Ministero dell'Università
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Università degli Studi
di Palermo

PhD Programme in
Genomics and Proteomics applied to Oncological and Endocrine-Metabolic research

Graduation year 2011

COMPARATIVE ANALYSIS OF HUMAN THYROID CARCINOMA CELL LINES

PhD Thesis by:
Dr. Rosa Musso

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Dottorato di Ricerca in
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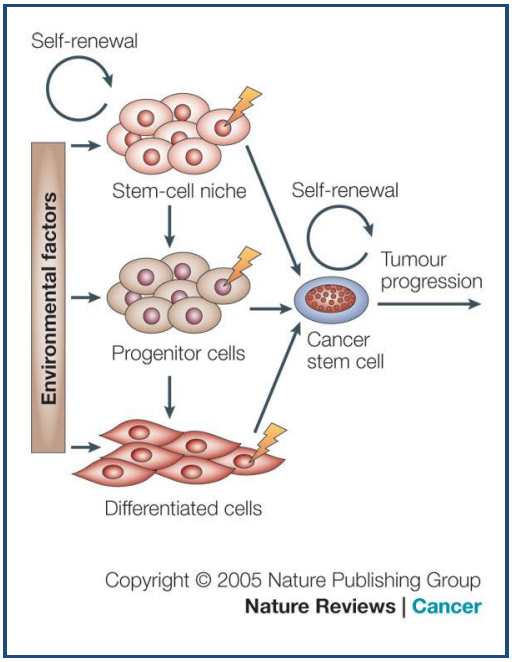
INTRODUCTION

Carcinogenesis

It is generally assumed that the neoplastic initiation leads to the appearance of a cell clone that, following an initial mutation, can prevail over the others because of its increased replicative activity and/or because of its being refractory to the pro-apoptotic stimuli. Furthermore, additional genetic alterations and selective mechanisms generate new different subclones, which are able to circumvent the tissue barriers and to invade the surrounding normal tissues.

Recently, the cancer stem cell model for explaining tumorigenic processes has received increasing attention (Figure 1). This model postulates that tumours are driven and maintained by a smaller subpopulation of cells that have the capacity to self-renew and differentiate generating a cell progeny which make up the bulk of a tumour (Clarke MF et al.2006). The former population has been called cancer stem cells (CSCs), tumorigenic cancer cells, or tumour-initiating cells, by various investigators, to point out that these are able to form new tumours when transplanted into immune-deficient animals (Clarke MF et al.2006).

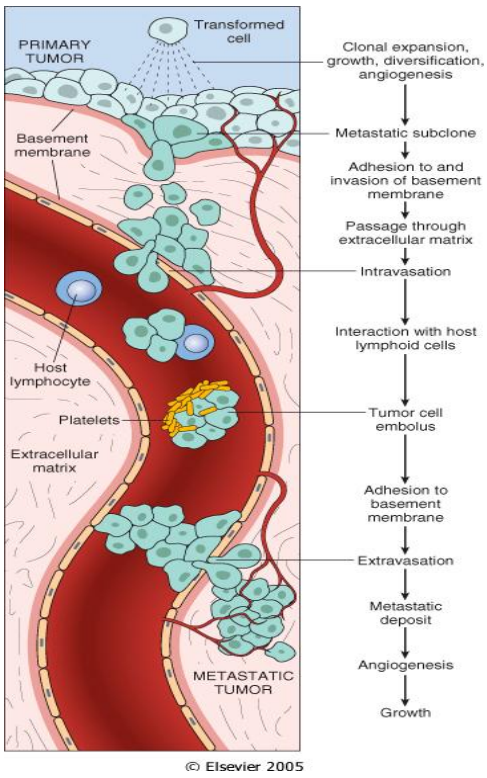


The cancer stem cell might appear after mutations in specific stem cells or early stem cell progenitors. It is also possible that cancer stem cells can be derived from differentiated cells. There might be numerous factors in the host microenvironment that trigger the initial steps of tumour formation (Nature 2005).

Early evidence for the existence of CSCs came from studies of acute myelogenous leukemia (AML), while their presence has been recently demonstrated in many solid tumours, including glioblastoma, medulloblastoma, breast cancer, melanoma, prostate cancer (Takaishi S et al. 2008) and thyroid (Zito G et al. 2008). The recognition of CSCs has profound implications for cancer biology and therapy since it is postulated that their eradication is a critical determinant in achieving appropriate therapeutic results. 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).

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

The tumor progression (Figure) is characterized by the expression of additional morphological and behavioural alterations leading the neoplastic cells to acquire a locally invasive phenotype. These cellular changes are most likely the result of a so-called epithelial-mesenchymal transition (EMT) that is an orchestrated series of events in which cell-cell and cell-matrix interactions are damaged or destroyed.



As a consequence, epithelial cells escape from the tissue border concurrently with the cytoskeleton reorganization which confers to the cells the ability to move through a three-

dimensional ECM, while a new transcriptional program is induced to maintain the mesenchymal phenotype.

Moreover, during progression, the detached epithelial cells acquire the ability to degrade the basal lamina and penetrate into the surrounding stroma, to form metastases. In particular, 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 are replaced by N-cadherin and integrins specific of the extracellular components. The actin cytoskeleton is remodelled into stress fibers which accumulate in the areas of cell protrusions. The epithelial intermediate filaments, cytokeratins, are replaced by vimentin. In the 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 for which they have been named cancer associated fibroblasts, CAFs, (Liotta LA et al. 2001). Likewise, previous work by our group demonstrated that fibroblasts, when co-cultured with the breast cancer cell line 8701-BC, induce considerable proteomic modulations, 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 a pivotal role 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 cell proliferation rate of and may have a strong 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 it has been described firstly by Otto Warburg.

It is only under anaerobic conditions that differentiated cells produce large amounts of lactate. In contrast, most cancer cells produce large quantities of lactate according to 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 O2 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 of HIF-1 (Hypoxia-Inducible Factors). 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.

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 activity of 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 its 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). Indeed, α-enolase could act as a hypoxic stress protein which may contribute to low oxygen 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 exposed to a microenvironment that may be 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.

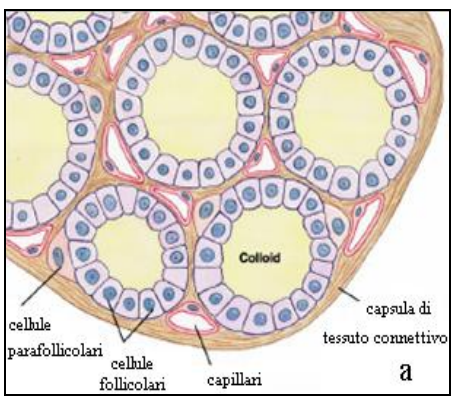
The hypoxic response is significantly controlled in most cells by HIF-1, a heterodimeric transcription factor composed of the nearly ubiquitous HIF-1α and its dimerization partner HIF-1β. HIF-1 activates approximately 200 genes encoding proteins that regulate cellular metabolism, proliferation, motility, 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).

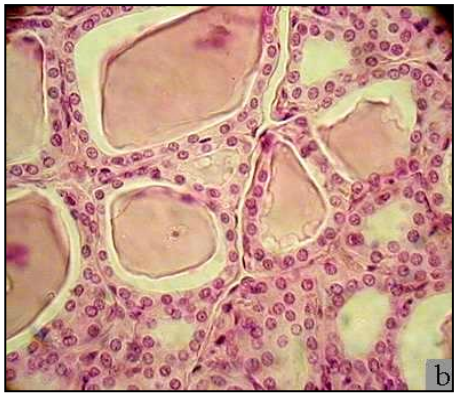
In response to various alterations in the microenvironment, and in particular, to intratumoral hypoxia, angiogenesis-stimulating factors produced by the cells themselves induce the formation of new blood vessels from pre-existing vasculature, to increase delivery of oxygen and nutrients to tissues. Many angiogenic genes, such as VEGF, angiopoietin-2, VEGF receptors (Flt1 and KDR), and neuropilin-1 (Hickey MM et al. 2006 and Simons M 2005) are induced by hypoxia through several mechanisms, including direct transcriptional activation by 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 target VEGF to enhance angiogenesis (Shie JL et al. 2004 and 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 structurally and functionally (expansion, basement membranes and endothelial surfaces incomplete or absent, irregular and tortuous architecture, arterial-venous shunts, blind ends and lack of contractile components of the wall) (Vaupel P et al. 1989), resulting in an irregular blood flow that sometimes reduce the transport of oxygen and nutrients to cancer cells.

Thyroid Cancer

The thyroid is a endocrine gland that controls heart rate, blood pressure, temperature and metabolism by producing of thyroid hormones, known as triiodothyronine (T3) and thyroxine (T4). The follicle represents the structural and functional unit of the thyroid gland and consists of two cell populations: *Follicular cells* (principal cells) which represent the thyroid parenchymal component and produce T3 and T4 and *Parafollicular cells* (C cells) located in the periphery of the follicles produce and secrete calcitonin hormone that plays a role in calcium homeostasis (Figure).





Thyroid cancer is the most common malignancy of the endocrine system and the incidence is increasing than to others types cancer. This fact could be due to the enhancement of diagnostic tecniques (thyroid ultrasound, neck imaging and fine-needle aspiration biopsy) and for the same reason it is recorded also an significantly increasing of survival rates for thyroid cancer.

Thyroid cancer can occur in any age group, with an incidence 3 times higher in women than in men (Hölzer S and Dudeck J 2000), its aggressiveness increasing significantly in older patients.

The etiology of most thyroid carcinomas is not well understood, but as in every type of cancer, they depend from combination of enviromental and genetic factors. Exposure to ionizing radiation, especially during childhood, remains the only enviromental factor clearly associated with benign and malignant thyroid tumors in humans (Schlumberger MJ 2000). Moreover epidemiologic, experimental and clinical evidences suggest that exposure to ionizing radiation may trigger a series of abnormalities that lead to genetic instability and thus cancer development (Ward LS et al.1998). It is been displayed also the involvement of

geographic, ethnic and dietary factors in the risk of sporadic thyroid cancer (Farid NR et al.1994, Schlumberger MJ 2000 and Kazakov VS et al.1992).

The genetic abnormalities play a crucial role in thyroid tumorigenesis and have been extensively explored over the last two decades due to their importance as potential targets for novel therapeutic approaches.

In spite of the progress in the treatment of thyroid malignant tumors by traditional methods (surgical and/or chemotherapy, radiotherapy), 10-20% of these patients die from advanced differentiated, poorly and undifferentiated thyroid tumors. Therefore, alternative therapies are of interest because of the possibility of developing selective targeting approaches, such as using of tissue-specific molecules directed against tumor cells.

Hystopathological classification

There are four histological types of thyroid cancer: papillary, follicular and anaplastic carcinomas (PTC, FTC and ATC respectively) which arise from follicular epithelium and medullary carcinoma (MTC) that arises from parafollicular epithelium. Moreover PTC, FTC and MTC are well-differentiated thyroid carcinomas, while ATC is characterized as undifferentiated**.**

PTC is the most common form of thyroid cancer, accounting for about 75-80% of thyroid malignancies (Thyroid Carcinoma Task Force 2001) with a 1% death rate and an approximately 5% recurrence rate (Hay ID 1990). It represents the most common pediatric thyroid malignancy and most papillary thyroid carcinomas (PTCs) in adults occur between 20 and 50 years of age (Davies L and Welch HG 2002). The PTC patient has the highest 20-year survival (99%) in the low-risk group, whereas survival is only 43% in the high-risk group; distant metastases are uncommon but if present, they are lymphatic and the prognosis is poor.

