007; Sotiriou C and Pusztai L, 2009). In contrast, basal-like breast cancers tend to express cytokeratins associated with basal types of cancers, as they arise from the outer basal layer. Basal-like breast cancers are typically high-grade and poorly differentiated when examined morphologically. It has also been reported that almost 82% of basal-like breast cancers express p53 compared with 13% in the luminal A subgroup (Sørlie T et al. 2001). Ongoing studies have recently identified a new and intriguing subtype called

“claudin-low”, characterized by the low to absent expression of luminal differentiation markers, high enrichment for epithelial-to-mesenchymal transition markers, immune response genes and cancer stem cell like features (Prat A et al. 2010).

The above mentioned five molecular subtypes of breast cancer are conserved across ethnic groups and are already evident at the ductal carcinoma in situ (DCIS) stage (Yu K et al.2004), suggesting distinct tumor progression pathways for each tumor type. Distinct cell of origin (e.g., cancer stem cells) and tumor subtype–specific genetic and epigenetic events are two possible, not necessarily mutually exclusive, explanations of this extensive intra- and intertumoral heterogeneity. Based on the cell of origin hypothesis, each tumor subtype is initiated in a different cell type presumably stem or progenitor cell (figure 6A), whereas according to the model depicted in figure 6B, the cell of origin can be the same for different tumor subtypes and the tumor phenotype is primarily determined by acquired genetic and epigenetic events.

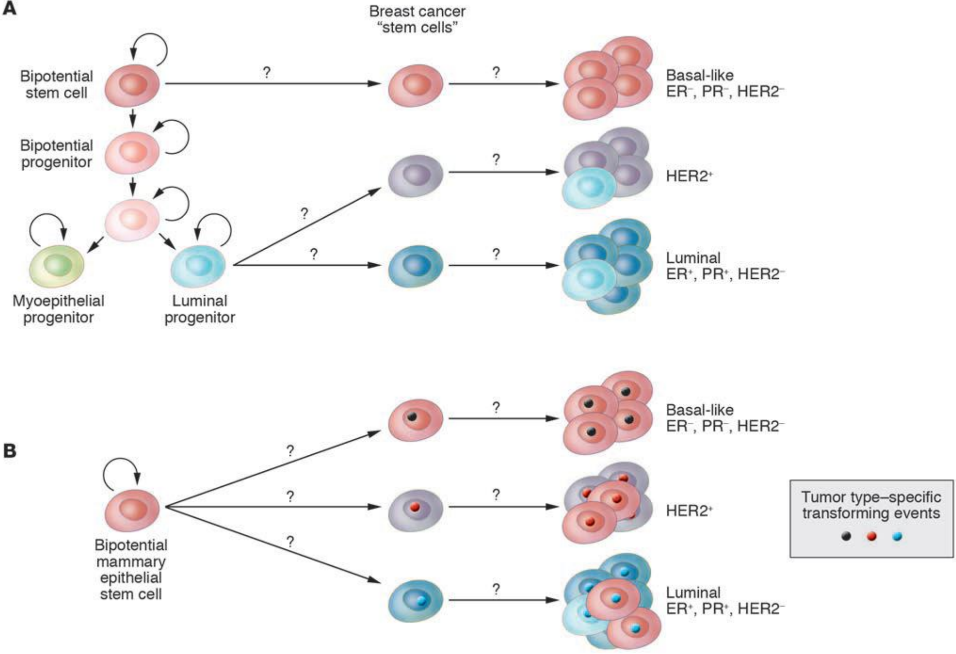


Figure 6: Hypothetical models explaining breast tumor subtypes. Cell of origin (A) and tumor subtype–specific transforming event (B) models.

GENE-EXPRESSION-BASED PROGNOSTIC ASSAYS FOR BREAST CANCER

Several gene signatures for prognostic purpose are now available for prognostication of patients diagnosed with breast cancer. The basic premise of these emerging tests is that they simultaneously quantify the expression of multiple genes and combine the gene expression measurements into prediction scores that may predict clinical outcome more accurately than any of the genes alone.

Mammaprint

MammaPrint® (Ach RA et al, 2007) was originally developed as a general prognostic test in premenopausal untreated patients. Original discovery of prognostic genes was from a microarray analysis of relative expression levels of 25,000 genes in relationship to a universal reference RNA in tumors from a cohort of 78 premenopausal patients younger than 55 years without nodal metastasis who received no systemic therapy. By associating expression of each gene with clinical outcome, a prognostic algorithm based on the expression levels of 70 genes was established (van 't Veer LJ et al. 2002).

Clinical utility of the MammaPrint® assay is currently being tested in a randomized clinical trial called MINDACT (Microarray In Node-Negative and 1 to 3 positive lymph node Disease may Avoid Chemo Therapy) (Cardoso F et al. 2008).

Oncotype DX® was developed specifically as a prognostic and predictive test for the benefit of chemotherapy in women with node-negative, ER positive breast cancer who have been treated with tamoxifen. This signature is a combined score of 16 genes of interest. Of note, this score is a gene expression signature derived using quantitative RT-PCR, on RNA extracted from paraffin- embedded tissues. The associated clinical trial for this test, is TAILORx—Trial Assigning IndividuaLized Options for Treatment (Rx) (Zujewski JA and Kamin L, 2008).

Several criticisms, questions and perspectives have been raised regarding the prognostic gene signatures reported so far. First, some argue that most of the signatures only add little information compared to an optimal clinical-pathological score that would include ER, Her2 and Ki67 in addition to the conventional clinical parameters (de Azambuja E et al. 2007). Another criticism relates to the fact that most of the predictors were generated using a mix of molecularly heterogeneous tumours.

In conclusion, although these tests allow an increase in the rate of patients who could be spared adjuvant chemotherapy while still correctly identifying the high-risk patients, they present some limitations that will have to be taken into account to generate more accurate ‘second generation' gene signatures (Desmedt C et al. 2008).

CANCER BIOMARKERS

Every cell type has a unique molecular signature, referred to as biomarkers, which are identifiable characteristics such as levels or activities (the abilities of genes or proteins to perform their functions) of a myriad of genes, proteins or other molecular features.

Tumor biomarkers are molecules often produced by the tumor itself or the host system in response to the tumor and provide the biological material to determine the risk of getting cancer, to detect cancer, to classify cancer, or to provide insight into prognosis and therefore a therapeutic advantage. Tumor biomarkers include cancer-specific mutations or changes in gene expression or promoter methylation, which can result in alterations in protein expression (Tainsky MA, 2009).

Establishment of biomarkers requires a comprehensive understanding of the molecular mechanisms and cellular processes underlying the initiation of cancer, especially focusing on how small changes in only a few regulatory genes or proteins can disrupt a variety of cellular functions.

CURRENT CLINICAL BIOMARKERS

Diagnostic and prognostic biomarkers may help clinical oncologists to (i) identify who is at risk, (ii) diagnose at an early stage, (iii) select the best treatment modality, and (iv) monitor response to treatment (Ludwig JA et al. 2005).

Genetic and epigenetic biomarkers

Duplication/ deletion/ mutation of genomic material is predominantly responsible for oncogenic transformation because induce gain/ loss of gene function. Unfortunately, random chromosomal abnormalities are not associated with a particular morphological change give rise to clinical cancer. On the contrary, non-random mutations, and translocations/ rearrangement within the regulatory region of the gene serve as highly specific tumour markers for unique clinical diagnosis. For example, the “Philadelphia chromosome” is associated with chronic myelogenous leukaemia due to a translocation between chromosomes 9 and 22. Other examples occur in Burkitt’s lymphoma and in follicular B-cell lymphomas.

In recent years it has become apparent that epigenetic events (occur directly through DNA methylation of genes or indirectly by methylation, acetylation, or phosphorylation of histones and other proteins around which DNA is wound to form chromatin) are potentially responsible for cancer initiation and progression as genetic abnormalities, with DNA hypo- and hyper-methylation promoting cancer development. Genomic hypomethylation may lead to both genomic instability and stronger gene expression. On the other hand, hypermethylation induces the functional silencing of tumour suppressor genes. For example, hypermethylation of APC gene is utilized to determine the stage of oesophageal cancer, detect recurrent disease, and monitor disease progression or treatment response (Arnold K et al. 2000). Moreover, high level of hypermethylated APC gene in the bloodstream is generally associated with poor survival (Puig PL et al.1998).

Protein biomarkers

The cancer proteome contains information on perhaps every biological process that takes place in cancer cells, cancer tissue microenvironment, and cancer cell-host interaction.

Cancer cells release many proteins and other macromolecules into the extra-cellular fluid through secretion that can also serve as biomarkers. Some of these products can end up in the bloodstream and hence serve as potential serum biomarkers. Some important cancer antigens that serve as diagnostic and prognostic biomarkers of cancer are summarized in the Table 1.

Table. Cancer biomarkers for diagnosis and prognosis of the disease

Biomarker	Tumour	Application	Sample type/ Method of detection
Cancer antigen (biomolecules) based biomarkers:			
Prostate specific antigen (PSA)	Prostate cancer	Diagnostic and prognostic	Serum/ Immunoassay
Alpha-foetoprotein (AFP)	Hepatocellular carcinomas (HCC)	Diagnostic and prognostic	Serum/ Immunoassay
Cancer antigen 125 (CA125)	Ovarian cancers Fallopian tube cancer	Diagnostic and prognostic	Serum/ Immunoassay
Cancer antigen 15-3 (CA15-3)	Breast cancer	Diagnostic and prognostic	Serum/ ELISA, Lymph node/ IHC, Bone marrow/ IHC
Cancer antigen 19-9 (CA 19-9)	Pancreatic cancer Bladder cancer	Diagnostic and prognostic	Serum/ ELISA Urine/ ELISA
BRCA-1, BRCA-2	Breast cancer	Diagnostic	Tumour samples/ RT-PCR
Carcinoembryonic antigen (CEA)	Colorectal cancer	Diagnostic and prognostic	Serum/ ELISA
Human chorionic gonadotrophin (hCG)	Germ cell tumours (ovarian and testicular)	Diagnostic	Serum/ ELISA
Thyroglobulin (Tg)	Papillary and follicular thyroid cancer	Diagnostic and prognostic	Serum/ ELISA or IHC with TPO Ab
Heat shock proteins (HSPs) Hsp27; Hsp70	Gastric, prostate carcinoma, osteosarcomas, uterine, cervical, and bladder carcinoma	Diagnostic and prognostic	Serum/ ELISA
TGFβ	Malignant tumours	Diagnostic and prognostic	Serum / ELISA

As already described above breast cancer treatment has experienced several changes in the past decades due to the discovery of specific prognostic and predictive biomarkers that enable the application of more individualized therapies to different molecular subgroups. Among the diagnostic biomarkers most commonly used for breast cancer we find:

- CEA Carcinoembryonic antigen is a 200 kDa glycoprotein that acts as a cell-cell adhesion molecule, normally expressed in fetal tissues and was among the first identified tumor biomarkers. CEA is a biomarker that is elevated in a variety of cancers including colorectal, breast, lung, or pancreatic cancer.

- CA 15.3 protein is a member of the family of proteins known as mucins, whose normal function is cell protection and lubrication. It plays a role in reducing cell adhesion and is found throughout the body. Elevated levels of this antigen are found mainly in breast cancer where it appears to be involved in metastasis (Duffy MJ 1999). Pre-operative concentration of CA15-3 is associated with worse prognosis than those with low concentrations (Park BW et al. 2008).
- MUC1 is a large, heavily glycosylated protein that is embedded normally in the apical membrane of many secretory organs, such as salivary glands, breast, and lung. Glycosylation of MUC1 reduce intercellular interactions between adjacent tumor cells because sterically impeded adhesion (Wesseling J et al.1995,1996). Moreover, the cytoplasmic domain of MUC1 could compete for and bind to the β-catenin molecule, inhibiting E-cadherin-mediated intercellular adhesion (Li Y et al. 1998). These evidence suggest that MUC1 may facilitate tumor invasion and metastases and, consequently, may be associated with biologically aggressive tumors and a worse prognosis.
- PSA Prostate specific antigen is a 33 kDa serine protease belonging to the family of "Kallikrein genes" and produced by both normal as well as neoplastic prostate epithelial cells is the most widely studied biomarker in prostate cancer. Being a protease, it appears to be involved in the initiation and growth of prostate cancer by abnormal release of growth factors or proteolysis of growth factor binding proteins. It may also have a role in invasion and metastases through the degradation of collagen and laminin.

POTENTIAL CLINICAL BIOMARKERS

Today there is a need to find new biomarkers able to predict a correct diagnosis and allow better stratification of patients for targeted therapy. An ideal tumor marker should have a high degree of diagnostic accuracy, and should be characterized by analytical reliability of assessment methods, diagnostic specificity (diagnosis of cancer, tumor localization) and diagnostic sensitivity (early detection of cancer). Prognostic factors intend to predict objectively and independently patient clinical outcome independent of treatment, while predictive factors aim to predict the response of a patient to a specific therapeutic intervention and are associated with tumor sensitivity or resistance to that therapy. In general, prognostic markers help to determine whether a patient requires treatment, and a predictive factor is useful in deciding which treatment will be the best.

Cells biomarkers

In advanced stages of tumours, cells starts appearing in bloodstream where it can be easily monitored. Advanced clinical practice in certain malignancy have effectively used tumour and immune cells where it served as a good biomarker of prognosis.

Circulating Tumor Cells (CTCs) :The presence of CTCs has been shown to predict survival in patients with metastatic breast cancer at multiple time points throughout the course of therapy (Ring A. et al. 2004). CTCs provide an early, reliable indication of disease progression and survival for patients on systemic therapy for metastatic breast cancer. Elevated CTCs at any time during therapy is a harbinger of progression, while elimination of CTCs indicates effectiveness of the therapy (Cristofanilli M. et al.2004).

CTCs have been shown to be superior to standard tumour markers in predicting prognosis. Furthermore, the efficacy or benefit to systemic therapy can also be predicted by the level

of CTCs as early as 3-4 week after initiation of therapy. Patients with persistent CTCs ($\geq$ 5) demonstrate lack of response to treatment or progressive disease at the time of restaging by standard imaging modalities, while objective remission have been reported in patients with < 5 CTCs. Available evidences clearly suggest that CTCs can be used as an early predictor of treatment efficacy and extremely useful in sparing patients from futile therapy early in the course of their treatment (Cristofanilli M. et al.2005).

Others important promising cell types as biomarker are the Cancer stem cells (CSCs): Identify and characterize CSCs is of paramount importance and will likely lead to new therapeutic avenues. Using mice models, Al-Hajj et al. found that only a minority of breast cancer cells had the ability to form new tumors. They were able to distinguish the tumorigenic (tumor initiating stem cells) from the nontumorigenic cancer cells based on cell surface marker expression. They prospectively identified and isolated the tumorigenic cells as $CD44^{+}CD24^{-}$ that show to express higher levels of proinvasive genes and have highly invasive properties (Al-Hajj M et al. 2003).

Another candidate marker of CSC is aldehyde dehydrogenase 1 (ALDH1), a detoxifying enzyme responsible for the oxidation of intracellular aldehydes (Duester G, 2000); ALDH may have a role in early differentiation of stem cells, through its role in oxidizing retinol to retinoic acid (Chute JP et al., 2006). Increased ALDH activity has also been found in stem cell populations in multiple myeloma and acute myeloid leukemia (AML) (Matsui W et al., 2004; Pearce DJ et al., 2005). ALDH activity may thus provide a common marker for both normal and malignant stem and progenitor cells. *Ginestier C* et al., showed that normal and cancer human mammary epithelial cells with increased aldehyde dehydrogenase activity (ALDH) have stem/progenitor properties (Ginestier C et al. 2007).