FTC cases account for about 15% of all thyroid cancer cases. FTC is less common in younger adults and children than papillary cancer, while it is characterized by a 20-year survival of about 97% in the low-risk group and 49% in the high-risk group; it is more common among elderly patients. This type of thyroid cancer is more aggressive than PTC and tends to spread through the bloodstream to other parts of the body (vascular invasion is characteristic of follicular carcinoma), and therefore distant metastasis is more common (Shaha AR et al.1996 and Shaha AR et al.1995).

ATC is the least common (accounting for 5%-15% of primary malignant thyroid neoplasms) and the most deadly type of thyroid cancer. It is characterized by sudden, rapidly growing thyroid mass, mostly arising from a benign thyroid disease or a pre-existing differentiated thyroid carcinoma. It tends to occur most often among elderly women and in iodine-deficient areas, or in a pre-existing goiter, while it is also rapidly fatal with a mean survival of 6 months after diagnosis (Giuffrida D and Gharib H 2000, Ain KB 1998). ATC is always refractory to conventional therapies including surgery, radioactive iodine and chemotherapy (Ain KB 1999).

MTC is a neuroendocrine malignancy that accounts for 3 to 10% of all thyroid cancer cases, but bears significant mortality (Kebebew E et al.2000). Among medullary thyroid carcinomas (MTCs), 25% are sporadic, whereas 75% are inherited as the predominant component of the multiple endocrine neoplasia type 2 (MEN2) syndromes (MEN2A, MEN2B and familial MTC) (Ball DW et al.2007). MTC is less common and more aggressive than PTC or FTC, and tumor tends to spread to lymph nodes very early and to distant sites via the blood stream with excessive calcitonin production (Ball DW et al.2007). This cancer is treated with difficulty and has a much lower cure rate than do the 'well differentiated' thyroid carcinomas (PTC and FTC) (Shaha AR et al.1996 and Shaha AR et al.1995), but cure rates are higher than they are for ATC. The recorded overall 10-year survival is about 60-70% ((Ball DW et al.2007 and Moley JF et al.2003).

Genetic Basis of Thyroid Cancer

Numerous genetic abnormalities are been found in different histotypes of thyroid tumors including rearrangements, point mutations or other gene disruptions, each of which plays a role in disease pathogenesis. Most of these genetic alterations have been used as diagnostic tools and molecular targets. Genes implicated in thyroid tumorigenesis are described below.

The **RAS oncogenes** were the first to be associated with thyroid cancer. Members of the RAS family are signal-transducing proteins that share properties with the G-proteins. Genetic alterations in the four isoforms of Ras proto-oncogene (H-RAS, K-RAS, N-RAS and B-RAS) have been described in many tumor types.

Point mutations of RAS determine the encoding of an active protein form and the constitutive activation of MAP kinase pathway that send cell proliferation signals. The complex RAS-

initiated MAPK-terminated cascade signaling is regarded as crucial for the development of thyroid cancer. Mutation of the phosphatidylinosibol-3-kinase catalytic alpha polypeptide (PIK3CA) gene (the product of which binds specifically with RAS and activates PI3K/Akt pathway) also has an important role in thyroid tumorigenesis (Wu G et al.2005 and Hou P et al.2007).

RAS point mutations are uncommon in PTCs, with an overall frequency of less than 10% (Namba H et al.1990 and Capella G et al.1996). Moreover, these genetic alterations appear to confer a predisposition to the development of both poorly differentiated and undifferentiated carcinomas (Nikiforova MN et al.2003).

The **p53 gene** encodes a nuclear transcription factor that plays a key role in cell cycle regulation, DNA repair, and apoptosis and acts a tumor suppressor gene. Alterations of the p53 gene are a peculiar feature in ATCs (Ito T et al.1992 and Donghi R et al.1993). Moreover, the accumulation of p53 protein, frequently caused by p53 mutations, characterizes tumoral areas with a less differentiated phenotype (Donghi R et al.1993 and Matias-Guiu X et al.1994). Conversely, p53 gene mutations are rare in PTCs or these genetic modifications represent a late mutational event (Salvatore D et al 1996). All the above strongly suggest that p53 inactivation is important in tumor progression e in development of a aggressive phenotype (Nikiforov YE 2004).

The **PTEN gene**, which acts as a tumor suppressor gene, encodes a protein that is involved in the regulation of cell division and plays a role in intracellular signaling. It should be noted also that PTEN acts in opposition to phosphatidylinosibol-3-kinase (PIK3), therefore it is considered as a regulator for cell cycle, translation, and apoptosis (Simpson L and Parsons R 2001). Mutations of this gene contribute to the development of a multitude of cancer types (Gimm O et al.2000).

In particular, Gimm et al. (Gimm O et al.2000), showed a 20-30% frequency of hemizygous deletions of the PTEN gene in PTCs and the loss of PTEN function is associated to an advanced stage of this cancer histotype (Garcia-Rostan et al.2006, Fagin JA and Mitsiades N 2008). Moreover, 60% of undifferentiated carcinomas show hemizygous PTEN deletions (Gimm O et al.2000).

The **RET gene** plays a critical role in cell differentiation, proliferation and cell survival of nerve cells. The gene encodes a tyrosine kinase receptor that is activated by interacting with a multicomponent complex that includes a soluble ligand family, the glial cell line-derived neurotrotrophic factors (GDNF), and also a family of cell surface bound coreceptors (LiVolsi VA et al.2004). This binding determinates the receptor dimerization leading to protein autophosphorylation on tyrosine residues and initiation of the proliferation signaling cascade. Subsequent studies have demonstrated a novel form of RET (RET/PTC) resulting from different gene rearrangements which are been identified in PTC. The most common rearranged forms of this gene are RET/PTC1, RET/PTC2 and RET/PTC3. Moreover, RET/PTC rearrangement leads to the costitutive activation of the MAPK pathway and thus, these genetic alterations are being actively explored as therapeutic targets for thyroid cancer.
In recent studies, RET rearrangements have emerged as the second most common genetic abnormality found in PTCs and these genetic alterations are particularly common in tumors in pediatric patients (50-60%) and in patients exposed to accidental/therapeutic radiation during childhood (60-70%) (DeLellis RA 2006). Therefore, ionizing radiation considered as the major risk factor for development of PTC, can directly induce RET recombination events. Finally, several studies have emphasized the fact that rearrangements of this gene are associated with PTCs lacking evidence of progression to poorly differentiated or anaplastic carcinomas (Tallini G and Asa SL 2001 and Quiros RM et al.2005).

The **PAX8-PPARγ** gene fusion has been identified in FTC cases in association with the presence of a recurrent translocation [t(2;3) (q13;p25)] (Kroll TG et al.2000). The consequence of this translocation is the fusion of the DNA binding domains of the thyroid transcription factor PAX8 to domains A-F of the PPARγ1 (Kroll TG et al.2000 and Nikiforova MN et al.2002). In a comparative study of RAS mutations and PPARγ rearrangements, 49% of conventional FTC had RAS mutations, 35% had PAX8-PPARγ rearrangements, only 3% had both abnormalities (Nikiforova MN et al.2003), while 12% were negative for both. The results highlight the crucial role of these genetic alterations in FTC progression.

The **NTRK1 gene** is located on chromosome 1q22 and encodes the receptor for the nerve growth factor (NGF). Similar to RET, NTRK1 undergoes oncogenic activation by

chromosomal rearrangement (Greco A et al.2004), but these mutational events are less frequently found in PTCs than RET rearrangements (Bongarzone I et al.1998). Moreover, the prevalence of NTRK1 rearrangements is approximately 3% in post-Chernobyl PTCs (Rabes HM et al.2000).

The **β-catenin protein** encoded by the CTNNB1 gene (chromosome 3p22- 21.3) has a role in cell adhesion via its interaction with the cytoplasmic tail of cadherins and in the Wnt signaling pathway involving a complex network of proteins that play a crucial role in carcinogenesis (Garcia-Rostan G et al.2001).
Garcia-Rostan et al. (Garcia-Rostan G et al.2001), have demonstrated that CTNNB1 mutations and nuclear (rather than plasma membrane) localization of β-catenin are restricted to poorly differentiated or undifferentiated carcinomas. These findings highlight the important role of CTNNB1 mutations in the progression of thyroid cancer.

The **Sodium Iodine Symporter** (NIS) is a membrane glycoprotein that mediates active iodide uptake in the thyroid gland and several extrathyroidal tissues. In thyroid tumours it is been observed a loss/reduced function of NIS and thus the reduced iodide uptake capacity that gives to tumor cells a proliferative advantage. In anaplastic thyroid carcinomas (ATCs) this altered capacity is mainly due to defects occurring mainly at gene expression level of NIS, but in some case involving post-transcriptional or other unknown alterations (Filetti S et al.1999 and Dohan O et al.2003).

Moreover, the lost ability to concentrate the radioiodine is used as very effective tool for diagnosis and treatment for both tumour remnants or distant metastases (Schlumberger M et al.1986).

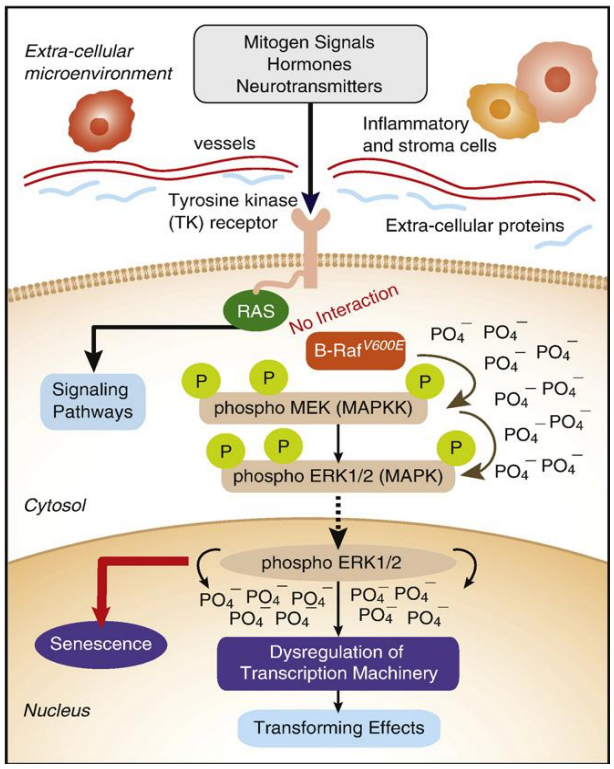
The **BRAF gene** (V-raf murine sarcoma viral oncogene homolog B1) encodes a serine/threonine protein kinase and it plays critical roles in cell proliferation, differentiation and apoptosis by MAPK signaling pathway. The RAF family includes three isoforms: ARAF, BRAF, and CRAF. In particular, CRAF is expressed ubiquitously, whereas BRAF is expressed at higher levels in hemopoietic cells, neurons, and testes (Daum G et al.1994). BRAF is also the predominant isoform in thyroid follicular cells and is more efficient

activator of the MAPK pathway by phosphorylating MEKs (Peyssonnaux C and Eychene A 2001).

In PTC, BRAF mutations have been identified in approximately 40-70% of the tumours (Ciampi R and Nikiforov YE 2005 and Trovisco V et al.2006), and can occur early (Nikiforova MN et al.2003). The most common BRAF mutation is the T1799A transversion mutation (formerly known as BRAF T1796A mutation) in exon 15 of the gene. This mutation causes a V600E (formerly known as V599E) amino acid substitution in the protein and confers the kinase oncogenic function through constitutive activation of the MAPK signaling pathway (Davies H et al.2002). The BRAF V600E mutation is the most common genetic change in PTC (Xing M 2005). In contrast, this genetic alteration is not found in any other form of well-differentiated follicular neoplasm or MTC (Xing M 2005). Practically, in PTCs there seems to be no overlap between RET/PTC, BRAF or RAS mutations, which altogether were found to be present in 66% of cases (Kimura ET et al.2003). Moreover, Pizzolanti G. et al. (Pizzolanti G et al.2007) have demonstrated that BRAF V600E mutation detected on fine-needle aspiration (FNA) specimens, more than RET/PTC rearrangerangements, is highly specific for PTC and its routine research might well be an adjunctive and integrative diagnostic tool for the preoperative diagnostic iter.

In addition a substantial body of data indicates that the BRAF mutational status is a significant predictor of poor clinical outcome and it has been shown that this genetic alteration is associated to extra-thyroidal invasion (Xing M et al.2005). BRAF V600E is also often found in ATC (Begum S et al.2004), suggesting that this mutation may be involved in thyroid cancer progression to poorly differentiated and aggressive phenotypes (Nikiforova et al. 2003, Nikiforov YE 2004 and Quiros RM et al. 2005). All the above strongly suggest that BRAF V600E may be used as tool for diagnosis and prognosis of thyroid cancer.

Finally, “in vitro” studies performed on thyroid cells suggest that BRAF V600E mutation promotes the invasion, by increasing the expression of certain metalloproteinases (MMP-3, MMP-9, MMP-13) (Puxeddu E and Moretti S 2007) and it reduces expression and localization on the plasmatic membrane of NIS (Riesco-Eizaguirre G et al.2006).



Two Hypotheses for ATC development

In order to explain the cascade of events that lead to ATC development two hypotheses are been developed, taking into consideration all the factors involved and their interaction with genetic susceptibility.

According to the first hypothesis, it is believed that ATC is derived from the follicular epithelial cells and it represents a terminal dedifferentiation of preexisting differentiated carcinoma. In dedifferentiation process oncogenes or tumor suppressor genes which foster the development of malignant phenotypes are altered.

Molecular genetic studies suggest that RAS oncogenes are frequently mutated in ATC as a result of progression from RAS-mutated FTCs (Lemoine NR 1989 and Suarez HG 1998). In contrast, RET/PTC rearrangements have not been detected in ATC (Tallini G et al.1998) and thus it is believed that this tumor histotype may derive from other oncogene-mutated PTC. Moreover, p53 frequently mutated in ATC may play a critical role in promoting dedifferentiation of PTC or FTC. BRAF V600E mutation that is peculiar for PTC is often found in ATC (Begum S et al.2004), suggesting that also this mutation may be involved in differentiation process (Nikiforova et al.2003, Nikiforov YE 2004 and Quiros RM et al.2005).

Finally, careful histopathologic examination of ATCs reveals that many of them contain a papillary structure or follicular components in focal areas.

The second hypothesis suggests that ATC may derive from thyroid fetal cells. A considerable number of researches has demonstrated that cancer cells derive from immature progenitor or stem cells, but not from well-differentiated cells (Takano T and Amino N 2000, Takano T 2004). Takano (Takano T 2004) has explained well the clinical and biological features of this hypothesis and there are the molecular evidences that support his theory for thyroid carcinoma. This hypothesis suggests that thyroid cancer cells are generated from fetal cells by proliferation without differentiation and that oncogenes play a crucial role in preventing the differentiation of fetal cells. Therefore, any defect or inhibition of differentiation may lead to cancer cell formation. ATC, PTC and FTC derive from thyroid stem cells, thyroblasts and prothyrocytes, respectively.

In conclusion, different clinical and molecular evidences support one or the other hypothesis, but the mechanism of thyroid cancer development is unclear. Therefore, it remains controversial as to whether ATC is derive from a terminal dedifferentiation of preexisting carcinoma or it arises from thyroid fetal cells.

Thyroid Biomarkers

Every cell type has a unique molecular signature, referred to as biomarkers, which are identifiable characteristics such as levels or activities 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). Therefore, the biomarkers are subject to dynamic modulation, and are expected to enhance our understanding of pathological processes but also drug metabolism. Herberman (Herberman, RB et al.1977) proposed five criteria for a biomarker to be useful in cancer detection: 1- measurement should be simple, reproducible, easily available and cost effective; 2- should detect a quantitative difference so that it can distinguish those with or without disease; 3- should have high sensitivity; 4- should be able to monitor for recurrence of disease, and 5- should have high specificity.

It has been over 40 years since biological markers were first introduced as a way to detect and manage thyroid cancer (Milhaud G et al.1974 and Melvin KE et al.1971). Thyroglobulin and calcitonin represent two of the earliest biomarkers discovered in thyroid cancer (Van Herle AJ et al.1973 and Tashijan AH et al.1970) and their discovery has helped revolutionize the management of patients with thyroid cancer in terms of earlier diagnosis and more sensitive follow-up. Biomarker studies have expanded to include genetic mutations and molecular changes to detect some of the earliest events in thyroid tumorigenesis. In conclusion, genomic and proteomic assays have become the latest methods for identifying a multitude of biomarkers which reflect a molecular profile associated to specific tumor type.

Serum-Based Biomarkers

Serum biomarkers represent the first generation of thyroid biomarkers. Ideally, a serum biomarker is one that is highly sensitive and specific, can establish diagnostic certainty and can be easily measured (Jensen MB et al.2008). Serum biomarkers for thyroid cancer detection are:

Calcitonin an anti-hypercalcemic hormone is secreted by the parafollicular cells and it is used as a serum-based marker for MTC (Holyoke ED et al.1981). The calcitonin detection and

FNA citology in fact has helped optimize the sensitivity and specificity of MTC diagnosing . However, it is not a foolproof test and calcitonin measurements differs depending on the assays used (Bieglmayer C et al.2007). Radioimmunoassays were initially used to detect calcitonin in the circulation. These assays are results less specific because they used polyclonal antibodies which detected several different monomers of this protein (Guilloteau D et al.1990). However, studies have demonstrated that immunoradiometric assays and immunochemiluminescent assays, which are based on monoclonal antibodies, can eliminate variation and improve reliability and sensitivity in the measurement of calcitonin (d'Herbomez M et al.2007 and Toledo SP et al.2009). In addition, the increased calcitonin levels are not exclusive to MTC but there are other conditions when the level of calcitonin is altered, such as C-cell hyperplasia, thyroid nodules of follicular cell origin, increasing age, increased body-mass index, cigarette smoking, breast feeding and small cell carcinoma of the lung (d'Herbomez M et al.2007 and van Veelen W et al.2009).

Thyroglobulin is a glycoprotein that is synthesized by thyroid follicular cells and is the precursor molecule for the production of T3 and T4 hormones (Wong J et al.2008). This protein is a valuable serum marker for detecting recurrent or persistent well-differentiated thyroid cancer of follicular cell origin (Whitley RJ and Ain KB 2004, Gupta M and Chia SY 2007). Although mainly localized in the colloid, thyroglobulin can be resorbed into the peripheral circulation and thus this protein can be detected in serum. However, there are some conditions that make it difficult to interpret the assays results. For example, the presence of anti-thyroglobulin antibodies can interfere with blood thyroglobulin levels (Spencer CA et al.2005 and Haugen BR et al.1999) and assays are not as reliable when patients are on thyroid hormone suppressive therapy. In addition, there are many benign thyroid conditions (e.g., thyroiditis, thyrotoxicosis, benign adenoma and iodine deficiency) which may cause false-positive results (Refetoff S and Lever EG 1983).

In conclusion, calcitonin and thyroglobulin biomarkers have helped improve the management of patients with thyroid cancer, but further advances are necessary because the assays have several limitations. Recent molecular studies have used reverse transcriptase polymerase chain reaction (RT-PCR) to measure mRNA levels in the circulation.

Mutation Based Biomarkers

The next step in the evolution of thyroid cancer biomarkers was the study of genetic mutations in thyroid tumors. The most common molecular alterations in thyroid cancer include BRAF and RAS point mutations and RET/PTC and PAX8/PPARγ rearrangements. These nonoverlapping genetic alterations described previously are found in more than 70% of PTC and ATC and can be detected in surgically resected samples and FNA samples from thyroid nodules. The detection of these molecular markers may to improve significantly the accuracy of cancer diagnosis and allow more specific surgical and postsurgical management of patients with thyroid cancer.

Moreover, the discovery of single genetic mutations is important for understanding cancer formation, but single mutations are not practical clinical biomarkers for thyroid cancer. Therefore, DNA mutations panels are being explored as thyroid cancer biomarkers. This shift in approach happened because cancer development is a complex event that involves multiple genetic and possibly epigenetic events.

Epigenetic Biomarkers

Epigenetics is defined as the study of heritable changes in gene expression which are independent from DNA sequence modifications. Moreover, one of the most exciting aspects of epigenetic regulation is that unlike genetic mutations, these processes are easily reversible with various therapeutic agents. Epigenetic mechanisms include DNA methylation, histone protein modification, nucleosome positioning, and microRNA silencing. In thyroid cancer DNA methylation, histone modifications and microRNA silencing have all been studied, but there is minimal data on nucleosome positioning.

In particular, hypermethylation of multiple genes has been identified in association with the PIK3/AKT pathway in FTC and with the MAPK pathway in PTC (Alvarez-Nuñez F et al.2006 and Hoque MO et al.2005). Moreover, most studies have demonstrated a miRNAs upregulation in PTC and FTC (Nikiforova MN et al.2009) A few trials using FNA samples have established miRNA as a possible diagnostic tool, but larger clinical trials are needed to confirm the utility of miRNAs as a clinical biomarker (Chen YT et al.2008).

Genomics and Proteomics

The terms “genomics and proteomics” refer to the analysis of gene sequence and protein production, respectively. Advances performed in these fields have contributed to the

understanding of signal transduction pathways that control important cellular processes, such as growth, differentiation and death of cells. Since defects in these processes may result in the pathological phenotypes expression, it becomes crucial the identification of candidate disease genes, the proteins study which are responsible of genetic function expression and consequently the detection of genetic or protein changes. This integrated approach, called "systems biology", aims to recognize early onset of disease, institute preventive treatment and identify new molecular targets for cancer therapy.

Moreover, “genomics and proteomics” are characterized from application of throughput or large- scale experimental methodologies combined with statistical and computational analysis. The fundamental strategy is to expand the biological investigation from study of single genes or proteins to analysis of genetic and proteomic profiles in a systematic fashion.

In conclusion, the hope is that the study of gene and protein expression profiling will lead someday to personalized medicine that allows to develop specific therapeutic strategies tailored of patient needs.

Genomics

The genomics field was focused initially on mapping the human genome and understanding the effects of single genes in a system. Subsequently, the attention has shifted to identification of disease genetic signatures. Many different gene expression profiling technologies are currently in use, including cDNA microarrays, oligonucleotide arrays and Serial Analysis of Gene Expression (SAGE) (Mandruzzato S 2007 and Hoheisel JD 2006).