T-regulatory cells: It is becoming increasingly clear that regulatory T cells (T-regs) are equally important in inducing and maintaining peripheral self-tolerance and thus

preventing immune pathologies (Sakaguchi S, 2004; Maloy KJ and Powrie F, 2001). Furthermore, studies in cancer patients suggests that increased T-regs activity may be associated with poor immune responses to tumour antigens and contribute to immune dysfunction resulting in tumour growth (Liyanage UK et al 2002; Siddiqui SA et al 2007). High numbers of T-regs have been found in lung, pancreatic, breast, liver and skin cancer patients, either in the blood or in the tumour itself (Woo EY et al. 2002; Wolf AM et al. 2003; Viguier M. et al 2004; Ormandy LA et al. 2005).

Serum autoantibodies

Immunosurveillance to cancer cells is triggered to initiate antigen-specific tumor destruction (Baldwin RW, 1966; Baldwin RW 1967). The autologous proteins of tumor cells, commonly referred to as TAAs, are thought to be altered in a way that renders these proteins immunogenic. These self-proteins could be overexpressed, mutated, misfolded, or aberrantly degraded such that autoreactive immune responses in cancer patients are induced (e.g. HER2⁄ neu, p53 and ras) (Ward RL et al 1999). TAAs that have undergone post-translational modifications (PTMs, e.g. glycosylation, phosphorylation, oxidation and proteolytic cleavage) may be perceived as foreign by the immune system (Anderson KS and LaBaer J 2005; Caron M et al. 2007).

Autoantibodies possess various characteristics that enable them to be valuable early cancer biomarkers. First, they can be detected in the asymptomatic stage of cancer, and in some cases, may be detectable as early as 5 years before the onset of disease (Fernandez Madrid F, 2005). Second, autoantibodies against TAAs are found in the sera of cancer patients where they are easily accessible to screening. Third, autoantibodies are inherently stable and persist in the serum for a relatively long period of time because they are generally not subjected to the types of proteolysis observed in other polypeptides. The persistence and

stability of the autoantibodies give them an advantage over other biomarkers, including the TAAs themselves, which are transiently secreted and may be rapidly degraded or cleared. Moreover, the autoantibodies are present in considerably higher concentrations than their respective TAAs; many autoantibodies are amplified by the immune system in response to a single autoantigen. Consequently, autoantibodies may be more readily detectable than their corresponding TAAs.

New emerging protein biomarkers

Cyclin D1 and Cyclin E: Cyclin D1 is overexpressed at the mRNA and protein level in over 50% of breast cancer cases including 15% in which a gene amplification occurs. While there is strong evidence that overexpression of cyclin D1 is a prognostic factor for better outcome in invasive breast cancer, in particular among ER-positive patients (Gillett et al. 1996, Hwang et al. 2003, Bilalović N et al. 2005), its amplification is associated with early relapse and poor prognosis (Michalides et al. 1996, Seshadri et al. 1996, Bieche et al. 2002). Cyclin E acts similarly to cyclin D1 as a positive regulator of cell cycle transition with peak levels of protein expression and enzymatic complex formation with cyclin-dependent kinase 2 in the G1 phase (Koff et al. 1992). Cyclin E gene amplification has been detected in several breast cancer cell lines, and there is strong evidence that cyclin E plays a role in tumorigenesis (Buckley et al. 1993).

S100 proteins: One group of proteins that is emerging as a potentially important group of markers in multiple tumour types is the S100 family. There is increasing evidence that altered expression of S100 family members is seen in many cancers including breast, lung, bladder, kidney, thyroid, gastric, prostate and oral cancers. S100 proteins are commonly up-regulated in tumours and this is often associated with tumour progression. The association between S100 family members and tumors can be explained for several

reasons: firstly, the region of human chromosome 1q21, where most of S100 genes are clustered, is prone to genomic rearrangements, likely supporting the tumor progression (Santamaria-Kisiel L et al 2006; Salama I, 2008); secondly, several S100 members show altered expression levels in cancer cells compared to normal cells and are differentially expressed in various malignancies, according to types and stages of cancer (Zhang H et al.2008; Wang G et al 2008). Finally, a number of S100 proteins have been shown to interact with and to regulate various proteins involved in cancer and exert different effects on p53 activity (van Dieck J et al. 2009).

S100 PROTEINS

The S100 genes were among the first genes detected as differentially expressed between normal breast and breast cancer cells (Barraclough R and Rudland PS, 1994), or between different stages of breast cancer (Emberley ED et al.2004), that show the characteristics of possible biomarker for breast cancer. These proteins, identified by B.W. Moore in 1965, are called S100 because of their solubility in a 100%-saturated solution with ammonium sulphate at neutral pH (Moore BW, 1965). The S100 proteins are small, acidic proteins of 10 and 12 kDa, found exclusively in vertebrates (Donato R, 2003). The S100 proteins are a multi-gene calcium-binding family comprising 20 known human members each coded by a separate gene. Many S100 proteins are located on chromosome 1q21, in the so-called epidermal differentiation cluster (Marenholz I et al, 2004; Schafer BW et al.1995).

Structure of S100 proteins

S100 protein are characterized by the presence of two Ca^{2+} binding motifs of the EF-hand type interconnected by an intermediate region often referred to as the hinge region. In each Ca^{2+} binding motif of the EF-hand type, a Ca^{2+}-binding loop is flanked by α-helices, resulting in a helix-loop-helix arrangement. Thus, helices I and II flank loop 1 and helices III and IV flank loop 2 (Salama I.et al. 2008) (figure 7).

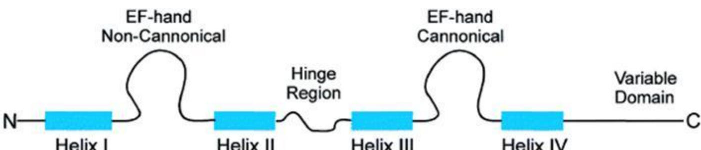


Figure 7: Scheme showing the structure of the protein S100.

In the case of S100 proteins, the first Ca^{2+}-binding loop is unconventional, in that it is longer and rearranged, whereas the second Ca^{2+}- binding loop is canonical. Hence, the two Ca^{2+}- binding sites in an S100 protein bind Ca^{2+} with different affinities, a higher affinity in the case of the C-terminal site and a much lower affinity in the case of the N-terminal site (Donato R, 2001).

The second Ca2+-binding site is followed by a C-terminal extension. As expected, the highest sequence identity among S100 members is found in the Ca2+-binding sites. Instead, the hinge region and the C-terminal extension display the least amount of sequence identity, suggesting the possibility that these two regions might have a role in the specification of the biological activity of individual S100 proteins (Hilt DC and Kligman D, 1991).

The S100 proteins have the ability to form homodimers, heterodimers and oligomers. In general, however, S100 proteins are suggested to function as Ca^{2+} sensor proteins that undergo Ca^{2+}-dependent conformational changes. These latter result in the exposure of a binding surface through which S100 proteins interact with target proteins. (Donato R,1999) (figure 8)

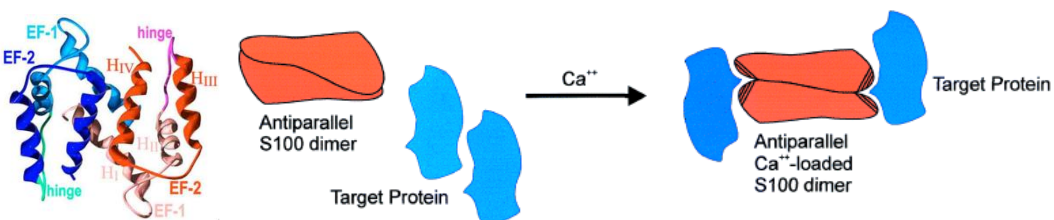


Figure 8: Three-dimensional structure of S100 proteins and model of interaction with target proteins.

Functions of S100 proteins

It is well documented that S100 proteins have a broad range of intracellular and extracellular functions [Donato R, 2001; Donato R, 2003]. Intracellular functions include regulation of protein phosphorylation and enzyme activity, calcium homeostasis, regulation of cytoskeletal components and regulation of transcriptional factors. When they are present extracellularly, they act in a cytokine like manner through the receptor for advanced glycation endproduct (RAGE) (Leclerc E et al. 2009). In particular some members of this family act as leukocyte chemoattractants, macrophage activators and modulators of cell proliferation (Hiratsuka S et al. 2006). These functions associate S100 proteins with a variety of pathologies such as inflammation and cardiomyopathies, and gradually their role in carcinogenesis is beginning to unravel.

S100 proteins and tumor

It is clear that some S100 proteins act as tumour promoters and others as tumour suppressors, and there is tissue specificity in their actions. Moreover a number of S100 proteins interact with p53 and can exert different effects on p53 activity (Mueller A et al. 2005; Grigorian M et al. 2001).

The tumour suppressor p53 is a crucial factor in the development of cancer. It acts as the central inducer of apoptosis and cell cycle arrest (Levine AJ 1997; Vousden KH 2000). Its activity is regulated by posttranslational modifications and interaction with other proteins (Appella E et al. 2000; Braithwaite AW et al. 2006; Lavin MF and Gueven N, 2006).

Both S100A4 and S100B are thought to inhibit p53 phosphorylation leading to inhibition of its transcriptional activity, thereby compromising p53 tumour-suppressor activity (Grigorian M et al. 2001). In contrast S100A2 promotes p53 transcriptional activity and interestingly S100A4 has also been documented as enhancing p53-dependent apoptosis.

Thus the balance of actions of different S100 proteins within a cell will determine P53 function. Many of the S100 family members have a role in modulating cytoskeletal dynamics. Again they display remarkable diversity of function, exhibiting direct interaction with tubulins, intermediate filaments, actin, myosin and tropomyosin (Santamaria-Kisiel L et al.2006). Some of these processes have been implicated in mediating metastasis. A number of members of the S100 family have also been shown to play a role in modulating proliferation, while both S100A1 and S100A11 shown to inhibit cell proliferation (Kriajevska M et al.1998).

S100A2

S100A2 protein was identified as tumor suppressor gene by subtractive hybridization between normal and tumor derived human mammary epithelial cells (Lee S et al. 1991). S100A2 gene is markedly down-regulated in several tumor tissues of various origins like melanomas (Maelandsmo G. et al. 1997) and breast carcinoma (Pedrocchi M et al. 1994). Repression of gene expression in tumor cells depended by site-specific DNA methylation of the S100A2 gene promoter (Wicki, R et al. 1996). Moreover, several growth factors were reported to alter the S100A2 gene expression at late G1/S-phase, indicating that S100A2 is cell cycle-regulated (Lee S et al.1992). Much attention has been paid to the expression of the S100A2 gene and gene product in head and neck squamous cell carcinoma (HNSCC), where is overexpressed (Nagy N et al 2001; Lauriola L et al. 2000).

S100A3

S100A3 binds Ca^{2+} with poor affinity but Zn^{2+} with exceptionally high affinity. This high affinity for Zn^{2+} is attributed to the unusual high Cys content of S100A3. The protein is highly expressed in fast proliferating hair root cells and astrocytoma pointing toward a function in cell cycle control. (Gunter F et al. 2002).

Previous studies suggest a close association of S100A3 with epithelial differentiation, leading to hair shaft formation, but its molecular function is still unknown.

S100A4

S100A4, also named metastasin, is a small molecule and can pass through the nuclear pores without any active transport mechanism being involved. S100A4 binds and inhibits phosphorylation of the p53 C-terminal peptide by protein kinase C. The tumor suppressor protein p53 has also been identified as an S100A4 interacting protein and may provide a link between S100A4 and apoptosis (Lombet A et al. 2003).

S100A4 is involved in several aspects of tumor progression including motility, invasion, metastasis and apoptosis (Takenaga K et al. 1994; Ambartsumain N et al.2001) (figure 9). The molecular mechanisms by which S100A4 exerts these functions remain unclear, and considerable functional diversity is created by the fact that extracellular, intracellular, tumor-derived, and stroma-derived S100A4 all contribute to metastatic dissemination. Intriguingly, S100A4-mediated induction of EMT may explain several of the prometastatic actions of S100A4 (Boye K and Maelandsmo GM, 2010).

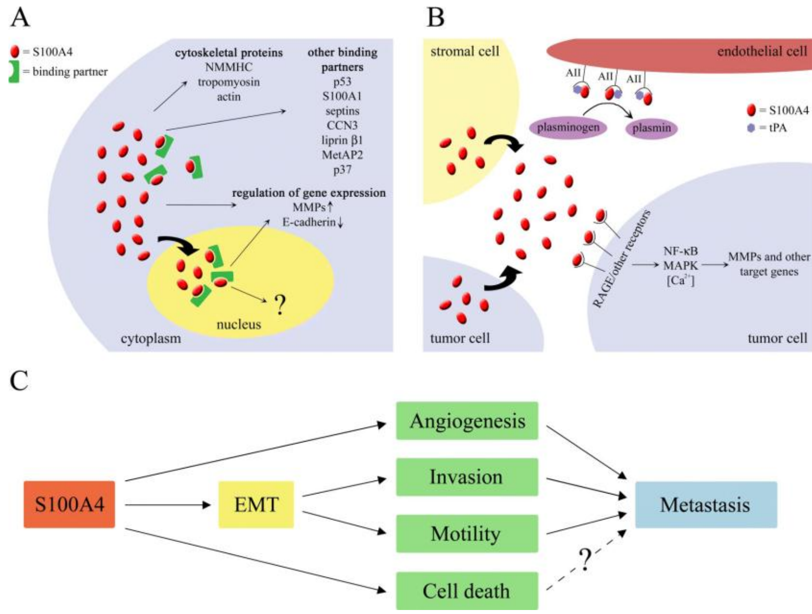


Figure 9: Mechanisms involved in S100A4-mediated metastatic progression. **A:** Molecular mechanisms associated with intracellular functions of S100A4. S100A4 interact with cytoskeletal proteins, particularly nonmuscle myosin heavy chain (NMMHC) IIA, and increase cell migration. Intracellular S100A4 expression is also associated with transcriptional regulation of certain genes, such as MMPs and E-cadherin, through so far unidentified mechanisms. **B:** Molecular mechanisms associated with extracellular S100A4. S100A4 is released from both tumor cells and stromal cells. Through interaction with annexin II (AII) and tissue plasminogen activator (tPA) on the surface of endothelial cells, S100A4 stimulates the conversion of plasminogen to plasmin, thus promoting angiogenesis. S100A4 also interacts with other cell surface receptors on both tumor cells and stromal, such as RAGE, to activate intracellular signal transduction cascades, including mitogen-activated protein kinases, increased intracellular [Ca^{2+}], and nuclear factor-κB. S100A4-activated signaling pathways may, in turn, result in regulation of several target genes probably involved in stimulation of metastasis. **C:** Biological processes associated with S100A4. S100A4 stimulates cell motility, invasion, and angiogenesis and participates in the regulation of cell death. Invasion and motility is probably promoted through induction of EMT. Cell motility, invasion, and angiogenesis all contribute to stimulation of metastasis.

It has also been reported that extracellular secreted S100A4 can affect cell differentiation and migration (Belot N et al. 2002). Numerous studies have investigated the potential use of S100A4 as a prognostic marker. An increase in S100A4 protein expression has been correlated with a worse prognosis for patients with different types of cancer including

colorectal, gallbladder, bladder, esophageal, breast, and non small lung cancer (Gongoll S et al. 2002).