In particular, DNA microarray analysis has allowed to define distinct expression profiles for the BRAF, RET/PTC and RAS mutation groups in PTCs (Giordano TJ et al.2005). Therefore, the data of this study suggest that approach may be useful for predict tumors with these mutations. Other gene expression profile studies have identified distinct genetic signatures which correlate with the PAX8/PPARγ translocation in FTC (Lui WO et al.2005) and with RET/PTC rearrangements in radiation-induced PTC. The correlation with RET/PTC rearrangements may help to evaluate the invasion risk in PTC (Detours V et al.2007).

However, contradictory findings are present in several studies; in fact genes which are involved in thyroid cancer and identified in individual studies are not consistently found across multiple studies. These experimental evidences suggest that the gene expression profile methodologies should be standardized to use these genetic signatures as biomarkers of thyroid cancer.

Proteomics

Proteomic application to cancer is a common effort by several laboratories in the world to integrate knowledge on structure, function and dynamics of proteins involved in cancerogenesis (Pucci-Minafra I et al.2006). Numerous progress has been made in the past few years in generating large-scale data sets for protein-protein interactions, organelle composition, protein activity patterns and protein profiles associated to cancer.

Before highthroughput proteomics was developed, the study of thyroid biomarkers is based on immunohistochemistry that combined with FNA cytopathology has improved the diagnostic accuracy in indeterminate thyroid nodule FNA samples.

Two immunohistochemical markers of thyroid cancer which have been extensively studied are galectin-3 and Hector Battifora mesothelial cell antibody (HMBE-1).

Galectin-3, a lectin-binding protein, has multiple functions, including cell–cell and cell–matrix adhesion, cell growth, neoplastic transformation and spread, cell cycle regulation and apoptosis, and cell repair processes. Galectin-3 has been found to be increased in several human malignant tumors, including well-differentiated follicular-derived thyroid carcinomas (Saggiorato E et al.2001).

HBME-1 was originally described as a marker of mesothelial cells and it recognizes an unknown antigen expressed by those cells. It was later shown to also stain most PTCs, and also a fraction of FTCs, while adenomas are generally negative (Casey MB et al.2003 and Cheung CC et al.2001).

However, the results for these markers were variables when each of these was examined individually. Some studies have demonstrated that these markers could differentiate between benign and malignant thyroid lesions and that the protein expression is varied depending on the PTC stage (Volante M et al.2004 and Rossi ED et al.2006), but they did not help for distinguish the histological variants of PTC.

Several studies have demonstrated that the combined detection of galectin-3, HBME- together with cytokeratin-19 determines an sensitivity and specificity increasing of cancer diagnosis on tissue or FNA samples (Rossi ED et al.2006 and Wiseman SM et al.2008). Consequently, these results suggest that panels or patterns of these and other markers may represent the

future of analyzing protein expression to improve the diagnosis of the indeterminate thyroid nodular lesion.

The study of thyroid biomarkers has now expanded to include the proteome analysis that investigates protein expression and modification, along with changes in protein activity and localization (Srinivas PR et al.2001). The information gained from the proteome reflects not only the genomic profile expressed from the cell, but also takes into account post-translational changes which are not detected at the mRNA level, as well as protein expression at different time points of neoplastic transformation (Srinivas PR et al.2001, Wilkins MR et al.1996 and Krause K et al.2009). Proteomic expression profiling high-throughput technologies include two-dimensional electrophoresis (2D-IPG), mass spectrometry analysis and ProteinChip technology (Wang JX et al.2006 and Wulfkuhle JD et al.2003).

In particolar, Giusti et al. (Giusti et al.2007 and Giusti et al.2008) was the first group to analyze the fluid obtained from FNA of thyroid cancer by applying of proteomic methodologies. They performed a comparative proteome analysis to examine the global changes of FNA protein patterns of two variants of malignant PTC with the controls. Changes in protein expression were identified using two-dimensional electrophoresis (2D-IPG) and peptide mass fingerprinting via MALDI-TOF mass spectrometry (MS), as well as Western blot analysis. They discovered 17 protein spots that could reliably distinguish between papillary thyroid cancer and normal thyroid tissue. These proteins included for example transthyretin precursor (TTR), ferritin light chain (FLC), proteasome activator complex subunit 1 and 2 (PSME1 and 2), glyceraldehyde-3-phosphate dehydrogenase (G3P), lactate dehydrogenase chain B (LDHB), annexin A1(ANXA1) and cofilin-1 (COF1) (Giusti et al.2008).

Moreover, a study has reported the presence of RET oncogene mutations in human MTC cell lines and thus it has compared expression profiles of downstream signaling elements by using proteomic analysis and immunological validation (Gorla L et al.2009). These results have demonstrated that 41 phosphotyrosine proteins were detected downstream following RET activation and the EGFR pathway was over-expressed.

In conclusion, although there are been numerous advances in thyroid biomarker research, an improvement of diagnostic tests and tumor aggressiveness predictors is necessary. Many thyroid cancer biomarkers have been identified, but few have made it into routine clinical

practice. Therefore, it is necessary the combined attendance of genomics and proteomics to find reliable and clinically helpful biomarkers. New fields of research, such as metabolomics or kinome, should be explored in thyroid cancer in order to implement the information known.

AIM OF THIS STUDY

Thyroid carcinomas are the 1-2% of all malignant tumors and, after those of the gonads, are the most common tumors of the endocrine system.

Moreover, it consists of several histotypes with different molecular profiles, biological behaviour and risk profiles as described previously. In particular, our interest is been focused on PTC and ATC because these thyroid hystotypes are characterized by opposite phenotypic features . In fact, PTC represent approximately 80% of all thyroid malignancies and are characterized by a good prognosis. Differently, ATC which account for less than 5% of all thyroid cancers, is the most malignant thyroid neoplasm and is almost invariably fatal.

Current clinical parameters with prognostic and predictive factors constitute important tools for the identification of thyroid cancer therapy and providing an efficient treatment, but an improvement of diagnostic tests and tumor aggressiveness predictors is necessary.

To contribute to the knowledge of thyroid tumorigenesis, we performed a differential analysis of two thyroid cancer cell lines, derived respectively from papillary (B-CPAP) and anaplastic (8505C) thyroid carcinomas. These cell lines represent important model for the study of in vitro differentiated and undifferentiated tumors.

The comparative analysis of the two cell lines has been structured on research different levels of and required the use of several experimental methodologies of cellular biology and proteomic analysis. Therefore, to characterize the phenotypes of B-CPAP and 8505C cell lines, the present study aims to:

- Analyze the morphological characteristics by optical and scanning electron microscopy;
- Evaluate the proliferative capabilities by application of MTS assays;
- Create the reference maps for each cell line and confirm the protein identities of the through MALDI-TOF and/or N terminal sequencing;
- Perform a comparative proteomic analysis between the B-CPAP and 8505C cell lines and thus find potential thyroid biomarkers and new functional pathways involved in thyroid cancer progression;
- Assess the invasive capabilities through detection of gelatinase activity;
- Evaluate the cell motility by Scrach Assay.

In conclusion, we suggest that this approach may be useful for increasing the knowledge and the typisation of these thyroid tumors. Moreover, the proteomic analysis may be useful for studying the mechanisms underlying the process of dedifferentiation and for identifying potential molecular markers associated with the PTC and ATC.

MATERIALS AND METHODS

Cell culture

Human papillary thyroid carcinoma cell line, B-CPAP, and human anaplastic thyroid carcinoma cell line, 8505C, were kindly provided by the Laboratory of Endocrinology (University of Palermo, Italy). The cells were cultured in RPMI 1640, supplemented with 10% foetal calf serum (FCS; GIBCO), L-glutamine 2 mM and antibiotics (100 units/ml penicillin and 100 ìg streptomycin) in a humidified incubator with 5% CO_2 in air at 37°C.

Cell proliferation assay

Cell proliferation was determined by the use of a colorimetric tetrazolium compound (CellTiter 96, Promega). Briefly, 20 μL of CellTiter 96 was added to 100 μL of medium into each well containing the cells. After 1 h of incubation in a humidified, 5% CO_2 atmosphere, the absorbance at 492 nm was read using a 96-well plate reader (Amersham).

Protein extraction from cell culture and conditioned media preparation.

Cell-lysates: After washing with ice-cold phosphate buffered saline (PBS), cells were carefully scraped and incubated on ice for 30 min with RIPA buffer (50 mM Tris pH 7.5, 0.1% Nonidet P-40, 0.1% deoxycholate, 150 mM NaCl, 4 mM EDTA) and a mixture of protease inhibitors (0.01% aprotinin, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1mM PMSF). The total cellular lysates were centrifuged at 14.000 rpm for 8 min to clear cell debris.

Conditioned media preparation: Conditioned media from confluent B-CPAP and 8505C cells were harvested after 24h culture in RPMI 1640 without serum, centrifuged to remove cell debris and sterile filtered.

Both cellular lysates and conditioned media were dialysed against ultrapure distilled water, lyophilised and stored at -80°C until analysis. The total protein concentration was determined by the Bradford method using bovine serum albumin as a standard (16).

Two Dimensional Gel Electrophoresis

The proteins extracted from B-CPAP and 8505C cell lines were solubilised in a buffer containing 4% CHAPS, 40 mM Tris, 65 mM DTE (1,4-dithioerythritol) and a trace amount of bromophenol blue 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).

Protein identification

N-Terminal microsequencing was performed by automated Edman degradation in a protein sequencer (Procise, 419 Applied Biosystems) (17).

Mass spectrometric sequencing was performed by Voyager DE-PRO (Applied Biosystems) mass spectrometer as described (18). 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.

Scratch assay

The confluent mono-layers of B-CPAP and 8505C cells were scraped with a p200 pipet tip. After removing cell debris with PBS, growth medium was added. The assays were monitored at different times (6h - 24h). from "scratch".

Zymography

Aliquots of conditioned media of B-CPAP and 8505C corresponding to 10 µg of cell proteins were separated in acrilamide gel containg 0.1% gelatin. After the electrophoresis run, gel was rinsed in 50 mM Tris-HCl (pH 7.5) with 2.5 % Triton X-100 for 1 h. and incubated at 37°C for 18 h in a buffer containing 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl and 10 mM CaCl2. to detect gelatinases activity. Finally, the gel was stained with a solution of 0.2% Coomassie Brilliant Blue G-250 in 40% methanol and 10% acetic acid and destained with a solution of 7% methanol and 5% acetic acid, without dye. Clear zones against the blue background indicated the presence of proteolytic activity.

Western Blotting

For immune detection the gel was 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-MMP-9 and MMP-2 (Santa Cruz). Following incubation with anti-mouse peroxidase-linked antibody (Santa Cruz sc-2005), the reaction was revealed by the ECL detection system, using high performance films (Hyperfilm ECL, Amersham).

RESULTS AND DISCUSSION

Morphological Analysis

B-CPAP cell line, isolated from a differentiated thyroid cancer, shows rounded morphology and absence of cytoplasmic extensions. In contrast, 8505C cell line is characterized by a quite irregular morphology with membrane perturbation and numerous cytoplasmic extensions generally associated with high invasive phenotype. Figure (A-B) shows representative optical micrographs of B-CPAP and 8505C cells. As it is possible to observe B-CPAP displays a regular-rounded morphology with a refractive cytoplasm. After substrate adhesion, B-CPAP cells acquire a follicle-like structures, which include a variable number of cells often delimiting the small central cavity corresponding to the "holes" described by Fabien *et al*. In contrast, 8505C cells acquire a very irregular morphology and organization after substrate adhesion. A common feature shown by these cells was their ability to connect with each others by very long cytoplasmic bridges or spikes.

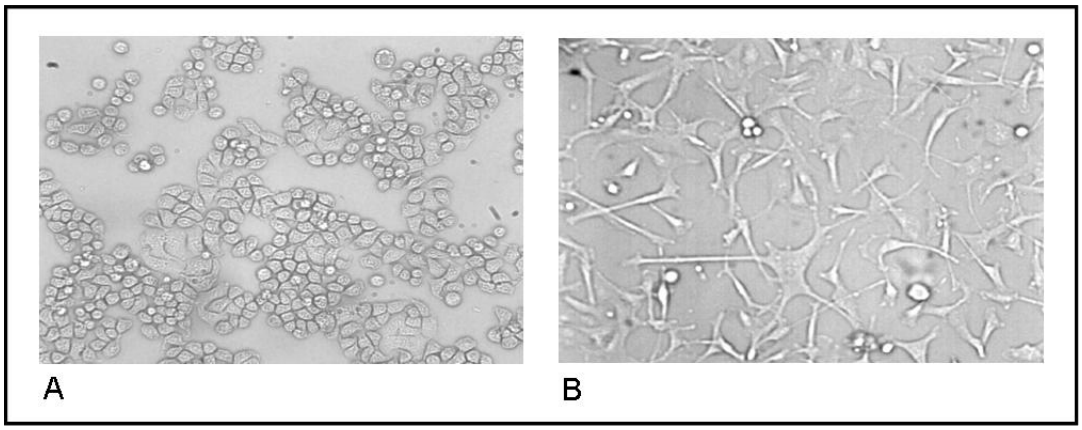


Figure . Representative optical micrographs (20x) of B-CPAP (A) and 8505C (B) cell lines at 72h from the seeding.

Electronic microscopy of B-CPAP and 8505C cells (Figure A-B) confirmed the different arrangements of the cells. B-CPAP cell line forms the follicle-like structures, previously described, and cell surface appears almost irregular. 8505C cell line shows a polygonal cell shape with numerous cytoplasmic extensions. The morphological features of these cell lines are indicative of different malignant potentiality, suggesting that the 8505C cells are a more aggressive phenotype respect to the B-CPAP cells.

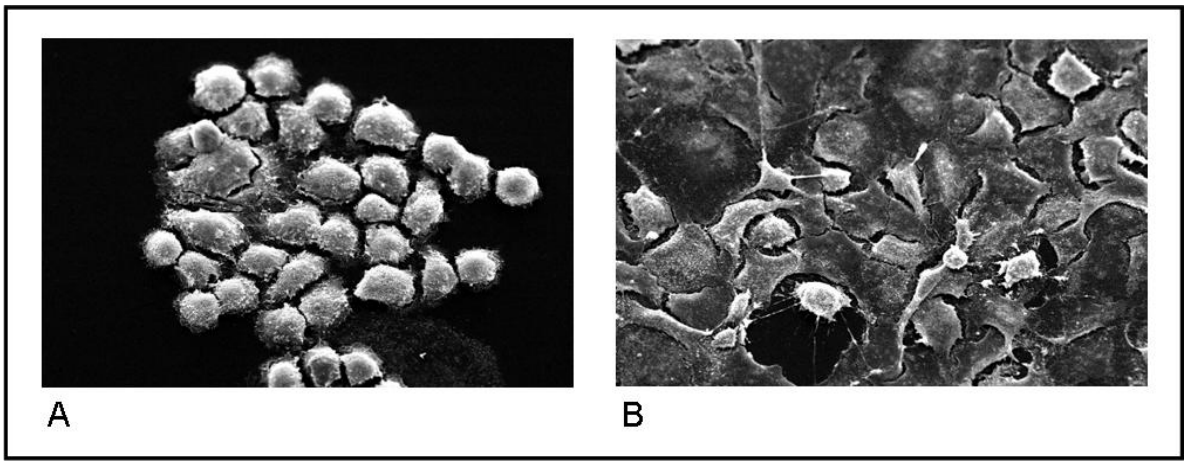


Figure . Representative electron micrographs (500x) of B-CPAP (A) and 8505C (B) cell lines at 72h from the seeding.

Cell Proliferation Assay

Cell proliferation ability was determined at 24h, 48h, 96h, 5, 6, 7 and 9 days from seeding, using the MTS colorimetric assay, which measures dehydrogenase activity found in metabolically active cells. The experiment was performed as described in materials and methods.

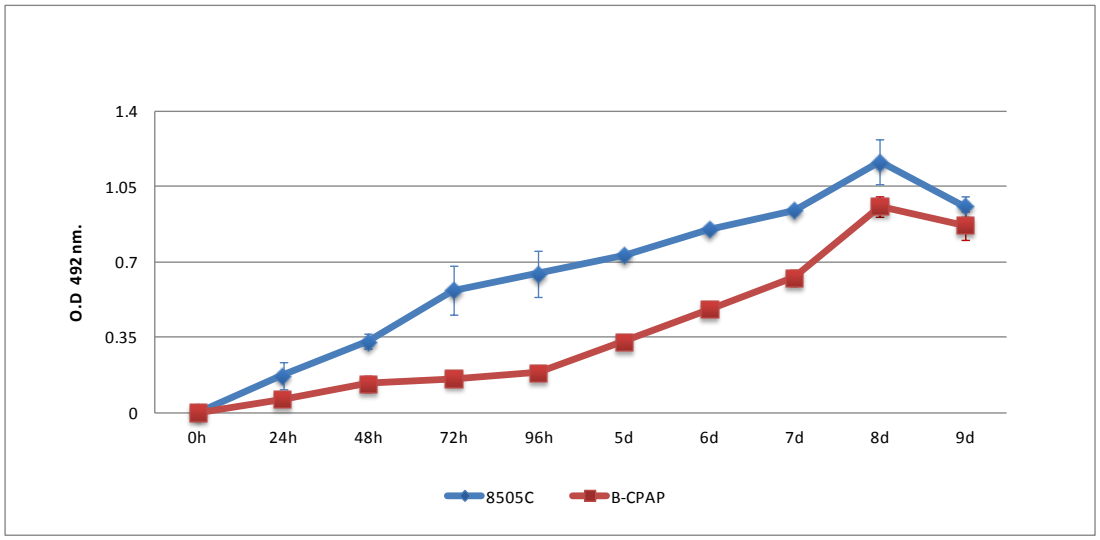


Figure Cell growth of B-CPAP and 8505C cell lines by MTS colorimetric assay.

The figure shows the growth curves of B-CPAP and 8505C cells where each point represents the mean of three replicates from three independent experiments (± SD). The results of the MTS assay show that in 8505C cells proliferation capacity is higher than in the B-CPAP cells.

Proteomic Analysis

The study performed in our laboratory on lysates of B-CPAP and 8505C cells demonstrated qualitative and quantitative differences in the proteomic profiles between papillary and anaplastic cell lines. Anaplastic proteome showed a greater abundance of cellular proteins when compared with papillary proteome.

Qualitative profiling of B-CPAP and 8505C cell lines

Figures show reference maps of B-CPAP (Fig.) and 8505C cell lines (Fig.), that have been analysed with Image Master 2D Platinum. The protein identities are marked with labels corresponding to the abbreviated name of Swiss-Prot database. 233 and 230 protein spots (including isoforms and variants) of B-CPAP and 8505C cells respectively, were identified in the maps. The protein identities were assessed by MALDI-TOF and/or N terminal sequencing.

The identified proteins were clusterized according to our previous criteria, confirmed by the DAVID Bioinformatics database. Database has been utilized 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, by 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, that correct the enrichment P-value to control false discovery rate.

The gene list corresponding to the 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, introduced new terms for further remodulation of protein clusters on the basis of the multiple functions for individual proteins.

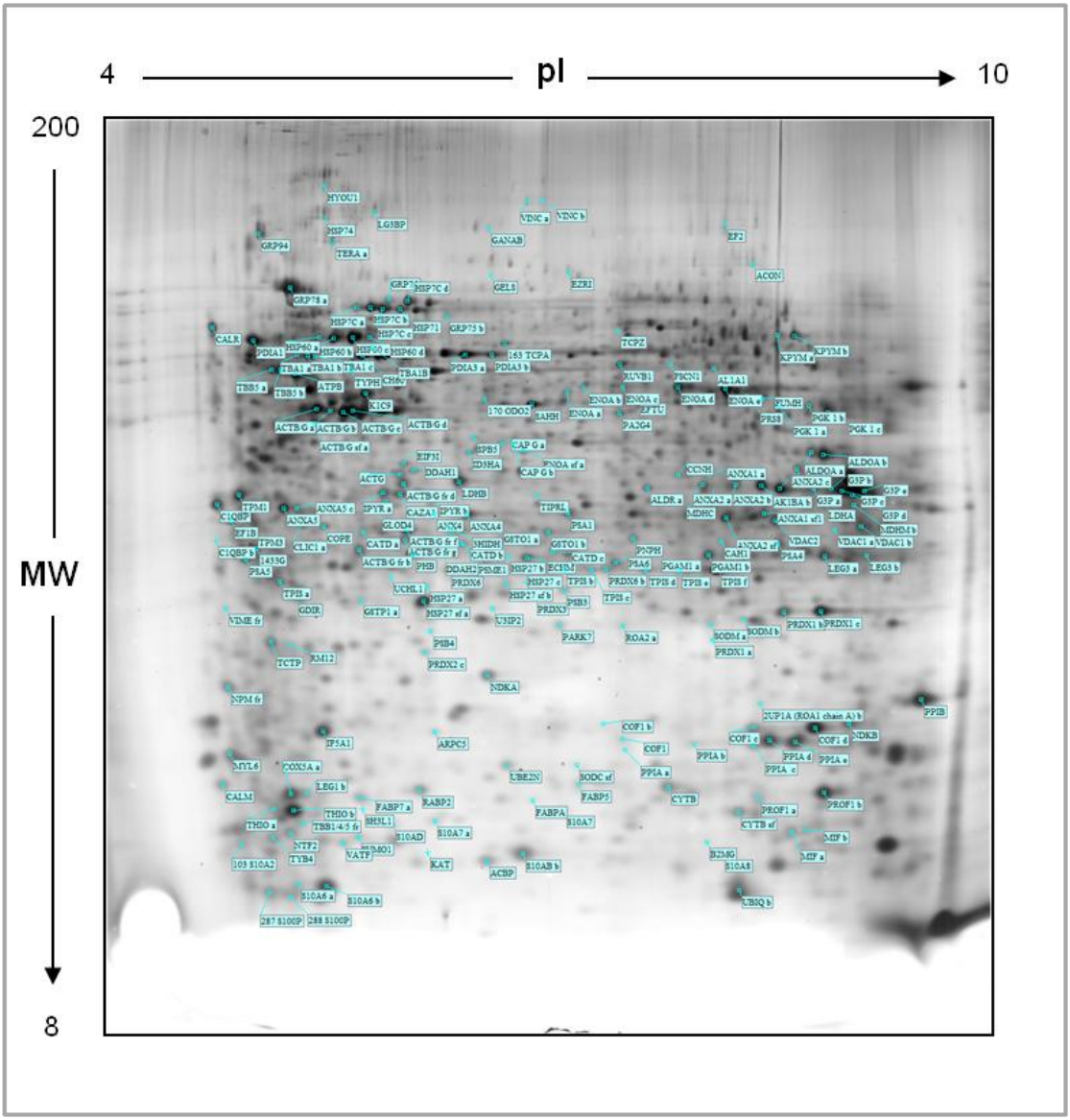


Figure . Representative proteomic map of the protein extracted from B-CPAP cells. 2-DE separation was performed on IPG gel strips (18 cm, 3.5–10 NL) followed by the SDS-PAGE on a vertical linear-gradient slab gel (9–16%T). Protein spots of known identity are labelled with the abbreviated name of the Swiss-Prot database. The different isoforms of the same protein are jointly labelled. Other abbreviations: sf = short form; fr = fragment.