S100A6

The S100A6 protein (calcyclin), similarly to other members of the S100 protein family, forms homodimers in solution in a Ca2+- independent manner (Wojda U and Kuźnicki J 1994; Potts BC et al. 1995). In addition, S100A6 may form a covalent dimer through a disulfide bridge between cysteine residues at position 2.

S100A6 is predominantly a cytoplasmic protein but in the presence of Ca^{2+} it associates with the plasma membrane and the nuclear envelope (Stradal TB and Gimona M, 1999), possibly as a result of its interaction with membrane-bound proteins. S100A6 expression at the protein and/or mRNA level can be upregulated by multiple factors including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), tumor necrosis factor (TNFa) (Tsoporis JN et al. 2008), retinoic acid (Tonini GP et al. 1991), estrogen (Hong EJ et al. 2006). S100A6 level also increases upon stress conditions such as ischemia (Lewington AJ et al. 1997), irradiation (Orre LM et al. 2007) or oxidative stress (Lesniak W et al. 2005).

Several early reports prompted the investigation of a potential role of S100A6 in the development of malignancy. For instance, high S100A6 mRNA levels were found in peripheral blood cells from patients with chronic myelogenous leukemia and in some human breast cancer cell lines.

Owing to alternations of the S100A6 expression level during cancer progression the S100A6 protein was recognized as a useful diagnostic tool for defining cancer stage and discriminating between various cancer types as in the case of cholangio carcinoma and

hepatocellular carcinoma (Kim J et al. 2002). Furthermore, differences in S100A6 level can be useful for the discrimination between primary liver tumors such as hepatocellular carcinoma and metastases derived from colorectal carcinoma (Melle C et al. 2008).

S100A7

Psoriasin (S100A7) was originally identified as one of the most abundant proteins in psoriatic keratinocytes (Madsen P et al. 1999). Subsequent studies showed that psoriasin expression is up-regulated in abnormally differentiating keratinocytes, squamous carcinomas of different organs, and in a subset of breast tumors (Alowami S et al. 2003; Emberley ED et al. 2004). Psoriasin was identified as one of the few genes that is highly and more frequently expressed in ductal carcinoma in situ (DCIS) than in invasive breast carcinomas, suggesting a potential role in tumor progression. In human invasive breast carcinomas, the expression of psoriasin has been shown to correlate with unfavourable histopathologic features, including estrogen receptor (ER) negativity and poor clinical outcome (Enerback C et al. 2002; Porter D et al. 2003). Psoriasin protein was found to be secreted but also present in the cytoplasm and the nucleus of cells expressing it. Psoriasin was found to interact with Jab1 (figure 10), a component of the COP9 signalosome that is involved in multiple signal transduction pathways, including the regulation of E3 ubiquitin ligases and the JUN/AP1 transcription factors. Overexpression of psoriasin in MDA-MB-231 breast cancer cells was shown to influence the intracellular distribution and activity of Jab1 and enhance tumorigenesis and metastasis (Emberley ED et al. 2003).

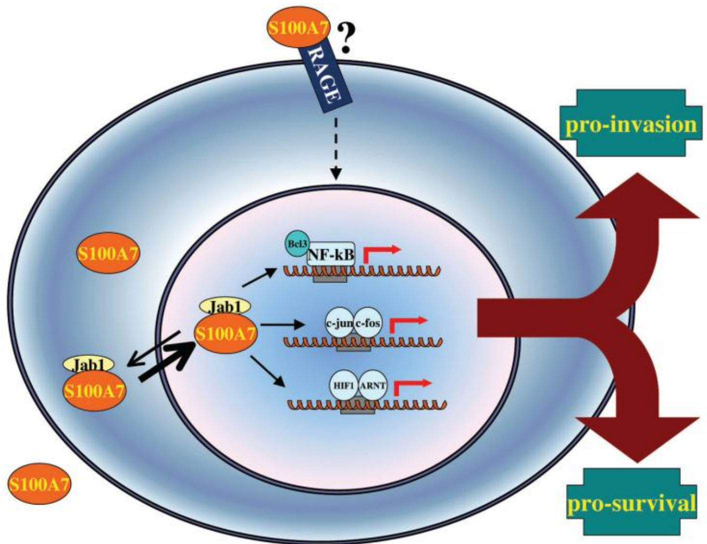


Figure 10: The influence of S100A7 on the prosurvival and proinvasive pathways is mediated through alterations in gene expression. The interaction of S100A7 with c-jun activation domain binding protein 1 (Jab1) results in a cellular redistribution of Jab1 to become predominately localized in the nucleus, where it stimulates gene transcription. Jab1acts as a cofactor to stimulate transcription of genes regulated by the NF-κB, activator protein- 1 and HIF1 transcription factors. Extracellular S100A7 may also interact with the receptor of advanced glycation of end products (RAGE) receptor, resulting in activation of signal transduction cascades and, ultimately, activation of gene transcription. S100A7 is believed to exert its effects through Jab1 and other proteins with which it forms an interaction, and one outcome is an alteration in gene transcription.

S100A8-S100A9

S100A8 and S100A9 are released from activated phagocytes and exerts antimicrobial activity as well as cytotoxicity against various tumour cells (Ghavami S et al. 2004). They are differentially expressed in a wide variety of cell types and are abundant in myeloid cells. High expression of S100A8 and S100A9 has been reported in disorders such as rheumatoid arthritis, inflammatory bowel disease and vasculitis (Nacken W et al. 2003).

The interaction with RAGE represent a novel proinflammatory axis involved in several inflammatory diseases. Evidence has accumulated that S100A8/A9 induces cell death

through a dual mechanism: one associated with zinc extraction from the target cells, the other through binding to the target cell surface, possibly via ligand-induced receptor activation (Kerkhoff C et al. 1998).

S100A11

In several studies, S100A11 was shown to be up- or down-regulated in different tumor entities (Melle C et al. 2006). S100A11 plays a dual role in growth regulation of human keratinocytes: inhibit the Ca2+- induced cell growth and stimulate the cell growth enhancement of the level of EGF protein family members (Sakaguchi M et al. 2003; Sakaguchi M et al. 2008). S100A11 through a p53 independent mechanism is able to mediate the stimulation of the activity of the cell cycle regulator $p21^{WAF1/CIP1}$ during cellular stress stimuli such as increase of extracellular Ca^{2+} concentration as well as induction of DNA damage (Murzik U et al. 2008). In particular, S100A11 by nucleolin-mediated translocation into the nucleus regulate p21 transcription (Figure 11).

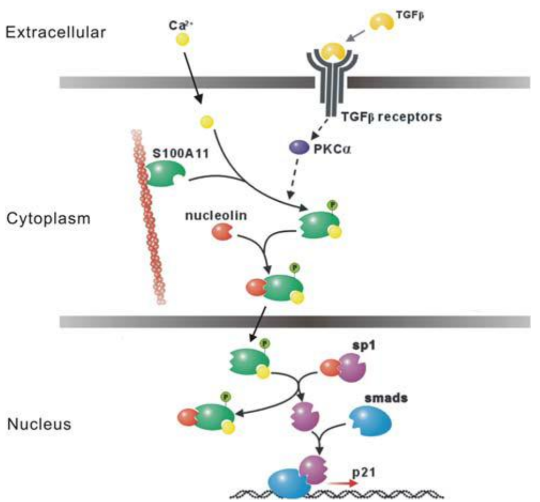


Figure 11: schematic representation of the interaction between nucleolin and S100A11; the complex translocate into the nucleus and regulate the p21 transcription.

Nucleolin was described as a shuttling protein which is involved in directed translocation into the nucleus of several proteins (Stepanova V et al. 2008; Jerke U et al. 2009).

It is also demonstrated that the accumulation of S100A11 in nuclei was more effective after DNA damage compared to the Ca^{2+} stimulus.

S100A12

S100A12 (EN-RAGE, Calgranulin C) is a members of the S100/calgranulin family such as S100A8 (MRP8, Calgranulin A) and S100A9 (MRP14, Calgranulin B).

The role of S100A12 has been implicated in the innate immune system in response to infection. The exact nature of S100A12 ligand interaction is unclear, it has been shown able to bind both itself and a range of other molecules and was suggested to act as a molecular chaperone in the presence of calcium (Hatakeyama T et al. 2004). RAGE has been identified as a receptor for S100A12 (Hofmann MA et al. 1999). The RAGE interaction leads to initiation of pro-inflammatory signalling cascades via MAP-Kinase and NFkB, resulting in increased adhesion molecules expression (Yang Z et al. 2001). Increased serum concentration of S100A12 has been shown in patients with severe bacterial infections (Kim MH et al. 2006), and therefore used as diagnostic marker in inflammatory bowel disease (Foell D et al. 2003), arthritis (Foell D and Roth J, 2004) and asthma (Yang Z et al. 2007). Moreover, S100A12 interaction with RAGE is able to activate endothelial, lymphocyte and macrophage cells directly (Roth J et al. 2003).

S100A13

Although S100A13 is similar to most S100 family members in that its homodimers possess two high- and low-affinity Ca^{2+} binding sites, it does not exhibit a calcium dependent exposure of the hydrophobic surface which is important for the interaction of other S100

proteins with their target peptides (Ridinger K et al. 2000). This protein has since been found to play a crucial role in the release of FGF-1 (Mouta Carreira C et al. 1998). The FGF family is known to be involved in angiogenesis (Presta M et al. 2005) and tumour metastasis and the growth is dependent upon tumour angiogenesis in vivo (Folkman J and Shing Y, 1992) Indeed, it is possible that S100 gene family members, including S100A13, support neoplastic and pro-inflammatory situations in vivo by their ability to participate in the release pathway for angiogenic and inflammatory signals such as FGF-1 and IL-1a (Mandinova A et al. 2003). S100A13 is widely expressed in many types of tissues, with particularly high expression in the thyroid gland. The localisation of S100A13 expression in human smooth muscle cells differs from that of all other S100 proteins. S100A13 is expressed in the perinuclear area of these cells, which suggests diverse functions for S100A13 in signal transduction (Ridinger K et al. 2000).

S100A15

The highly homologous human S100A15 (hS100A15) was recently identified in psoriasis and found to also be upregulated in lesional skin (Wolf et al. 2003). Genomic analysis of the S100A7 and S100A15 encoding chromosomal regions reveals that both must have evolved by gene duplications during primate evolution, forming an innate subfamily in man (Kulski et al. 2003).

The co-expression of the hS100A7 and hS100A15 in psoriasis suggests that both proteins participate in keratinocyte maturation, proliferation and/or skin inflammation. However, the existence of two highly similar paralogs (>90% identity) sets certain limits in discriminating between them when co-regulated. Furthermore, little is known about the

functional role of the S100A7/A15 proteins in cutaneous pathology, yet they are frequently dysregulated in a variety of pathological conditions including cutaneous cancer.

AIM OF THIS STUDY

Breast cancer is still one of the leading causes of cancer death in women. Indeed, it consists of several different subtypes with different molecular profiles, biological behaviour, and risk profiles. Current clinical parameters with prognostic and predictive factors constitute important tools for the identification of breast cancer therapy providing an efficient treatment. However, these parameters remain inadequate for appropriate stratification of patients, given that more than 1% of all human genes are implicated via mutation in cancer. Proteomic screening for differential protein expression in tumor samples represents a fundamental tool to understand biological pathways involved in breast cancer. In addition, it could be useful for the identification of biomarkers also in early detection of cancer. Moreover immunoproteomics, that defines the subset of proteins involved in the immune response, hold considerable promise for the discovery of tumor-associated antigens (TAAs) and autoantibodies targeting specific tumor-associated antigens as potential group of serum biomarkers in early cancer diagnosis.

Preliminary study performed in our laboratory demonstrates quantitative and qualitative differences in the proteomic profiles between ductal infiltrating breast cancer tissues and the non-tumoral adjacent counterparts. Subsequently, from the analysis of comparative proteomic profiles of breast cancer tissues, it has been shown that a large proportion of the tumor-derived proteins are present in all patients, including glycolytic enzymes, detox and heat shock proteins, and calcium binding proteins; conversely some proteins, including psoriasin (S100A7), cofilin1 and galectin, showed sporadic and high expression level, which suggest their possible role for patients stratification (Pucci-Minafra I et al. 2007). In particular, a group of proteins that has emerged as potentially important markers in multiple types of cancer is the S100 family. The altered expression of S100 family

members was demonstrated in several types of cancer (breast, lung, bladder, kidney, thyroid, stomach and prostate) and in general the overexpression of these proteins is associated with tumor progression.

Although the knowledge about cancer biomarkers is increasing enormously in the last years, however much still remains to be comprehend for a better stratification of patients and to improve the management of cancer patients by enhancing the efficiency of detection and efficacy of treatment.

In order to identify new protein clusters involved in breast cancer progression and to find potential diagnostic, prognostic and therapeutic biomarkers, the present study aims to:

- Expand the number of breast cancer tissues analysed, increasing the number of identified proteins and find new functional pathways involved in breast cancer progression.
- Extended the research to S100 proteins family that are potentially involved in tumor progression.
- Identify circulating antibodies direct against specific tumor antigens as putative biomarker through SERPA approach.

PRELIMINARY DATA

Preliminary study performed in our laboratory demonstrates quantitative and qualitative differences in the proteomic profiles between ductal infiltrating breast cancer tissues and the non-tumoral adjacent counterparts of 13 pairs of surgical samples (figure 12). Tumor tissues showed a greater abundance of cellular proteins compared with normal counterparts, enriched instead in extracellular amount (Pucci-Minafra et al. 2007). The healthy tissue of adult women mammary gland contain a reduce amount of parenchyma with respect to the tumor mass.

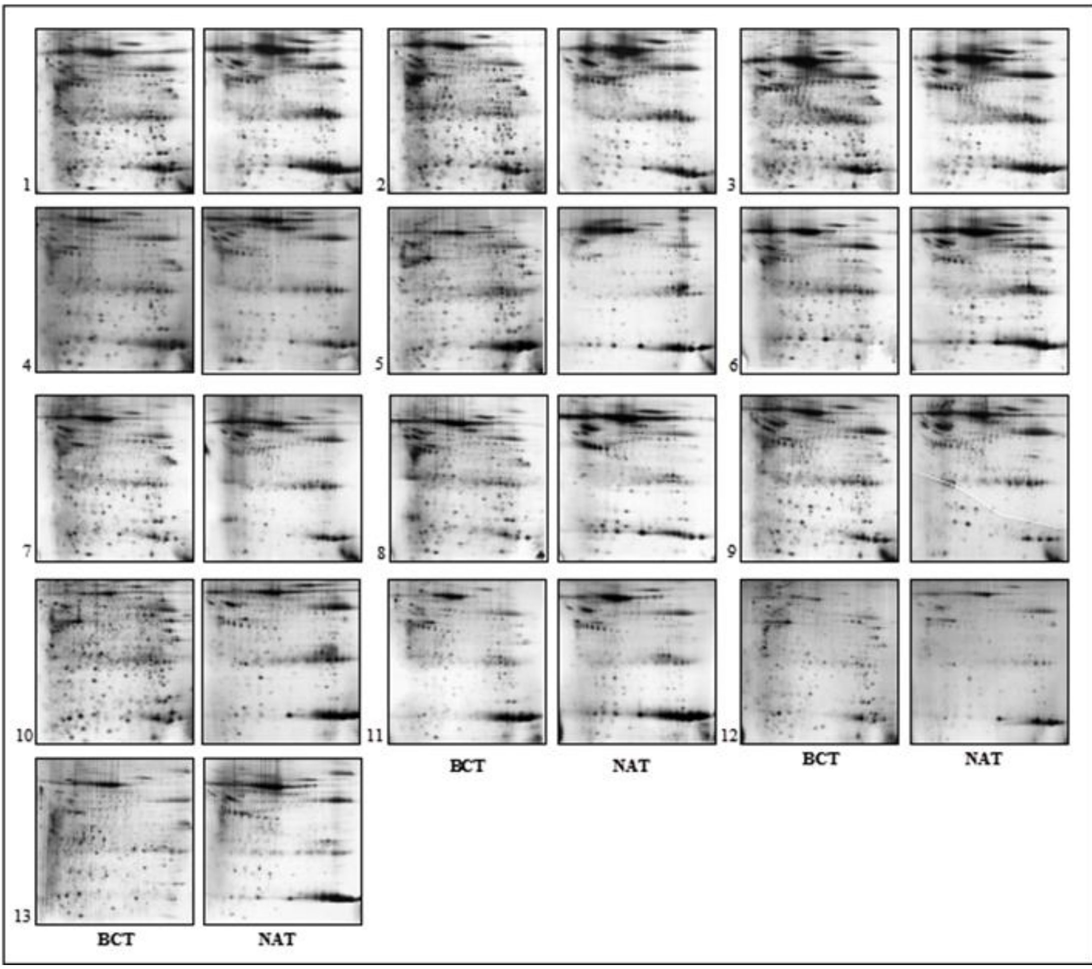


Figure 12: Panel showing the miniatures of the 2-D matching maps from 13 surgical specimens of BCT and its NAT.