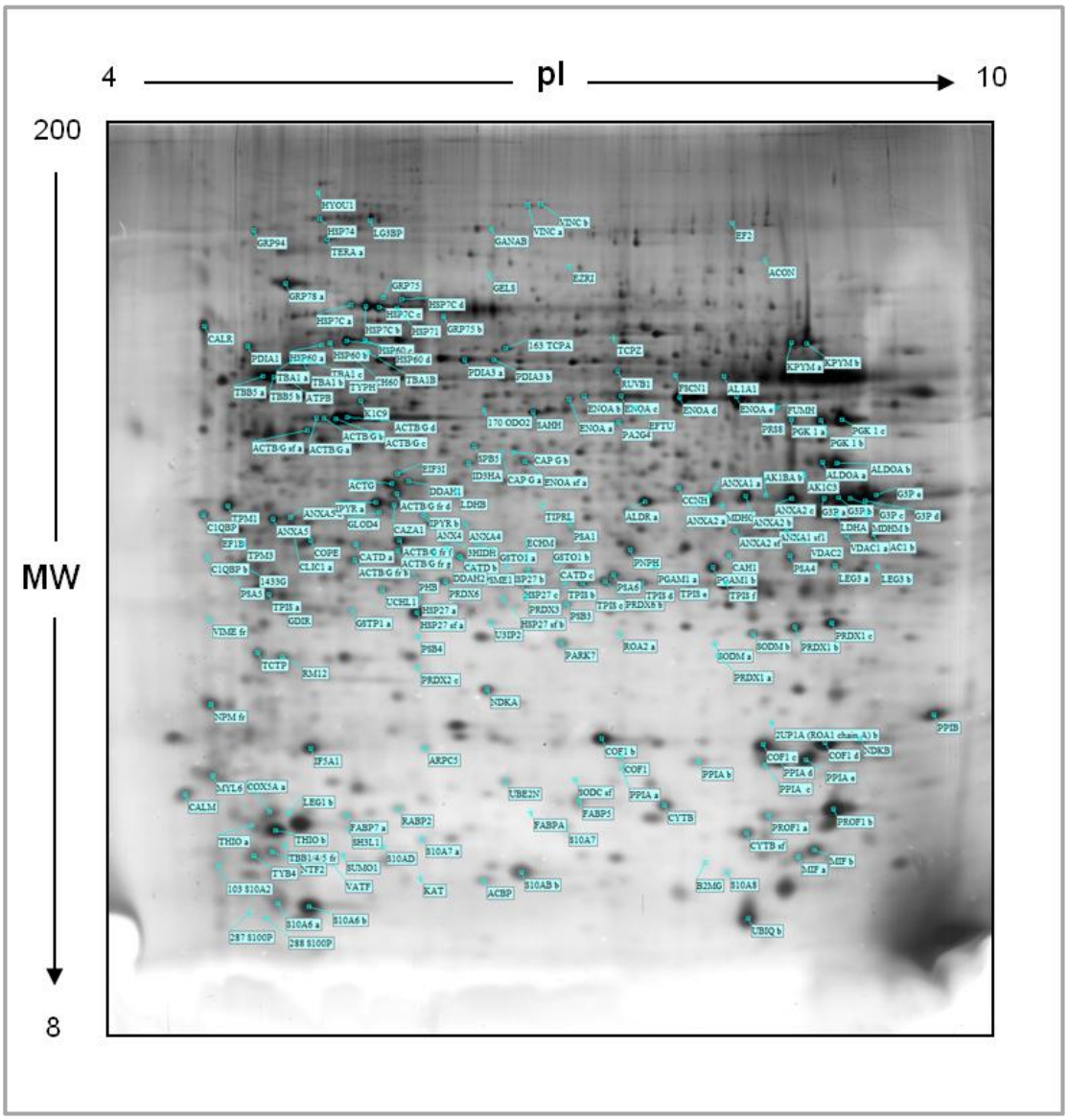


Figure . Representative proteomic map of the protein extracted from 8505C cells. 2-DE separation was performed on IPG gel strips (18 cm, 3.5–10 NL) followed by the SDS-PAGE on a vertical linear-gradient slab gel (9–16%T). Protein spots of known identity are labelled with the abbreviated name of the Swiss-Prot database. The different isoforms of the same protein are jointly labelled. Other abbreviations: sf = short form; fr = fragment.

The identified proteins were clusterized into ten groups which are the followings: 1) Cytoskeleton and associated proteins; 2), Metabolic enzymes; 3) Molecular Chaperones/Heat Shock Proteins; 4), Membrane-associated proteins with multiple activities, 5) Calcium binding proteins with EF domain, 6) Proteins with binding function, 7) Cell growth and proliferation regulators, 8) Protein degradation, 9) Detoxification and redox proteins and 10) Proteins with multiple activities.

Quantitative profiling of B-CPAP versus 8505C cell lines

To compare the protein expression levels of 8505C versus B-CPAP cells, we carried out triplicate experiments to ensure reproducibility of results (Figure), and we applied the densitometric algorithm of the Image Master software, normalizing the data to the sum of all spot volumes on gels (% Vol).

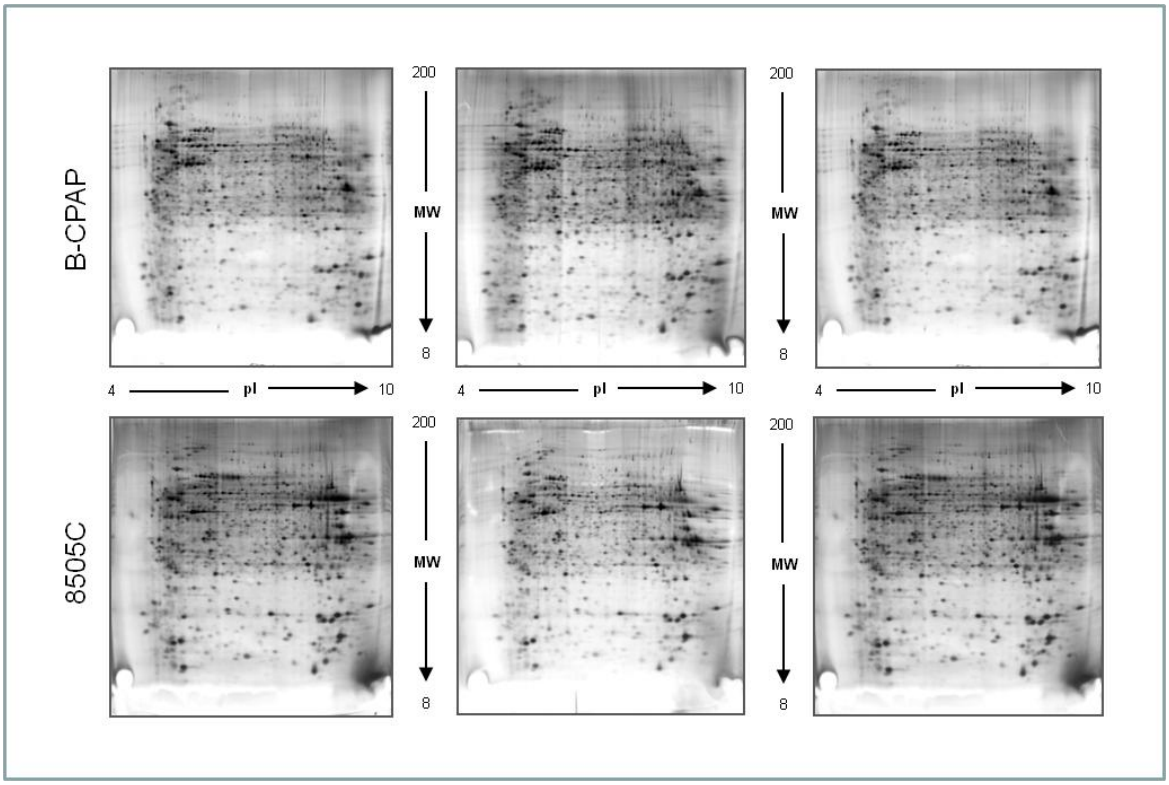


Figure . Miniature of triplicate 2D gels from B-CPAP and 8505C cell lines, used for comparative analyses.

The quantitative analysis was performed on 229 identified protein spots (Figure). The average of three spot values from three different gels for each cell line was utilized, and expression levels of cellular extracts were considered significantly different for ≥ 30% variations.

The diagam in figure shows the density profiles of B-CPAP (blu curve) and 8505C cells (red curve). As it can be observed the two profiles are clearly dissimilar in terms of expression levels of individual proteins.

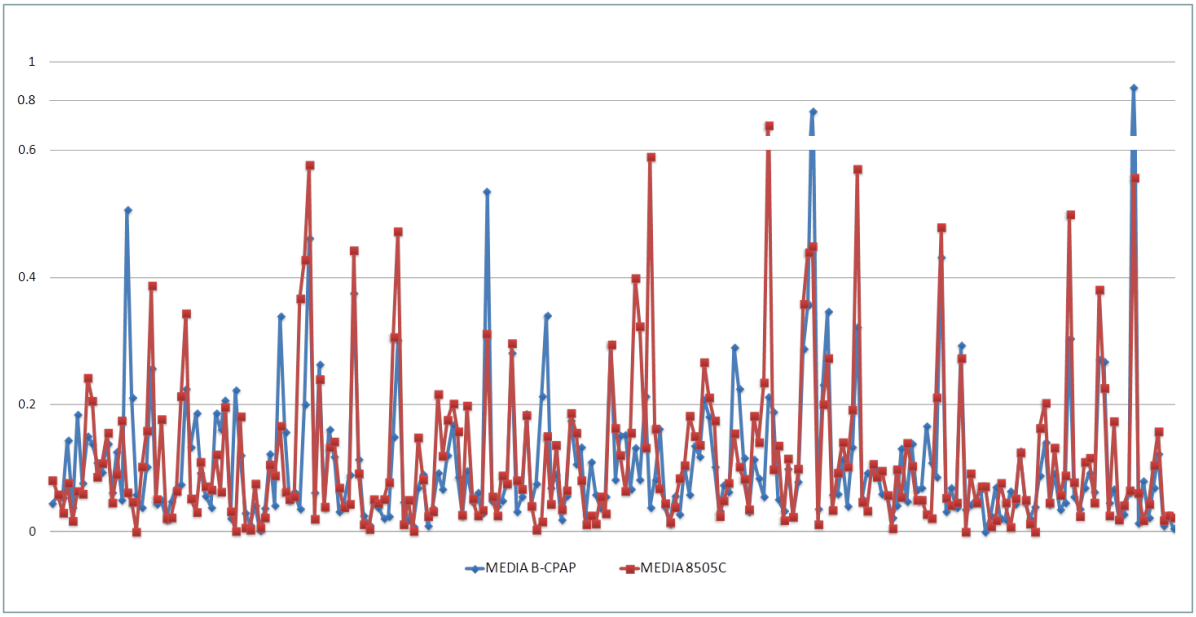


Figure . Diagram of comparative proteomic profiling of B-CPAP and 8505C cells.