In figure 12, 13 proteomic maps of breast cancer tissue and their adjacent healthy counterparts are shown. These maps have been analysed with Image Master 2D Platinum and compared for differentially expressed proteins. These were grouped based on the closest affinity for their major biological functions in: 1) cytoskeleton and associated proteins; 2) metabolic enzymes; 3) molecular chaperones; 4) proliferation and differentiation regulators; 5) detoxification and redox proteins; 6) protein degradation; 7) serum proteins.

Subsequently, proteomic profiles from 37 breast cancer surgical tissues, with ductal infiltrating diagnosis, were compared for proteomic screening of differential expression proteins. This approach indeed may contribute to the knowledge of biological pathways and allow a molecular classification of cancer for patients stratification and biomarkers discovery (Pucci-Minafra et al. 2008).

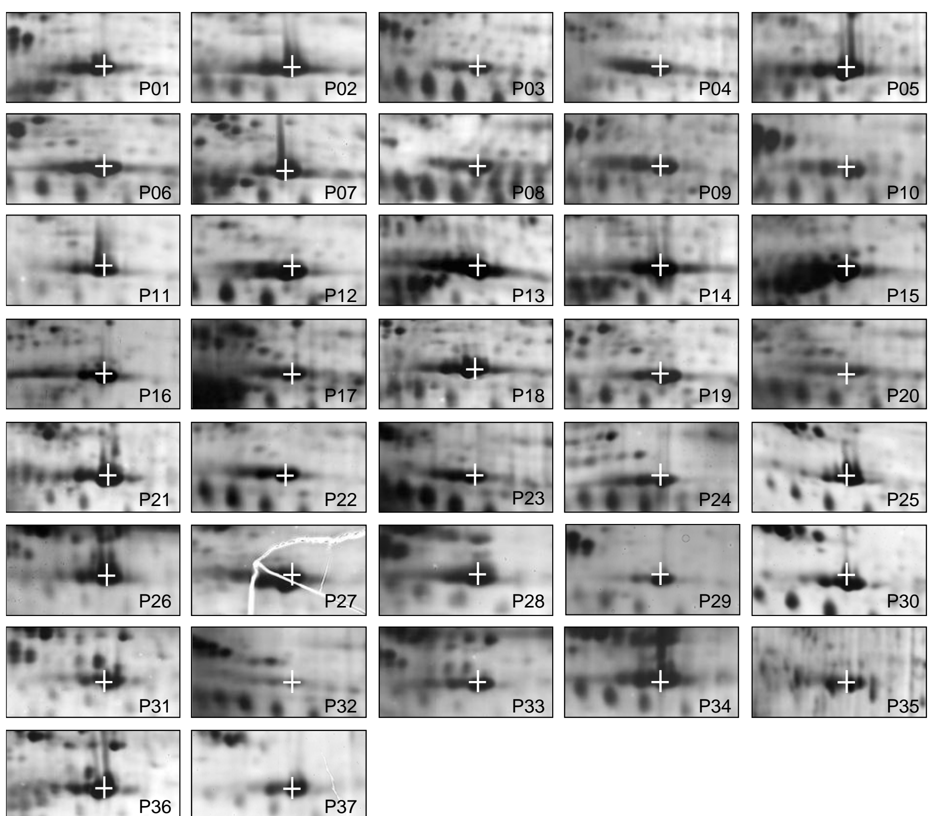


Figure 13: Panel showing an experimental window from ImageMaster 2D Platinum software containing the sections of actin spots (white crosses) from the matched two-dimensional gels of the 37 breast cancer surgical tissues.

In this study, since proteomic profiling may be influenced by extension and amount of neoplastic cells within the host stroma, an objective criterion to compare proteomes of different tissues has been introduced: the expression levels of individual proteins have been normalized for actin content. Figure 13 shows cropped windows containing the actin spot from 37 2D-gels, attesting the variability of actin amount among different tissues, most likely as an effect of different cellularity (that is, cell densities within an area of the surgical sample), in the examined samples, under the same quantity of protein loading in the 2D-IPG.

Analysing the distribution of the identified protein among the group of 37 breast cancer tissues, it is clear that some proteins are ubiquitously present in all examined tissues, whereas others appear sporadically. Among the sporadic one, some proteins when present, showed high expression levels. For instance Psoriasin (S100A7) is one of the most variable proteins among the 37 patients. It is highly expressed in 15/37 patients.

RESULTS

In order to identified novel protein clusters in breast cancer tissues potentially involved in tumor progression, the first part of my thesis regarded the enlargement of previous cohort. Indeed the number of processed and studied samples was increased, up to 100 breast cancer tissues as shown by miniature of the 2D gels (figure 14).

Figure 14: miniature of 100 2D gels from ductal infiltrating breast cancer tissues.

The proteomic expression profiles among 2D gels resulted comparable, even if was evident the heterogeneity of protein expression levels among different tumoral tissue, under the same quantity of proteins loaded in 2D-IPG, due to a different content in cellularity of tumor mass.

The number of identified proteins, was also enhanced through mass spectrometry and/or N-terminal sequencing, as describe in MM, identifying 213 protein spots corresponding to 133 genes.

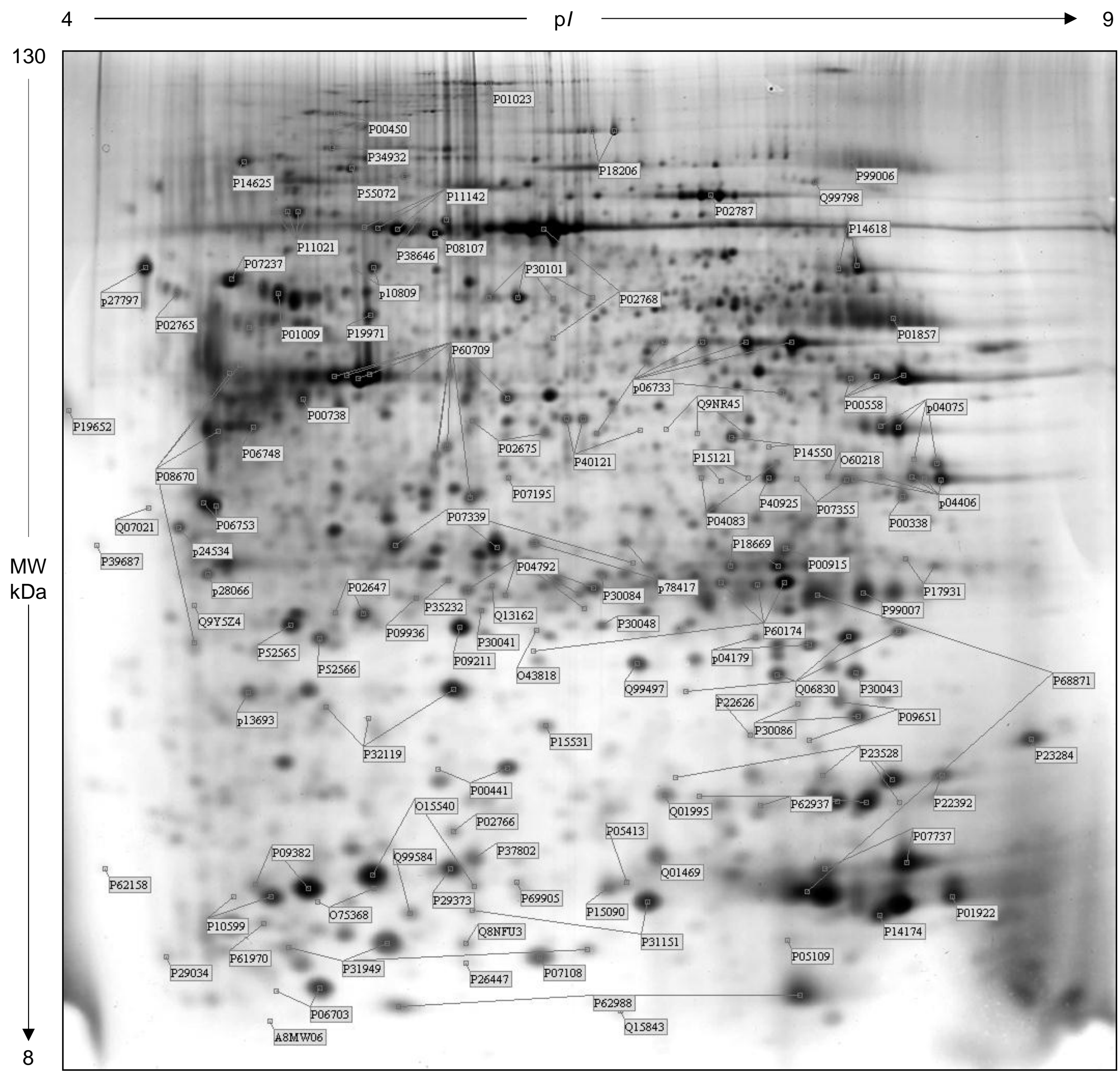


Figure 15: prototype of a proteomic map of breast tumor tissue, used as reference map in this study. It shows the protein species identified by the access number of the Swiss-Prot/TrEMBL database.

In order to rationalize our data and to find functional pathways in which the identified proteins are involved, a gene ontology approach has been used. To this aim DAVID (Database for Annotation, Visualization and Integrated Discovery) Bioinformatics Resources, has been utilized as an excellent tool to provide functional interpretation of large lists of genes derived from genomic and proteomic studies. The program recognizes gene lists and displays results in terms of significance, correlating to each group of genes two values that are Fisher exact P-value index and Benjamini FDR: False Discovery Rate.

The first is a variant of the *p-value* that indicates whether the association of a gene in a given cluster is reliable or due to chance; the second parameter of significance is *Benjamini*, who represents the percentage of false positives that you would expect from a list of genes that DAVID has grouped in the same cluster.

The gene list corresponding to the 213 proteins was uploaded into DAVID Bioinformatics database that identified, with a certain degree of significance, the molecular pathways in which these proteins are involved, by clustering them in functional classes. The application of the powerful Bioinformatics Resources for gene/protein classification provided by DAVID knowledgebase, while confirming our previous protein classification, introduced new terms for further remodulation of protein clusters on the basis of the multiple functions for individual proteins. In particular we found a high number of proteins with specific biochemical functions, converging towards common pathways. Overall, three predominant pathways the gene cluster of regulation of programmed cell death, comprising 23 genes, cell motility cluster, comprising 17 genes, and glycolysis, comprising 11 genes, were at first places for robustness (figure 16). It is known, that proteins belonging to these clusters are involved in different biological processes and play different cellular functions but may be deregulated during cancer progression.

Annotation Cluster 1	Enrichment Score: 12.5		Count	P_Value	Benjamini
GOTERM_BP_FAT	regulation of programmed cell death	RT	31	8.6E-13	2.3E-10
Annotation Cluster 2	Enrichment Score: 6.96		Count	P_Value	Benjamini
GOTERM_BP_FAT	glycolysis	RT	11	3.1E-12	5.8E-10
Annotation Cluster 3	Enrichment Score: 5.35		Count	P_Value	Benjamini
GOTERM_BP_ALL	response to stress	RT	31	1.6E-5	3.7E-4
Annotation Cluster 4	Enrichment Score: 5.02		Count	P_Value	Benjamini
GOTERM_BP_ALL	cell motion	RT	17	1.2E-6	3.8E-5

Figure 16: functional cluster of identified protein obtained through DAVID Bioinformatics Resource.

Regulation of programmed cell death

The processes related to programmed cell death are essential for turnover of cells in organs and tissues, as well as to remove cells whose genome would be irretrievably altered by chemical, physical and environmental factors. The alteration of expression and activity of proteins belonging to this cluster may cause an imbalance between pro-apoptotic and anti-apoptotic stimuli, leading to a neoplastic transformation.

The genes corresponding to the identified proteins belonging to this cluster were: CALR, 2 isoforms of ANXA1, ANXA5, 3 isoforms of COF1, DDAH2, GDIR, GRP94, GSTPI, 5 isoforms of HSP27 , 2 isoforms of HSP60, 2 isoforms of LEG1, MIF, NDKA, NDKB, NPM a, 2 isoform of PDIA3, PHB, 3 isoforms of PRDX2, PSA5, SODC, SODM, TCTP, 2 isoforms of TERA. About the 50% of the genes belonging to this category, codify for proteins with anti-apoptotic functions. The cluster of anti-apoptotic proteins, corresponding to 12 genes was compared among the 100 proteomics maps and the 13 reference non-tumoral tissue maps. The comparative proteomic profiling showed: 1) a highly significant overexpression of several members of the anti-apoptotic protein cluster in the cancer tissues vs non tumoral counterparts; 2) a relative variability of the expression levels of the normalized proteins within patients. The distribution among the whole group of 100 patients of the cell death cluster was significantly high, with an average of 90% of protein

expression (figure 17). The general expression pattern of proteins involved in anti-apoptotic pathways confirms the cell survival-dependence for primary tumor growth.

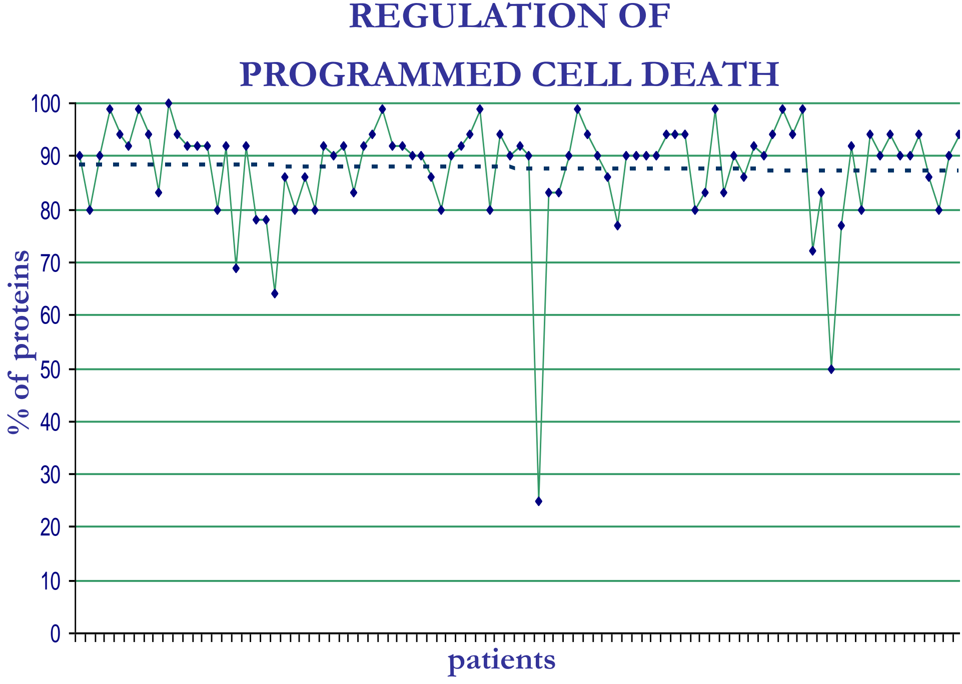


Figure 17: The regulation of programmed cell death graph show for each patients, the distribution of clusters that express.

Among proteins of this category reaching the high levels of expression, we observed: *nucleophosmin* (NPM), a crucial regulator of p53; *translationally-controlled tumor protein* (TCTP1), a protein involved in calcium binding and microtubule stabilization; *cofilin* (COF1), an actin-binding protein responsible also for the signal translocation from cytoplasm to nucleus; *annexin A1* (ANXA1) a calcium/phospholipid-binding protein which promotes membrane fusion and ruffling, *glutathione s-transferase* (GSTP1), which plays important roles in detoxification but having also a role in susceptibility to cancer; *heat shock protein 27* (Hsp27) that regulates apoptosis through its ability to interact with key components of the apoptotic-signaling pathways, particularly those involved in caspase activation; *peroxiredoxin 1* (Prx I) regulates cell proliferation and apoptosis by its

interaction with oncogene products such as c-Abl; *NDKA* also named nm23-H1 plays complex roles in the development of diverse cancers including breast carcinoma, high-grade lymphomas, and acute myeloid leukemia (AML). High levels of nm23 gene expression has been noted in the advanced stage of thyroid carcinomas and associated with significant reductions in survival for neuroblastoma and osteosarcoma patients.

Cell motion

Cancer cells usually undergo to profound remodelling during neoplastic transformation, and this lead to acquisition and manifestation of the invasive phenotype of tumor cells. Among the proteins belonging to cell motility cluster there are ACTB, ANXA1, ANXA1 a, COF1 b, COF1 c, COF1 d, GDIR, GDIS, HSP27 a, HSP27 b, HSP27 c, HSP27 d, HSP27 e, VIME a, VIME b, VIME c, VIME d, VIME fr, VINC a, VINC b several of them are implicated in different diseases, such as: the actin binding protein called *Cofilin* is involved in the invasive and metastatic phenotype of tumour cells (Wang W et al. 2007). In many cell types, the formation of membrane protrusions and directional migration depend on the spatial and temporal regulation of this protein; *Annexin A1* (AnxA1) is a candidate regulator of the epithelial- to mesenchymal (EMT)-like phenotypic switch, a pivotal event in breast cancer progression. AnxA1 promotes metastasis formation by enhancing TGFbeta/Smad signalling and actin reorganization, which facilitates an EMT-like switch, thereby allowing efficient cell migration and invasion of metastatic breast cancer cells (de Graauw M et al. 2010); *Vimentin* is one of the cytoplasmic intermediate filament proteins which are the major component of the cytoskeleton. Elevated vimentin expression level well correlates with up-regulated migration and invasion of cancer cells (Gilles C et al. 2003).

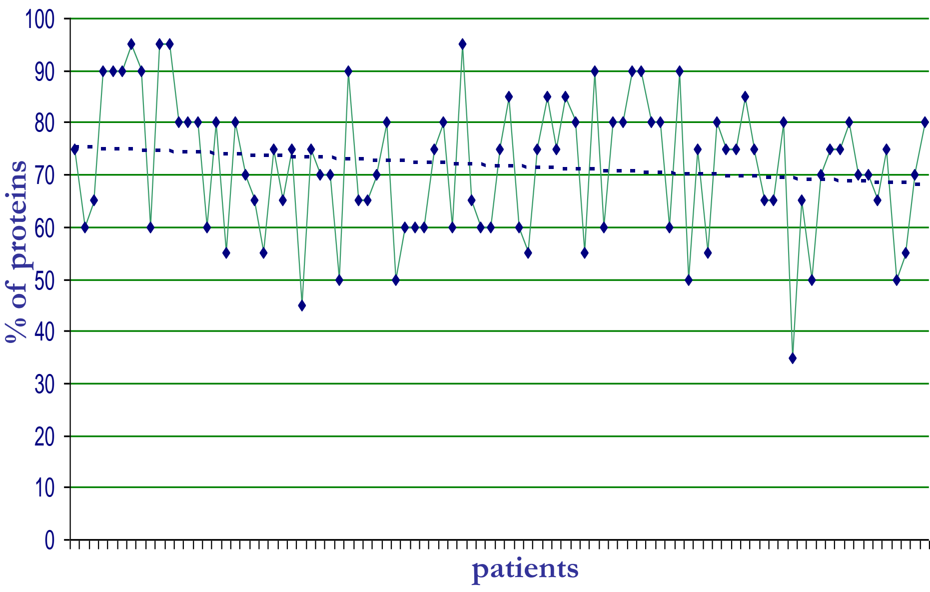


Figure 18: The cell motion graph show for each patients, the distribution of clusters that express.

The distribution of the cell motility cluster was significantly lower rather the regulation of programmed cell death cluster and dissimilar among patients, with an average of 70% of protein expression among patients (figure 18).

The more irregular expression of proteins involved in cell motility, clearly demonstrated the heterogeneity of metastatic potentialities among patients and suggests the possibility of using some of the motility cluster proteins as prognostic factors for metastasis propensity.

Glycolysis

Cancer cells alter their metabolism in order to support their rapid proliferation. Glucose deprivation can activate oncogenes and these can upregulate proteins involved in aerobic glycolysis. In turn, proteins implicated in increased glycolysis make tumor cells more resistant to apoptosis. Aerobic glycolysis induces acidification of the tumor environment,

by favouring the development of a more aggressive and invasive phenotype (Annibaldi A and Widmann C, 2010). It is well established that the process of cancer development and growth involves major alterations of cells' metabolism, leading to increase of proteins levels involved in this process.

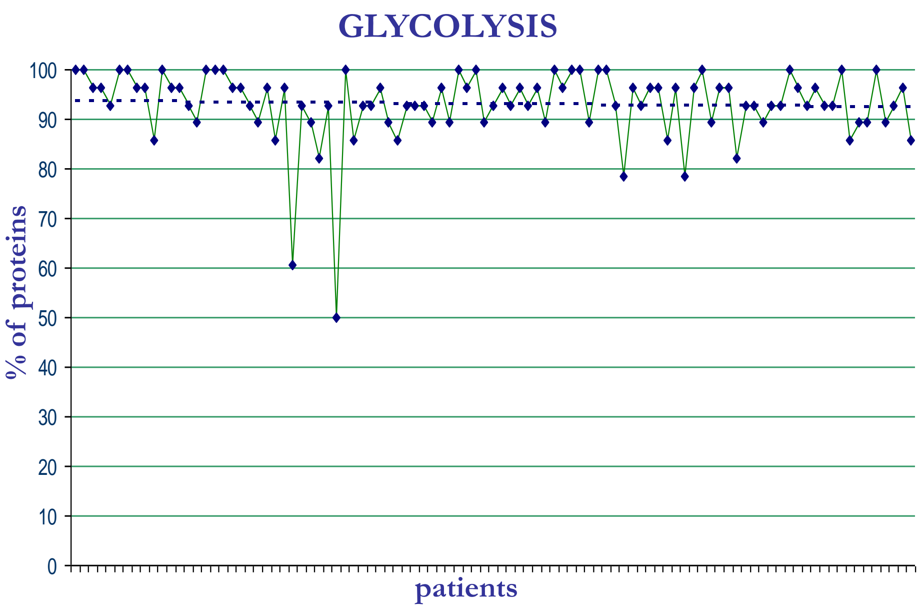


Figure 19: The glycolysis graph show for each patients, the distribution of clusters that express.

This cluster contain 2 isoforms of ALDOA, 4 isoforms of ENOA, 2 isoforms fragment of ENOA, ENOG, 5 isoforms of G3P, 2 isoforms of KPYM, LDHA, LDHB, MDHC, 2 isoforms of PGAM1, 3 isoforms of PGK 1, 4 isoforms of TPIS.

The distribution of protein belonging to glycolysis cluster among patients, is quite high, with an average of almost 95% of protein expression (figure 19). Among the glycolytic enzymes, some of them have different roles: *α-enolase* for example, acts also a plasminogen receptor (Redlitz A et al. 1995) and thus mediates activation of plasmin and extracellular matrix degradation. In tumor cells α-enolase is up-regulated and supports anaerobic proliferation (Warburg effect), it is expressed at the cell surface, where it

promotes cancer invasion, and is subjected to a specific array of post-translational modifications, namely acetylation, methylation and phosphorylation. Both α-enolase overexpression and its post-translational modifications could be of diagnostic and prognostic value in cancer; *GAPDH* recently is described as a regulator of cell death; whereas some studies point toward a proapoptotic function, others describe a protective role and suggest its participation in tumor progression.

RESULTS II

Since S100 family can be consider as a potentially promising group of markers in cancer development and progression, we extended our research to identified other member of S100 proteins family also because, as described in our preliminary data, are expressed sporadically and with high level in breast cancer tissues (Pucci-Minafra et al., 2008).

Proteomic screening for S100 members identification

In this study is shown, for the first time, a large-scale proteomic investigation on breast cancer patients for the screening of multiple forms of S100 protein.

Figure 20 shows the region covering a pI/kDa range of 4.5-7/15-9 kDa of a breast cancer tissue reference map, in which the majority of the S100 proteins are expected to localize. After a comprehensive mass-screening of protein spots included in this area, in different proteomic maps, we identified by MALDI-TOF the following S100 protein members: S100A2 (protein S-100L), S100A4 (metastasin), S100A6 (Calcyclin, Prolactin receptor-associated protein), two isoforms, S100A7 (psoriasin), two isoforms, S100A8 (Calgranulin-A), S100A11 (Calgizzarin), three isoforms and S100A13 (S100 calcium-binding protein A13). A protein number appeared as multiple spots on 2-D gels, corresponding to isoforms of the same protein, possibly after post-translational modifications.

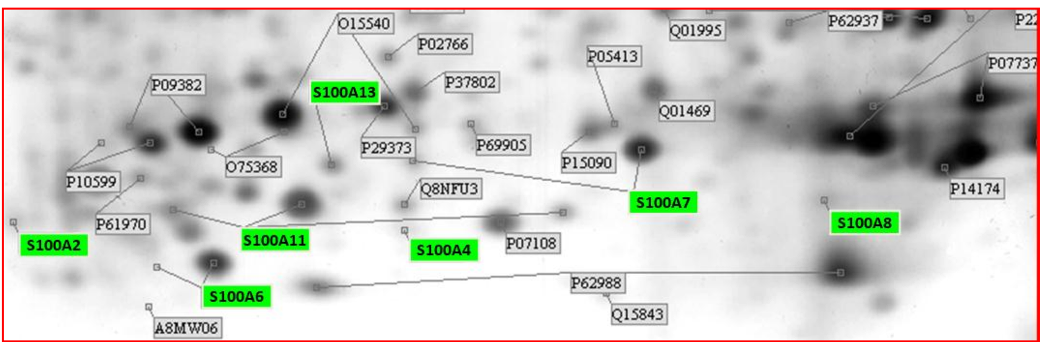


Figure 20: region of 2D gel covering a pI/kDa range of 4.5-7/15-9 kDa where localize the majority of S100 proteins

Comparative proteomic analysis of S100 proteins between breast cancer tissues and normal adjacent tissues

Comparative proteomic analysis performed in a pilot group of 10 patients showed the high significant expression of S100 members in the cancer tissues vs non tumoral counterparts. As shown in cropped windows of 2D gels, the S100 proteins are almost exclusive expressed in tumor tissues compared to adjacent healthy counterparts, even though the expression of some members (for example S100A2, S100A4, S100A7a and S100A8) is very low also in the tumor tissue (figure 21 A). The quantitative analysis of S100 protein expression level between breast cancer and normal adjacent tissues, performed by Image-Master software as described in MM, showed significant differences for all S100 protein members (figure 21 B), except for S100A4 (expressed only in 5 patients) and for S100A6 (expressed also in normal tissues).

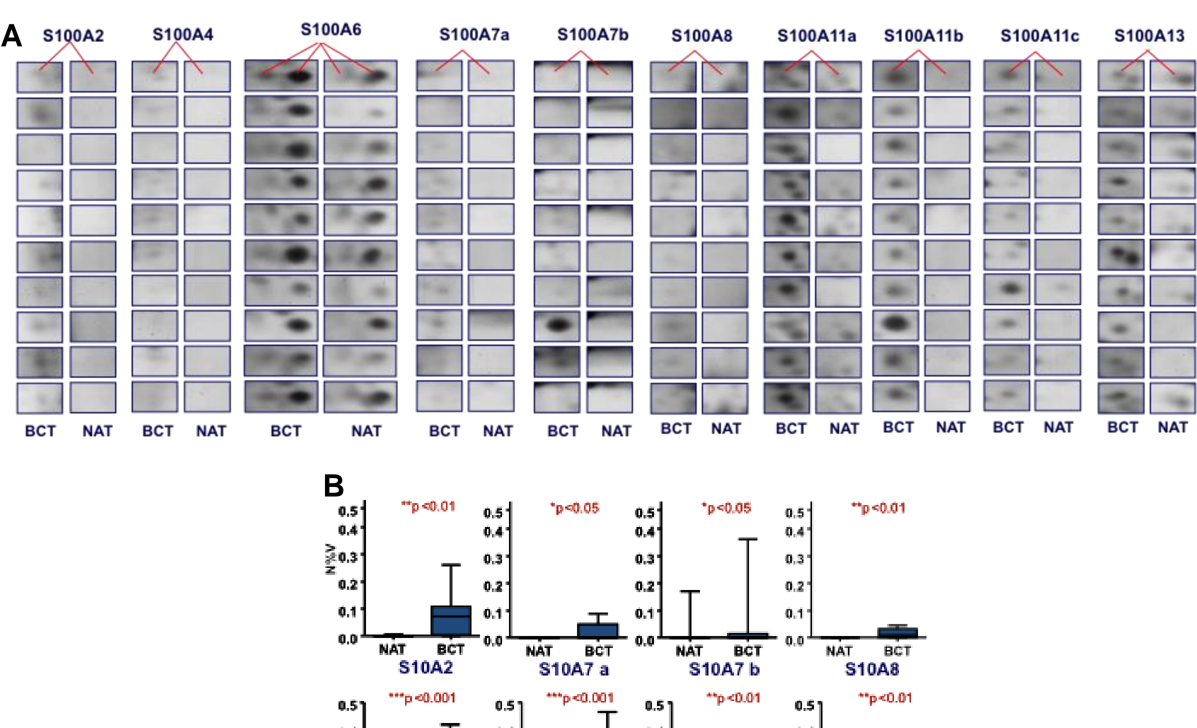


Figure 21: A. Panel of cropped areas of individual S100 protein spots from matched breast cancer tissues (BCT) and non-tumoral adjacent tissue (NAT). **B.** Quantitative analysis of S100 proteins, given as boxplot graphs, of matched breast cancer tissues (BCT) and non tumoral adjacent tissues (NAT).