In particular the majority of identified proteins, i.e. 63% of the total, are differentially expressed while the remaining 37% are unvaried. Among the differentially expressed 53% are increased in 8505C proteome while and 47% are reduced in the same maps (Figure a-b).

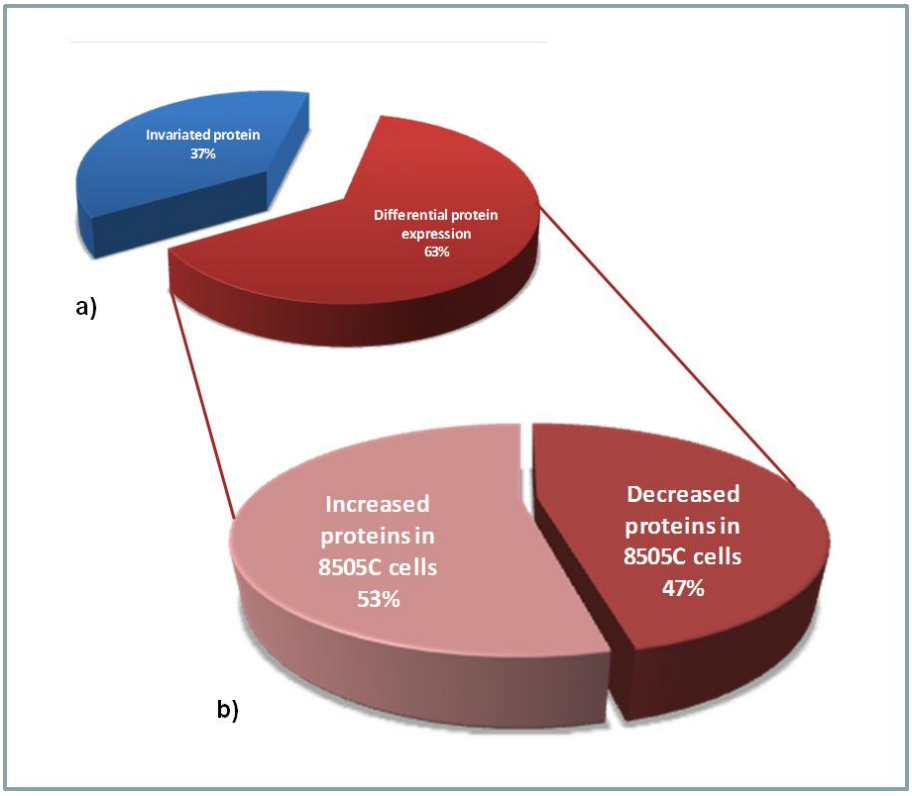


Figure. Diagram A shows invariated and differential proteins, while diagram B displays increased and decreased proteins in 8585C respect B-CPAP cell line, expressed in percent.

Concerning the identity of the differentially expressed proteins, it was observed that the majority of them belong to the group of metabolic enzymes, followed by the other groups, that in order of representativity are: Cytoskeleton and associated proteins, Molecular Chaperones/Heat Shock Protein, Cell growth and proliferation regulators, Membrane-associated proteins with multiple activities, Detoxification and redox proteins, Calcium binding proteins with EF domain, Proteins with binding function, Protein degradation and Proteins with multiple activities .

Metabolic enzymes

The elevated levels of anaerobic metabolism, even in the presence of oxygen, known as the "Warburg effect", is a frequent clinical syndrome in oncologic patients, an observation

recognized by several authors, including our group (13-15-19), as a frequent feature of neoplastic cells both in vivo and in vitro.

In both B-CPAP and 8505C proteomic profiles the category of metabolic enzymes includes 38 protein spots, expressed at different intensity levels between the two cell types. The cluster contains anaerobic glycolysis, Krebs Cycle and six proteins of other metabolic pathways (AL1A1, DDAH 1, IPYR, 3HIDH, PNPH and VATF). Figure displays the diagram of protein fold variation between B-CPAP and 8505C cell lines belonging to metabolic enzymes cluster and expressed in a logarithm scale. Relative intensity of protein spots was calculated normalizing the data to the sum of all spot volumes on gels (vol%). Each value is the mean of three independent determinations and the expression levels of cellular extracts were considered significantly different for ≥ 30% variations. In this cluster we observed the number of proteins belonging to the glycolysis pathway is quite high (26 protein spots) and many of them show higher levels of expression in 8505C than B-CPAP cell line. Aerobic glycolysis induces acidification of the environment cancer by stimulating the development of a more aggressive and invasive phenotype that is characteristic of 8505C cell line. The increased proteins in 8505C cell line are: 2 isoforms of ALDOA, 4 isoforms of ENOA, 1 short form of ENOA, 4 isoforms of G3P, 1 short form of G3P, 3 isoforms of PGK 1, 2 isoforms of KPYM, 3 isoforms of TPIS and 1 isoform of PGAM1 and LDHA. In contrast other glycolytic enzymes decrease, these are: LDHB, 1 fragment of ENOA and 1 isoform of TPIS.

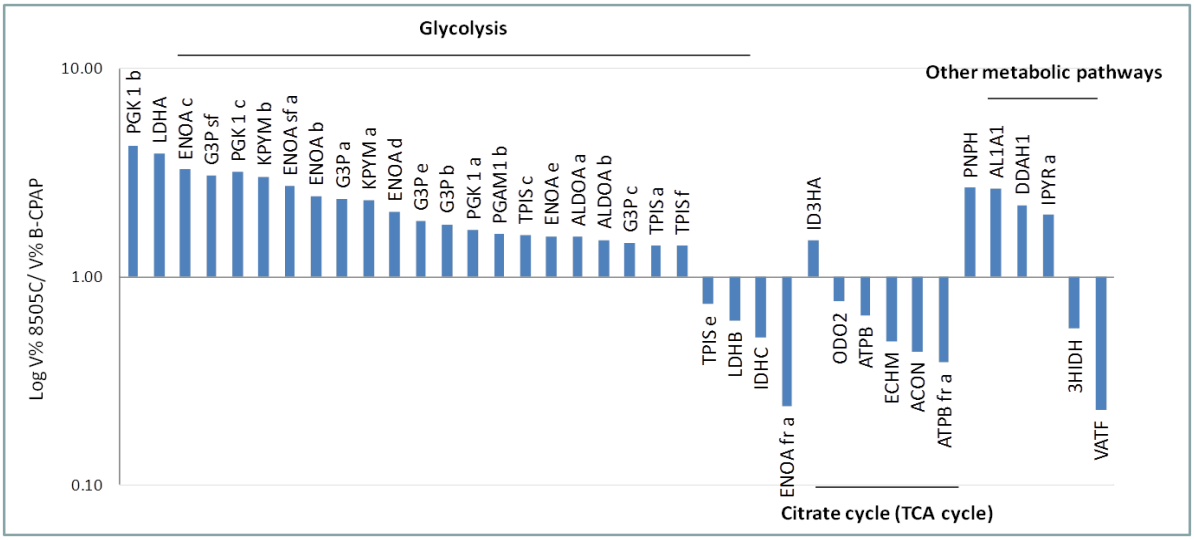


Figure. Diagram shows the distribution of metabolic proteins among B-CPAP and 8505C cell lines and breakdown of enzymes in metabolic pathways.

Among these enzymes two proteins are representative for the glycolytic process:
α-enolase (ENOA) This protein may acts as 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. α-enolase is expressed at the cell surface, where it promotes cancer invasion, and it 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;

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyzes an important energy-yielding step in carbohydrate metabolism, the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate in the presence of inorganic phosphate and nicotinamide adenine dinucleotide (NAD). The enzyme exists as a tetramer of identical chains. Glucose metabolism has a central role in carcinogenesis; among the enzymes controlling this energy production pathway, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is of particular interest. This enzyme is actually tightly regulated and is involved in numerous cellular functions, such as the regulator of cell death; some studies point toward a proapoptotic function, others describe a protective role and suggest its participation in tumor progression.

Finally, other proteins (6 protein spots) of this group are Krebs Cycle enzymes. IDH3A increase in 8505C cell line, while ODO2, ECHM, ACON, 1 isoform and 1 fragment of ATPB.

Cytoskeleton and associated proteins

Cytoskeleton represents a dynamic structure, that is capable of organizing cytoplasmic organelles and intracellular compartments, defining cell polarity and generating both pushing and contractile forces. In cell cycle, this structure plays a pivotal role in chromosomal separation and cell division. During morphogenesis, cytoskeleton determines cell shape and polarity, and promotes stable cell-cell and cell-matrix adhesions. Finally, during cell migration it generates protrusive forces at the front and retraction forces at the rear. These are all aspects of cell behavior than often go awry in cancer.
In the group of cytoskeleton and associated proteins (24 protein spots) the increased levels were detected for: ACTG, 2 isoforms of ACTB, 2 isoforms of COF1, 2 isoforms of PROF1, 2

isoforms of TBB5, 1 fragment of VIME, 1 isoform of VINC, ARPC5 and TYB4. Decreased levels were found for 2 isoforms of ACTB, 1 short form of ACTB, 2 isoforms of CAP G, EZRI, K1C9, K2C8, TPM3, TBA1B and LMNB1. Figure displays the diagram of protein fold variation between B-CPAP and 8505C cell lines belonging to cytoskeleton and associated proteins cluster and expressed in a logarithm scale.

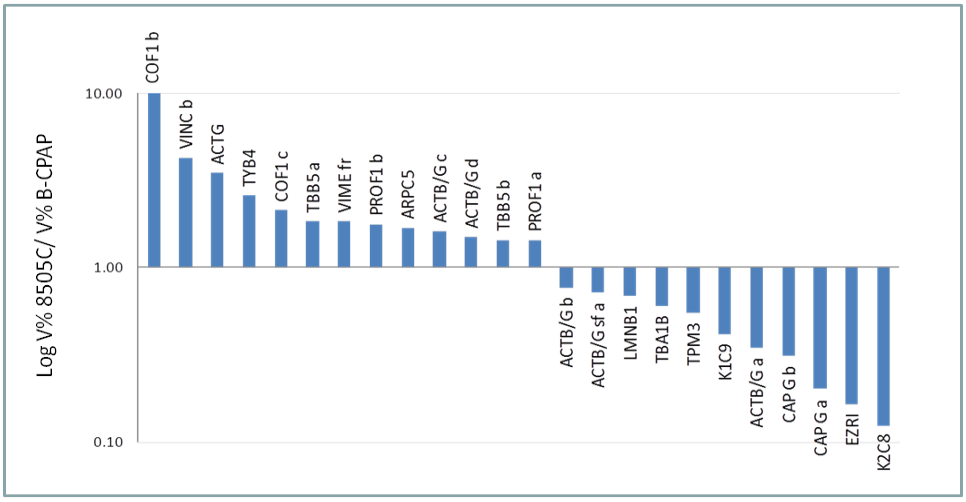


Figure. Diagram shows the distribution of cytoskeleton and associated proteins among B-CPAP and 8505C cell lines.

The proteins of this category, implicated in different diseases, are briefly described below.