The differential expression of S100 proteins was also confirmed by western blotting on three different patients for each S100 proteins(figure 22).

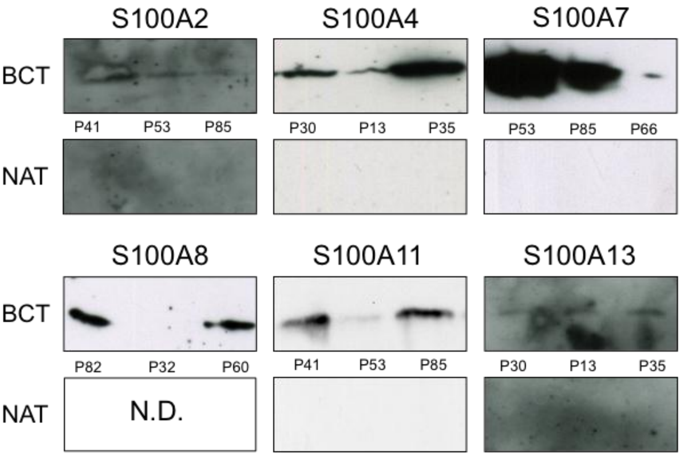
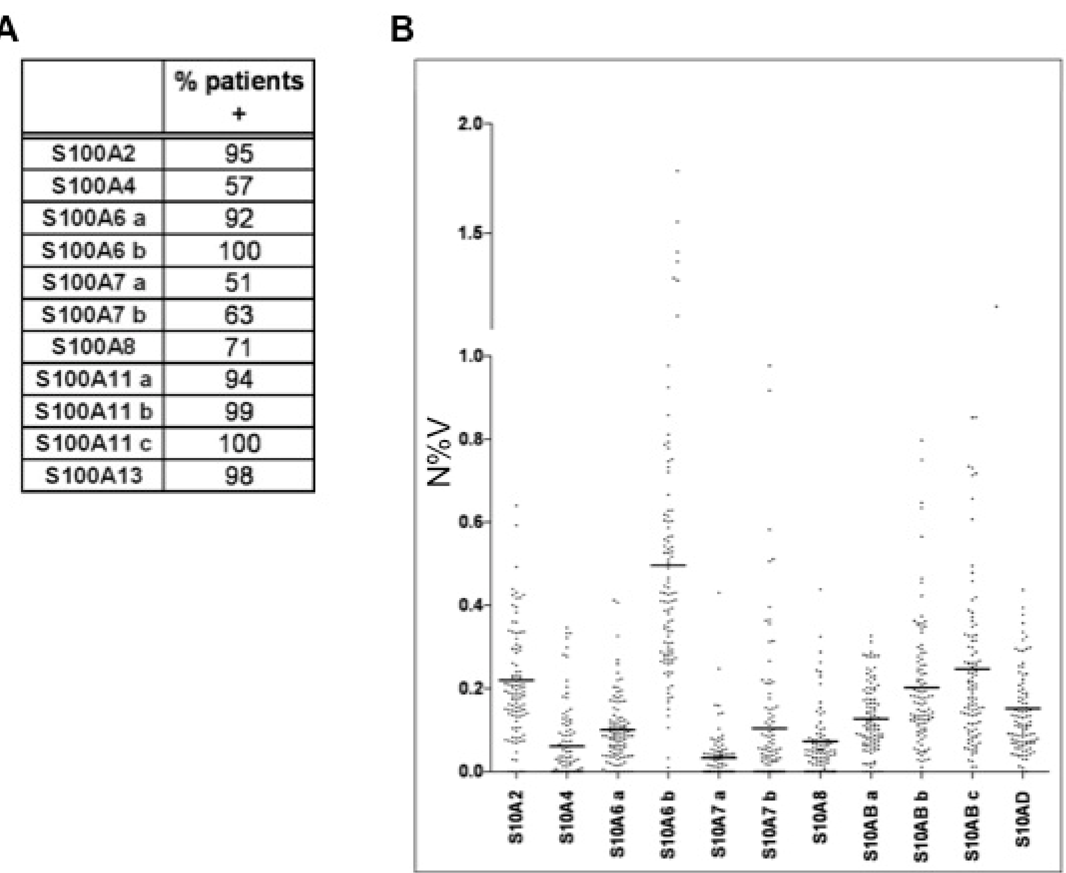


Figure 22: Panel of Western Blot reaction of S100 antibodies, performed on three selected breast cancer tissues and their non tumoral counterpart.

Occurrence of S100 proteins in a large sample-set of breast cancer patients

We performed quali/quantitative proteomic screening in a cohort of 100 patients. Qualitative analysis (presence-absence) showed that some proteins are expressed in almost all patients (S100A6, S100A11, S100A2 and S100A13), while others are expressed in a variable number of patients, i.e.: S100A8 in 71% of the patients, S100A4 in 57% and S100A7 in 51% (isoform a) and 63% (isoform b) (figure 23 A). The quantitative evaluation showed that the expression levels of each S100 member was quite different from one to the others and among patients (figure 23 B), but collectively, most of the S100

protein forms were statistically correlated to each others. This adds complexity to the role of this protein category in the breast cancer and suggests possible common pathway of (dys-)regulation.



A

	% patients +
S100A2	95
S100A4	57
S100A6 a	92
S100A6 b	100
S100A7 a	51
S100A7 b	63
S100A8	71
S100A11 a	94
S100A11 b	99
S100A11 c	100
S100A13	98

Figure 23: A. Qualitative analysis, table reporting the percentage of different S100 proteins among 100 patients. **B.** Quantitative analysis, diagram of the relative intensities of the eleven S100 protein forms among 100 patients, in abscissa are indicate the protein name and in ordinate the density values.

Prognostic value of S100 proteins in breast cancer

In order to investigate the prognostic value of S100 proteins to predict metastatic relapse during a time lapse of three years from the surgical intervention, patients were grouped into metastatic and disease free patients. Patients with 3-year follow-up were 57: 22 had developed distant metastases while 35 were disease-free. The expression level of each

S100 protein (expressed as average among patients of each group) was higher in the metastatic group compared to disease free-group. The most robust correlation with metastases (calculated by unpaired F-test statistical method) regarded primarily, the protein S100A4 also named Metastasin for its presumed role in metastases promotion. Indeed, in different studies, S100A4 protein is involved in certain types of cancer (such as breast cancer, bladder cancer and lung adenocarcinomas) suggesting that S100A4 may play a role in cancer progression (Ismail NI et al. 2008; Agerbaek M et al.2006; Matsubara D et al. 2005). Also in our analysis, S100A4 expression level shows an increase of more than 2 fold in the metastatic group.

Surprisingly the second most robust correlation involved with metastases is the protein S100A7 (S100A7a isoform), also named Psoriasin that was identified as highly expressed in psoriatic keratinocytes and is also highly and more frequently expressed in ductal carcinoma in situ (DCIS) (Krop I et al. 2005), thus suggesting a possible role in early stages of breast tumor progression in association with the development of the invasive phenotype.

Western blot validation of S100A7 expression

In agreement with the last result, we extended our analyses regarding S100A7 expression in breast cancer patients. We firstly confirmed, by 2D- Western blot, the proteomic expression of two different isoforms of S100A7, the more acid one and the more intense and basic second one (figure 24).

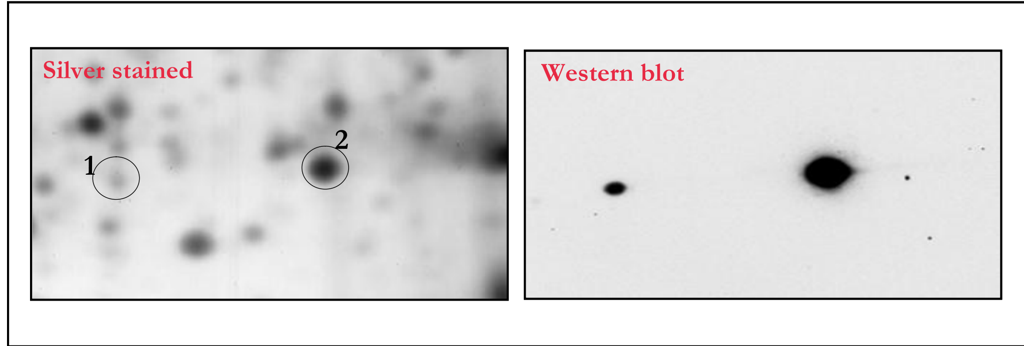


Figure 24: two different S100A7 isoform in silver stained (left) gel confirmed by 2D western blotting (right).

Next we investigated the occurrence of the S100A7 isoforms among patients. Quantitative evaluation showed that the expression level of the acidic form is always low or very low (according to an arbitrary scale), while the other isoform is expressed at very high level in 11% of patients (Figure 25).

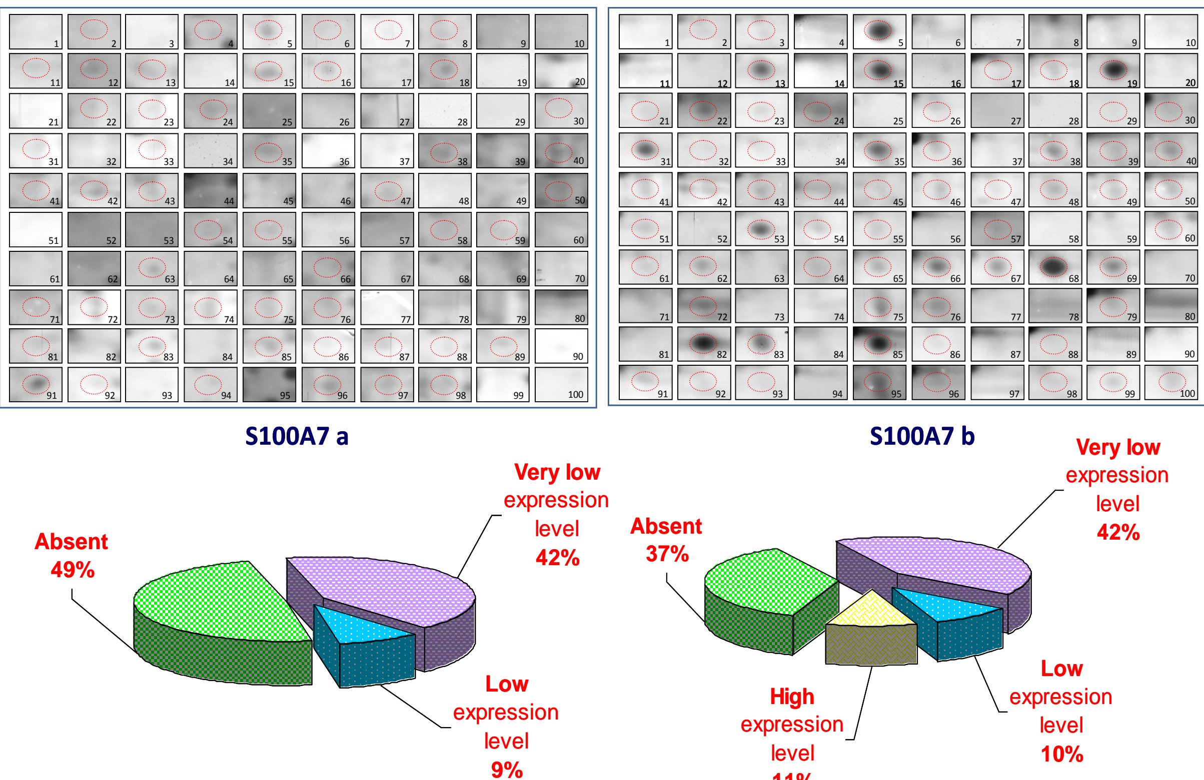


Figure 25: quantitative evaluation of S100A7 isoforms in 100 breast cancer tissues.

The immunological validation of the S100A7 expression in the patients, performed by mono-dimensional WB, overlaps with the expression pattern of the S100A7 basic isoform in 2D gels, confirming also its sporadic expression (figure 26)

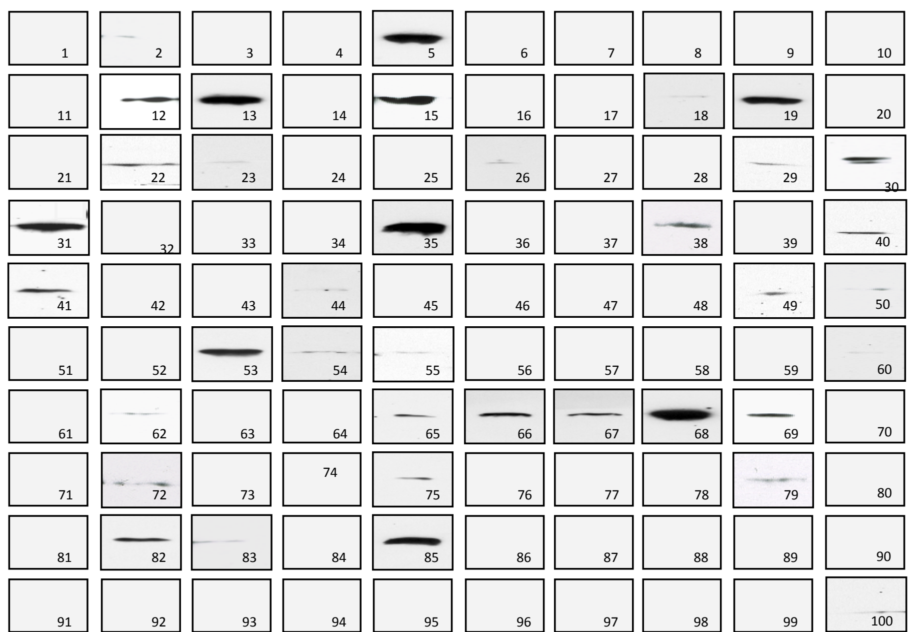


Figure 26: 1D-western blotting on 100 breast cancer tissues performed with anti S100A7 antibody.

The immunohistochemical evaluation on paraffin-embedded tissue sections for S100A7 on random selected breast cancer tissues showed that S100A7 expression, when present, is confined only to tumoral cells. S100A7 immunoreactivity was scored from moderate to strong in analyzed samples. Moreover, the expression of psoriasin was focal (≤ 10% of tumor cells) in some cases, heterogeneous (> 10 to < 75% of tumor cells) or marked (≥ 75% of tumor cells) in other cases. All positive tumors showed cytoplasmic expression, and among these, nuclear expression of psoriasin was also observed (figure 27).

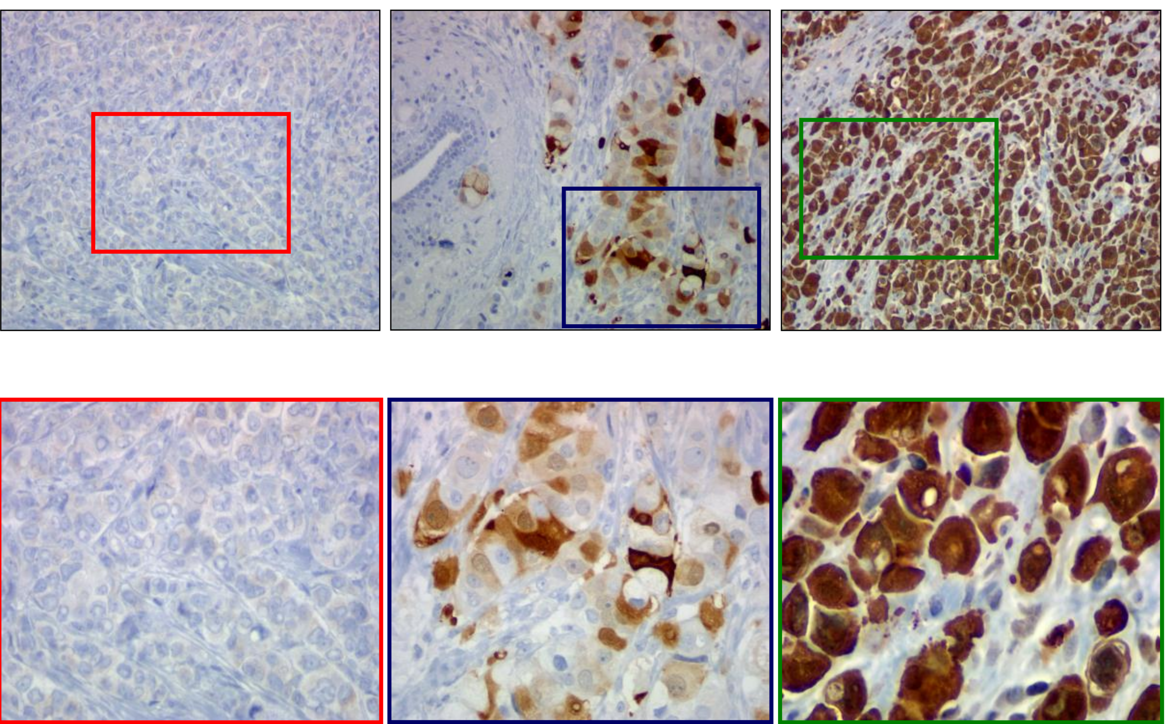


Figure 27: immunohistochiemical evaluation of S100A7 in three different breast cancer tissues; below: enlarged view of the same tissues, in which is evident the nuclear localisation of the protein.

Functional correlation of S100A7 and pathways analysis

Finally, in order to investigate biochemical functions of S100A7 into the cells and understand the possible role in breast cancer progression, we performed proteomic correlation analysis with other proteins identified in the proteomic maps. The association was determined using Person correlation. A p value less than 0.05 was considered significant, a p value less than 0.01 was considered high significant and a p value less than 0.001 very high significant. The expression of S100A7 proteins was positively correlated with 45 protein spots. In particular 20% of proteins were associated with the more acidic form of S10A7, while 80% showed correlation with the isoform b. (Table 2)

	S10A7 a	S10A7b
ACON	NO	p<0.001***
ALDR a	NO	p<0.005**
ALDR b	NO	p<0.05*
AN32A	NO	p<0.05*
ANXA2 b	p<0.05*	p<0.05*
ANXA5	YES	p<0.05*
CALM	NO	p<0.005**
CALR	NO	p<0.05*
CATD b	NO	p<0.05*
EF1B	NO	p<0.05*
ENOA a	NO	p<0.005**
ENOA d	p<0.05*	p<0.005**
G3P b	NO	p<0.05*
GDIR	p<0.001*	NO
GDIS	NO	p<0.05*
GSTP1	NO	p<0.005**
HSP60 b	p<0.05*	NO
HSP74	NO	p<0.05*
LDHA	NO	p<0.05*
LDHB	NO	p<0.005**
LEG1 b	NO	p<0.05*
NDKB	NO	p<0.005**
NTF2	NO	p<0.005**
PHB	NO	p<0.05*
PPIA c	p<0.005*	NO
PPIA e	NO	p<0.05*
PRDX1 c	NO	p<0.05*
PRDX6	p<0.05*	NO
PSA5	NO	p<0.005**
RABP2	p<0.005*	NO
S10A2	NO	p<0.05*
S10A4	NO	p<0.001***
S10A6 b	NO	p<0.05*
S10A7 b	NO	p<0.001***
S10A8	NO	p<0.001***
S10AB	NO	p<0.005**
SODM b	NO	p<0.001***
TAGL2 sf	p<0.001*	NO
TPM4 b	NO	p<0.005**
TYPH	NO	p<0.05*
UBIQ a	p<0.05*	NO
UBIQ b	NO	p<0.05*
VINC a	NO	p<0.05*
VINC b	NO	p<0.05*

Table 2: Proteomic correlations analysis between S100A7 isoforms and proteins identified in breast cancer tissue map

Since proteomic organisation into functional protein categories is fundamental to understand biochemical functions from a global point of view, the list corresponding to the

AC in Swiss-prot of S100A7-correlated proteins was running into DAVID Bioinformatics database for functional clustering. The program found 4 different clusters for the S100A7 correlated proteins. These are: calcium binding proteins, antiapoptotic proteins, antioxidant enzymes and glycolitic enzymes. This is an important result because represents the first link between S100A7 expression with these important pathways, correlated to cancer progression. The high correlation among S100A7 and calcium binding proteins such us S100A4 and S100A8/9 has previously described also by other authors (Zibert JR et al. 2010; Liu H et al. 2007).

Molecular classification of tumors for patient stratification

In order to identify biologically and clinically significant subgroups of patients with a high risk of progression to invasive disease, the set of 45 S100A7 associated proteins were used to perform hierarchical clustering of patients based on Person correlation measure of the expression pattern and complete linkage. This revealed that the patients consistently separated into three major groups with distinguishable protein expression profiles.

We analysed patients who underwent to cancer progression, and interestingly, the majority of the patients with tumor progression belonging to cluster 1, the 28% of the patients who underwent tumor progression are in the second cluster, while cluster 3 contain a smaller % of the patients with horse prognosis. So we can speculate that cluster 1 identify patients with high risk of progression, cluster 2 identify patients with moderate risk of progression and cluster 3 contain patients with low risk of tumor progression.

RESULTS III

Previously in my thesis, DAVID bioinformatics has been used to identify functional pathways involved in breast cancer such as glycolysis cluster that results among the clusters at first places for robustness. Indeed it is well known that transformed cells display increased levels of glycolytic enzyme mRNA and protein, that, can act as tumor associate antigens (TAA). If these antigens undergone post-translational modifications (PTMs), may be perceived as foreign by the immune system that is induced to product autoantibodies. The generated sera autoantibodies targeting TAAs could be useful as early molecular signatures for diagnostics and prognostics of cancer patients.

Immunoproteomics, which defines the subset of proteins involved in the immune response, holds considerable promise for the discovery of tumor-associated antigens, suggesting that this approach may be a useful tool for identification of prognostic, diagnostic markers and novel therapeutic targets. Recently, various different approaches has been developed to identify autoantibodies against tumor-associated antigens from tumor patients' sera.

In this study, in order to identify circulating antibodies direct against specific tumor antigens as putative biomarker, SERological Proteome Analysis (SERPA) was initially performed as preliminary study in breast cancer sera.

Proteins lysate from breast cancer tissue were separated using 2D electrophoresis, transferred into a nitrocellulose membrane and incubated with immunoglobulins (Ig) purified from serum of the same patients that are used as primary antibodies. Immunoglobulin isolation from serum was conduct by immunoprecipitation technique, standardized in our laboratory, with protein A sepharose that binds specifically the Fc domain of Ig (as described in MM).

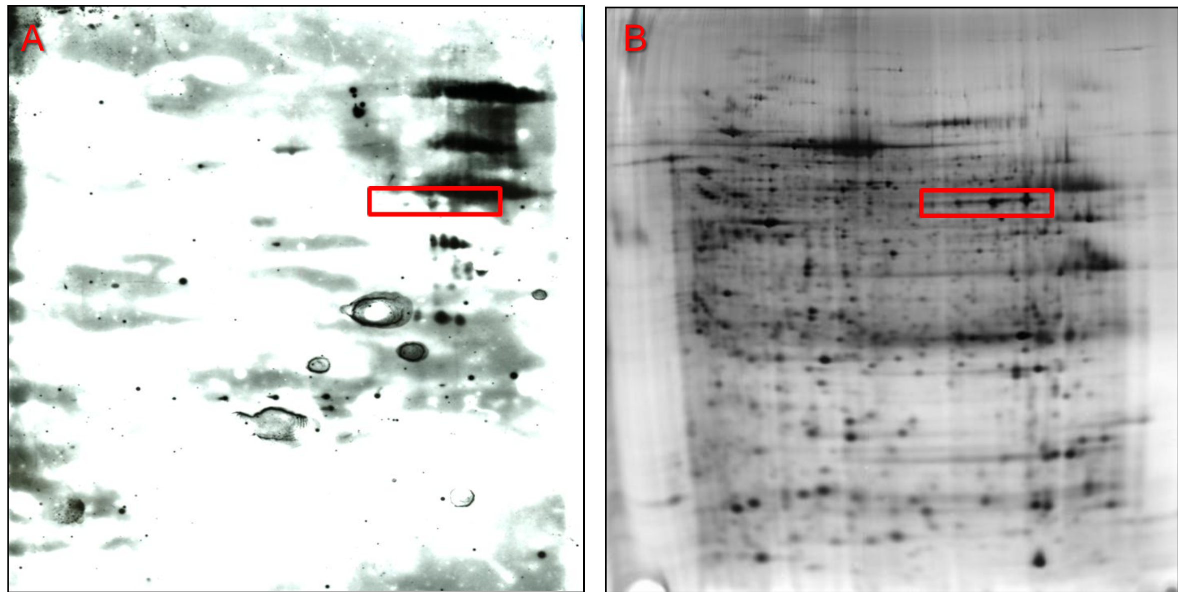


Figure 28: A Two-dimensional Western blot performed using the autologous serum purified by protein A; **B** correspondent silver stained breast cancer tissue. In red is indicated the region where the protein enolase migrates.

Figure 28 A shows the outcome of 2D-western blot as described above. Through the matching with reference map of breast cancer tissue (figure 28 B) generated in our laboratory, we identified a large amount of immunoglobulin, but also a specific signals derived from different proteins, including putative acidic isoforms of α-enolase (in red box).

Enolase was originally characterized as an enzyme involved in glycolytic metabolism. More recent evidence, however, shows that enolase is a multifunctional protein. In mammalian cells, three isoforms of enolase have been found. They are designated as a-(ENO1), h-(ENO3), and g-(ENO2)

enolases. The expression of these isoforms is developmentally regulated in a tissue-specific manner. ENO1 is widely distributed in a variety of tissues, whereas ENO2 and ENO3 are exclusively found in neuron/neuroendocrine and muscle tissues, respectively (Pancholi V 2001). Enolases form heterodimers or homodimers to convert 2-phosphoglycerate into

phosphoenolpyruvate in glycolysis. In addition to its glycolytic function, as previously described in this thesis, ENO1 exist on the cell surface working as one of the plasminogen receptors and it may play a role in tissue invasion. In hypoxic conditions, it also acts as a stress protein that is up-regulated and speculated to provide protection to cells by increasing anaerobic metabolism (Jiang BH et al. 1997). By using an alternative translation start codon, ENO1 transcripts can be translated into a MBP1-like p37, which is localized to nucleus and binds to the c-myc P2 promoter (Feo S et al. 2000).

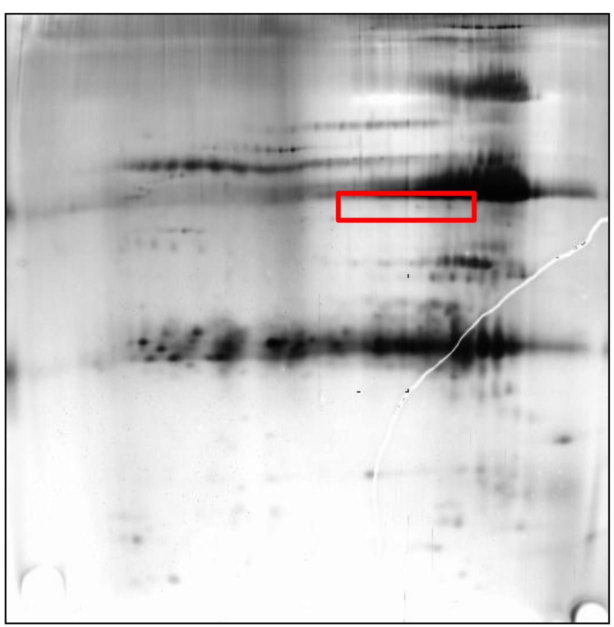


Figure 29 : 2D IPG of proteins extracted from tumor tissue and immunoprecipitated using protein A. In red is indicated the region where the protein enolase migrates.

To validate further these data and to better comprehend if linkage between Ig and its ligand is specific, we performed tissues immunoprecipitation with corresponding sera Ig. At first protein A sepharose was incubated with serum to isolate Ig and then tissue lisate derived from the same patient, was added to isolate immunoprecipitated proteins. With this approach is possible to appreciate which tissue proteins are recognized from the enriched

fraction Ig of the serum. Finally we separated the immunoprecipitated proteins by 2D electrophoresis and stained with silver nitrate. Also in this silver stained gel (figure 29) , among all the proteins detected, is possible to discriminate the putative acidic isoform of α-enolase (in red box).

To confirm the identity of these immunoreactive antigens, the spots were excised from breast cancer tissue (figure 30 A) and subjected to in-gel tryptic digestion and MALDI-TOF mass spectrometry analyses. Figure 30 B shows the MS spectra obtained from these acidic isoform spots, confirming the enolase identities. This data was further confirmed by a 2D western blotting utilizing the purified antibody anti-enolase (Santa Cruz) on breast cancer tissue. (figure 30 C)

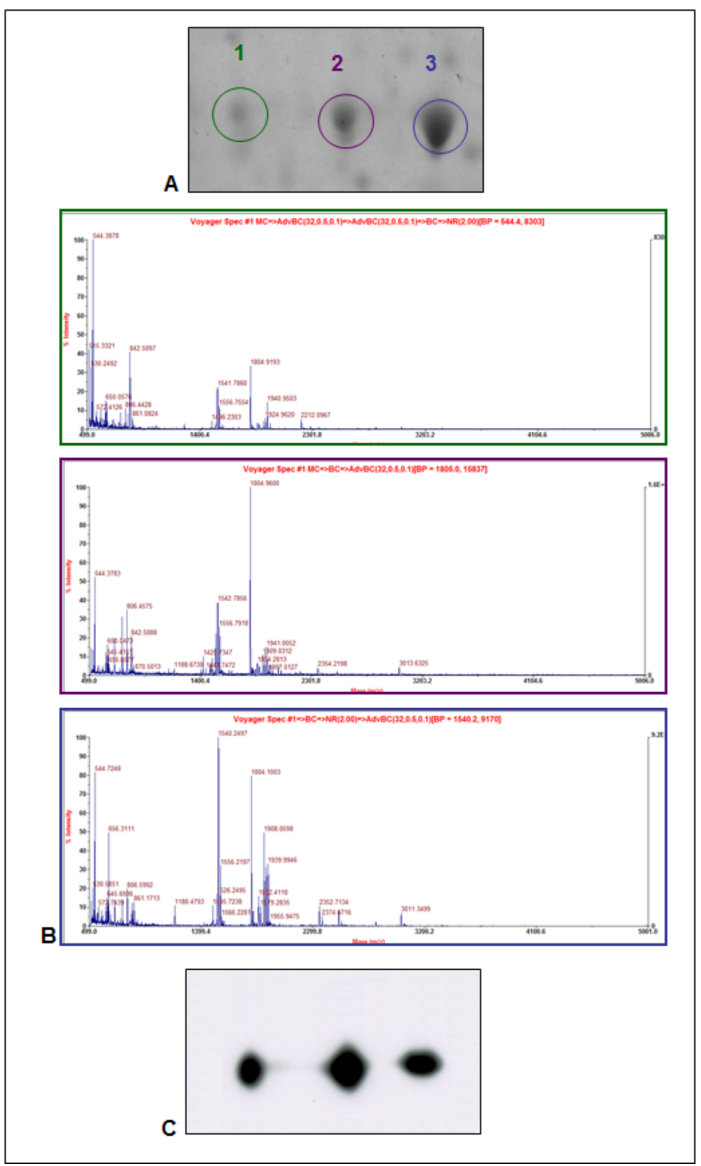


Figure 30: A Identification of immunoreactive spots present in a parallel gel stained with blue comassie **B** α Enolase by MALDI-TOF **C** by two-dimensional Western blot.

To appreciate the reproducibly of these data, we analysed other Ig purified of serum specimens from additional breast cancer patients, with counterpart protein tissue. Therefore proteins from these breast cancer tissues were processed as describe above. As shown in cropped window (figure 31) among the proteins recognized from breast cancer Ig, the presence of three acidic α-enolase isoform has been confirmed and demonstrated by matching with the correspondent silver stained tissue.

Development of high titers of autoantibody against enolase has been reported to be associated with a plethora of systemic or organ-specific autoimmune diseases (Gitlits VM et al. 2001), but also with different subtypes of lung cancer (Jankowska R et al. 2004, Li C et al. 2006), with melanoma (Suzuki A. et al. 2010) and endometriosis suggesting that it can be an immunogenic target in different typology of disease.

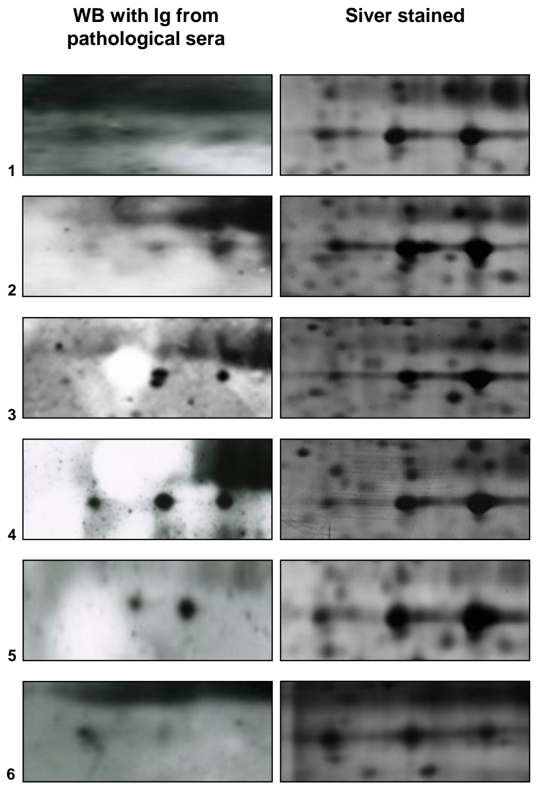


Figure 31: 2D western blot with purified serum of 6 different breast cancer patients to confirm the presence of enolase autoantibodies.

In addition to comprehend if auto-antibodies anti-enolase proteins were exclusive present in breast cancer serum and not in the normal one, we analysed sera from disease-free patients. Ig from healthy volunteers were also purified by our immunoprecipitation technique and used as primary antibodies for 2D WB with the same protein breast cancer tissues used for previous assays.

As shown from the cropped images (figure 32),Ig isolated from healthy volunteers sera did not contain auto-antibodies direct against α-enolase proteins.

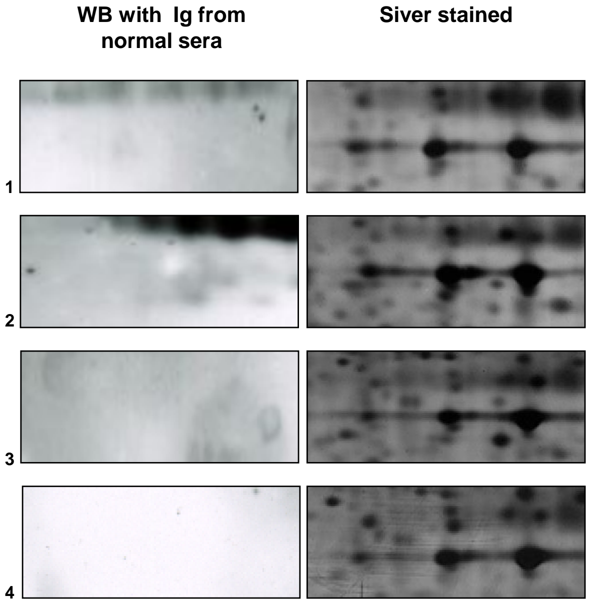


Figure 32: 2D western blot with normal sera on 4 different breast cancer tissues.

In fact free disease patients do not contain auto-antibodies against α-enolase, while sera from cancer patient contain auto-antibodies that strongly react with breast cancer tissue proteins.

In conclusion, although Immunoproteomics remains of difficult interpretation, it allows us to identify different isoform of proteins, thus suggesting that this technique has the advantage to recognized the post-translational modification of the proteins.

Finally, this results strongly suggest to consider that auto-antibodies direct against α-enolase as potential biomarkers, as already seen for different neoplastic disease (Jankowska R et al. 2004, Suzuki A. et al. 2010) facilitating, in the future, the early diagnosis of breast cancer.

MATERIALS AND METHODS

Clinical specimens

The present study was conducted on 100 surgical tissues of ductal infiltrating breast cancer collected between 2003 and 2007 in the Breast Unit of the La Maddalena Hospital. Research was carried out in compliance with the Helsinki Declaration with the patients' written consent.

The study used leftover specimens, that is, aliquots of specimens collected for routine clinical care, and immediately frozen at -80°C until used. The specimens were not individually identifiable.

The patients of this study did not receive any cytotoxic/endocrine treatment prior to surgery. Diagnosis of ductal breast cancer (G2/G3) was confirmed histopathologically. Post-operative monitoring to define whether or not distant metastases were present, was performed by conventional imaging follow-up, consisting of chest radiography, bone scintigraphy, magnetic resonance imaging (MRI) and positron emission tomography using 2-[fluorine-18] fluoro-2-deoxy-D-glucose (FDG-PET), as clinically indicated at La Maddalena Hospital.

Sample preparations

The frozen breast tissue samples were washed several times with phosphate-buffered saline and homogenized in RIPA buffer (50 mM Tris pH 7.5, 0.1% Nonidet P-40, 0.1% deoxycholate, 150 mM NaCl, 4 mM EDTA) containing a mixture of protease inhibitors (0.01% aprotinin,10 mM sodium pyrophosphate, 2 mM sodium orthovanadate,1 mM PMSF). The extraction was carried out overnight at 4°C with the same buffer. The total cellular lysate was centrifuged to remove tissue debris, and the resulting supernatant

dialysed against ultrapure distilled water, lyophilized and stored at -80°C until use. The total protein concentration was determined by the Bradford method using bovine serum albumin as a standard.

Two Dimensional Gel Electrophoresis

The proteins extracted from breast cancer tissue and normal adjacent tissue were solubilised in a buffer containing 4% CHAPS, 40 mM Tris, 65 mM DTE in 8 M urea. Aliquots of 45 μg (analytical gels) or 1.5 mg (preparative gels) of total proteins were separately mixed with 350 μL of rehydration solution containing 8 M urea, 2% CHAPS, 10 mM DTE and 0.5% carrier anpholytes (Resolyte 3.5-10), and applied for IEF using commercial sigmoidal IPG strips, 18 cm long with pH range 3.0-10. The second dimension was carried out on 9-16% linear gradient polyacrylamide gels (SDS-PAGE), and the separated proteins were visualized by ammoniacal silver staining. Stained gels were digitized using a computing densitometer and analyzed with Image Master software (Amersham Biosciences, Sweden). Gel calibration was carried out using an internal standard and the support of the ExPaSy molecular biology server, as described [16].

Protein identification

N-Terminal microsequencing was performed by automated Edman degradation in a protein sequencer (Procise, 419 Applied Biosystems), as previously described (Pucci-Minafra I et al. 2002).

Mass spectrometric sequencing was performed by Voyager DE-PRO (Applied Biosystems) mass spectrometer as described. Briefly, proteins were digested using sequencing-grade trypsin (20 μg/vial). The tryptic peptide extracts were dried and redissolved in 10 μL of 0.1% trifluoroacetic acid (TFA). The matrix, R-cyano-4-hydroxycinnamic acid (HCCA),

was purchased from Sigma-Aldrich. A saturated solution of HCCA (1 µL) at 2 mg/200 µL in CH3CN/H2O (50:50 (v/v)) containing 0.1% TFA was mixed with 1 µL of peptide solution on the MALDI plate and left to dry. MALDI-TOF mass spectra were recorded in the 500-5000 Da mass range, using a minimum of 100 shots of laser per spectrum. Delayed extraction source and reflector equipment allowed sufficient resolution to consider MH+ of monoisotopic peptide masses. Internal calibration was done using trypsin autolysis fragments at m/z 842.5100, 1045.5642, and 2211.1046 Da. Peptide mass fingerprinting was compared to the theoretical masses from the Swiss-Prot or NCBI sequence databases using Mascot http://www.matrixscience.com/. Typical search parameters were as follows: (50 ppm of mass tolerance, carbamidomethylation of cysteine residues, one missed enzymatic cleavage for trypsin, a minimum of four peptide mass hits was required for a match, methionine residues could be considered in oxidized form.

Western Blotting of S100 proteins

For immune detection the gels were electrotransferred onto nitrocellulose membrane (HyBond ECL, Amersham) and stained with Ponceau S (Sigma). The membranes were then probed with one of the following monoclonal antibodies: anti-actin (Oncogene), anti-S100A2, anti-S100A4, anti-S100A6, anti-S100A7, anti-S100A8 (Santa Cruz), or polyclonal antibodies: anti-S100A11, anti-S100A13 (SantaCruz). Following incubation with the appropriate peroxidase-linked antibody, the reaction was revealed by the ECL detection system, using high performance films (Hyperfilm ECL, Amersham).

Quantification and normalization methods

Quantitative expression levels were calculated as the volume of the spots (i.e. integration of optical density over the spot area). In order to correct for differences in gel staining, spot

volumes relative to the sum of the volume of all spots an each gel (%Vol) were calculated by the software. Since the cell densities within an area of the surgical sample, may be very variable among the different patients, measurements of relative expression levels of individual protein spots were normalized in each map for actin content (Pucci-Minafra et al. 2008) and the final value was designated as N%V. For statistical analyses Ms Excel and Graph Pad Prism 4 software were used. Correlation of S100 protein members for breast cancer patients was performed using the Pearson correlation test. The difference in S100 expression between metastatic versus disease free patients was analyzed by unpaired F test. In all cases, $p < 0.05$ was considered significant (*), $p < 0.01$ highly significant (**) and $p < 0.001$ very highly significant (***).

Purification of Ig from serum with Protein A

To favourite the bond between protein A and serum Ig, 10 µl of Protein A (GE Healthcare) has been incubated with 50 µl of H_2O, 50 µl of serum and 50 µl of Immunoprecipitation Buffer (2% Triton X-100, 300 mM NaCl, 20 mM Tris pH 7.4, 2 mM EDTA, 2 mM EGTA, 0.4 mM sodio ortho vanadato, 0.4 mM PMSF e 1% NP-40) for 30' at 4°C.

After centrifugation of 2 minutes at 13,000 rpm, the pellet was washed 3 times in Immunoprecipitation Buffer to remove the non-specific interactions. Protein A bounded to Ig was then resuspended in 30 µl of Eluition Buffer (0.1 M citric acid pH 3.0) for 15 min at 4 ° C to allow the separation of IgG and then centrifuged for 2 minutes at 13,000 rpm. The eluate was then diluted in 1% milk (in TBS-T 0.05% Tween) and used as primary antibody.

Immunoprecipitation of tissue proteins

In order to immunoprecipitate antigens, contained in the cell lysate, from tissues of breast

cancer, immunoglobulins from autologous serum were bound to protein A, as described previously.

To the protein A-conjugated Ig of the serum were added 300 μg of total protein extracted from tumor tissue of the same patient with 50 μl of Immunoprecipitation Buffer. The suspension was incubated 1 hour at 4 ° C and then centrifuged for 2 minutes at 13,000 rpm, removing the supernatant. Then three washes were performed with 50 μl each of Immunoprecipitation Buffer and centrifuged each time for 2 minutes at 13000 rpm. Finally, were added 30 μl of Eluition Buffer for 15 minutes at 4 ° C with shaking and centrifuged for 2 min. at 13000 rpm, each time recovering the supernatant. The operation was repeated 3 times. The eluate was dialyzed, lyophilized and resuspended in rehydration solution and loaded into two-dimensional electrophoresis.

CONCLUSIONS

In order to identify novel protein clusters in breast cancer tissues potentially involved in tumor progression, the first part of my thesis regarded the increase, up to 100 breast cancer tissues, of samples studied, followed by a gene ontology study performed with DAVID Bioinformatics Resources. A redundant list of categories was generated, including the gene cluster of regulation of programmed cell death, cell motion cluster and glycolysis were at first places for robustness.

The combination of experimental proteomics with bioinformatics has produced information of great interest. In particular, the general expression pattern of proteins involved in anti-apoptotic and glycolysis pathways confirms respectively the cell survival-dependence for primary tumor growth and the altered metabolism of tumoral cells, while the irregular expression of proteins involved in cell motility, clearly demonstrated the heterogeneity of metastatic potentialities and suggests the possibility of using some of the motion cluster proteins as prognostic factors for metastasis propens.

Subsequently my study focalized on the S100 family, that is emerging as a potentially important group of markers in multiple tumour types. we verified a significant deregulation of most S100 member in breast cancer tissues compared with non-tumoral tissues adjacent to the primary tumor in which these proteins are very low or absent. Most of the detected S100 members appeared reciprocally correlated suggesting therefore common pathways of (dys)-regulation. Finally patients which developed distant metastases after a three year follow-up showed a general tendency of higher S100 protein expression, compared to the disease-free group resulting be associated with breast cancer progression. Among these, S100A7 (isoform a) showed the most robust correlation with metastases, a sporadic protein, expressed only in about 20% of the patients but when present, shows a remarkable

intensity, suggesting therefore its potential role as biomarker for patient stratification. Then, although the function of these proteins into the cells is still unknown, we believe that this information may substantially contribute to the progress of protein profiling of breast cancer for clinical applications.

Finally we use SERPA approach to identify autoantibodies circulating in cancer sera, that recognize antigenic protein and may serve as novel markers in screening, diagnosis or in prognosis.

In this study we have combined a proteomic approach with an immunological method to find autoantibodies direct against tumor antigens that elicit humoral immune response in the sera derived from breast cancer patients. Through SERological Proteome Analysis (SERPA) approach indeed we identified antibodies direct against α-enolase in several serum of breast cancer patient suggesting that autoantibodies have potential as biomarkers and may in future facilitate the early diagnosis of breast cancer. Moreover multiple studies demonstrated that autoantibodies are tumor-associated and the molecules recognized as autoantigens in cancer sera are frequently overexpressed in tumor tissue.

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