Actin, cytoplasmic 1 and 2 (ACTB and ACTG) are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells. Actin and tubulin form highly versatile, dynamic polymers that are implicated in the maintenance of cell polarity, in cell cycle, cell division and cell motility. It is interesting to note that isoforms of ACTB in 8505C cells are characterized by opposite expression, probably due to a different post-translational regulation of the native protein, which require different skills for each isoform;

Tubulin alpha-1B and beta-5 chain (TBAIB and TBB5) are the major constituents of microtubules and participate in mitotic spindle formation. Therefore, TBB5 increased expression in 8505C cells may be related to the rate of cell proliferation. Furthermore, TBAIB mRNA overexpression was observed in anaplastic carcinomas (Takano T. 2011). In contrast

we have seen low protein expression of TBAIB in 8505C cells and this levels difference between mRNA and protein may be due to a post- trascriptional regulation;

Cofilin (COF1) is extremely important for the formation of membrane protrusions and directional spikes, through actin-binding activity, involved in the invasive and metastatic phenotype of tumour cells (Wang W et al. 2007). We observe the increase of cofilin in 8505C cells and this high expression is correlated with the appearance of cytoplasmic extensions, showed in these cell line.
The protein is responsible also for the signal translocation from cytoplasm to nucleus. However it can also function as anti-apoptotic factor by binding to the 1433-G protein;

Profilin (PROF1) is involved in the maintenance of cell morphology, cell motility, promoting the metastatic process and in signal transduction induced by growth factors;

Vimentin (VIME) 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);

Vinculin (VINC) is a focal adhesion protein, involved in the mechanical coupling between the actin cytoskeleton and the extracellular matrix. The protein is a key protein regulating the transmission of contractile forces. High expression of this protein is observed in 8505C cells and it has been shown that amplification and overexpression of vinculin are associated with increased tumour cell proliferation and progression in advanced prostate cancer (Ruiz C);

Thymosin β4 (TYB4) is known as a 4.9-kDa mass polypeptide that interacts with G-actin and functions as a major actin-sequestering protein in cells . It stimulates tissue remodeling, cell differentiation, cell proliferation and cell motility. Furthermore, TYB4 induces angiogenesis (49) and is involved in the formation of metastasis (28). Enhanced thymosin β4 expression is observed in medullary thyroid carcinoma (26) and renal tumor (27) as compared with their normal counterparts;

Actin-related protein 2/3 complex subunit 5 (ARPC5) functions as component of the Arp2/3 complex which is involved in regulation of actin polymerization and together with an

activating nucleation-promoting factor (NPF) mediates the formation of branched actin networks. Once activated, theArp2/3 complex nucleates new actin filaments and also crosslinks actin filaments from end to side-branch, thus creating new barbed ends, which drive the extension of the cell's leading edge (Mullins et al, 1998).Increased expression of Arp2/3 complex in 8505C cells may be related to the creation of cytoplasmic extensions;

Keratin, type I cytoskeletal 9 and type II cytoskeletal 8 (K1C9 and K2C8) are intermediary filaments involved in cell motility and cancer progression. In a variety of organs, the expression of distinct intermediary filaments are associated with patient prognosis. Moreover, K1C9 and K2C8 are considered epithelial markers and decreased of these proteins in 8505C cell line may be indicative of the process of epithelial-mesenchymal transition (EMT) correlated with invasive and aggressive phenotype;

Ezrin (EZRI) is involved in connections of major cytoskeletal structures to the plasma membrane. In epithelial cells, required for the formation of microvilli and membrane ruffles on the apical pole.
The protein is also implicated in the redistribution of intercellular adhesion molecules and the organization of cell membrane structures. The up-regulation expressions of ezrin and moesin and the down-regulation expression of E-Cadherin were associated with the invasion and metastasis of PTC. Combined detection of Ezrin, Moesin and E-Cadherin in PTC might served as an important predictor for the invasion, metastasis and prognosis of PTC (Tao XF 2009);

Tropomyosin alpha 3 chain (TPM3) is a member of the tropomyosin family of actin-binding proteins involved in the contractile system of striated and smooth muscles and the cytoskeleton of non-muscle cells. TPM3 provides stability to the filaments and regulates access of other actin-binding proteins. Increased expression of this protein in B-CPAP cell line may be indicative of greater structural stability associated with a benign phenotype. Moreover, rearrangements between TPM3 and NTRK1 genes producing TRK oncogenes in papillary thyroid carcinomas. TRK oncoproteins display constitutive tyrosine-kinase activity, leading to in vitro and in vivo transformation (Greco A. 2010);

Lamin-B1 (LMNB1) is involved in most nuclear activities including DNA replication, RNA transcription, nuclear and chromatin organization, cell cycle regulation, cell development and differentiation, nuclear migration, and apoptosis (Gruenbaum et al., 2003).

It was found reduced nuclear lamin expression to be an early and frequent finding in gastrointestinal cancer, often accompanied by aberrant cytoplasmic immunolabelling. Although the cause of this phenomenon is currently not known, it is assumed that reduced nuclear lamin expression may be useful as a marker of malignancy (S F Moss 1999);

Macrophage-capping protein (CAPG) is a ubiquitous actin-modulating protein involved in cell signalling, receptor-mediated membrane ruffling, phagocytosis, and motility. CapG has generated great interest due to its oncogenic function in the control of cell migration or invasion in a variety of cancer cells (Hitomi Nomura 2008).

Molecular chaperones/heat shock proteins

In group of molecular chaperones/heat shock proteins (22 protein spots) we show the increased levels of CARL, 1 isoform of HSP7C, 1 isoform of TERA, HSP74, HSP71, TCTP and HSP47. Decreased levels were found for 5 isoforms of HSP60, 1 isoform of HSP7C, GRP75, GRP78, CH10, 2 fragments of HYOU1, PDIA1, 2 isoforms of PDIA3 and PPIB. Figure displays the diagram of protein fold variation between B-CPAP and 8505C cell lines belonging to molecular chaperones/heat shock proteins cluster and expressed in a logarithm scale.

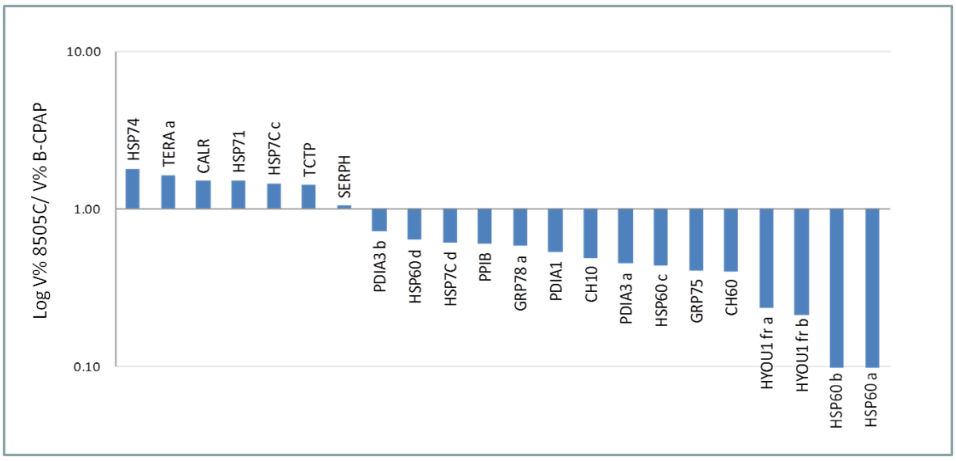


Figure. Diagram shows the distribution of molecular chaperones/heat shock proteins among B-CPAP and 8505C cell lines.

The proteins of this category, implicated in different diseases, are briefly described below.
Calreticulin (CARL) is an intracellular calcium-binding chaperone, that when localizes in the endoplasmic reticulum (ER) plays important functions in directing proper conformation of proteins and glycoproteins, as well as in homeostatic control of cytosolic and ER calcium levels. Moreover, it acts as a critical regulator of extracellular functions, particularly by mediating cellular migration and phagocytosis of apoptotic cells. The association of calreticulin with pathological conditions, such as autoimmune disorders and certain types of cancer have been reported. Lwin ZM et al. showed that calreticulin expression is associated with more advanced tumors and is a potential prognostic biomarker (Lwin ZM et al. 2010). Therefore, the increased levels of CARL may be correlated with aggressive phenotype showed in 8505C cells;

Transitional endoplasmic reticulum ATPase (TERA) is necessary for the fragmentation of Golgi stacks during mitosis and for their reassembly after mitosis. Involved in the formation of the transitional endoplasmic reticulum (tER);

Heat shock proteins (HSPs) are implicated in tumor cell proliferation, differentiation, invasion, metastasis, cell death and recognition by the immune system. HSP60 is implicated in mitochondrial protein import and macromolecular assembly. It may facilitate the correct folding/refolding and assembly of neosynthetized proteins, and also of unfolded/misfolded polypeptides generated under stress conditions in the mitochondrial matrix. This protein also influences apoptosis; thus HSP60 changes in expression level have been shown to be useful new biomarkers for diagnostic and prognostic purposes (Cappello F. 2006). There is some inconsistency in that some research shows a positive expression while other shows a negative expression, and it seems to depend on the type of cancer. There are different hypotheses to explain the effects of positive versus negative expression. Loss of HSP60 expression indicates a poor prognosis and the risk of developing tumor infiltration specifically with bladder carcinomas (Lebret T. 2003). Moreover, ovarian tumors research has shown that over expression is correlated with a better prognosis while a decreased expression is correlated with an aggressive tumor (Lebret T. 2003). Finally, it is possible that levels decreased of HSP60 in 8505C cells than to B-CPAP cells may be a feature associated to thyroid tumor histotype and more aggressive tumor;

Translationally-controlled tumor protein (TCTP), a protein involved in calcium binding and microtubule stabilization; another study indicate that the interaction between TCTP and p53 prevents apoptosis by destabilizing p53. Thus, TCTP acts as a negative regulator of apoptosis in lung cancer (Rho SB et al. 2011) and it is assumed that this protein may be involved in ATC;

Hypoxia up-regulated protein 1 (HYOU1) has a pivotal role in cytoprotective cellular mechanisms triggered by oxygen deprivation. May play a role as a molecular chaperone and participate in protein folding (Ref.5). Highly expressed in tissues that contain well-developed endoplasmic reticulum and synthesize large amounts of secretory proteins. Decreased levels of HYOU1 in 8505C cells may be correlated to loss of normal secretory function showed in ATC;

Protein disulfide-isomerase (PDIA1 and PDIA3) are involved in the quality control of the glycoprotein folding, also by interacting with calreticulin and calnexin;
PDIA1, also namely cellular thyroid hormone-binding protein, may be involved with other chaperones in the structural modification of the tireoglobulin precursor in hormone biogenesis (20). 8505C cells express lower levels of protein PDIA1 than B-CPAP cells, and this result suggest a lower capability of 8505C cells in maintenance of cell function respect B-CPAP cells.
PDIA3 was found significantly overexpressed in both invasive and borderline ovarian cancers (Chay D et al. 2010):

Peptidyl-prolyl cis-trans isomerase B (PPIB) is thought to be essential for protein folding in the cells and functional studies also confirmed the role of PPIB in migration and invasion of cancer cells. Moreover, modulated TCPZ (T-complex protein 1 subunit zeta) and PPIB are two novel indicators for evaluating lymph node metastasis in colorectal cancer (Fei Yue 2009).

Cell growth and proliferation regulators

In group of cell growth and proliferation regulators (16 protein spots) the increased levels were detected for: 1 isoform of MIF, 2 isoform of CATD, RM12, HNRPM, SPB5, CCNH, GDIR and NTF2. Decreased levels were found for: 1 isoform of CATD, 1 isoform of ROA1,

ROA2, RUVB1, PA2G4, PHB and AGR2. Figure displays the diagram of protein fold variation between B-CPAP and 8505C cell lines belonging cell growth and proliferation regulators cluster and expressed in a logarithm scale.

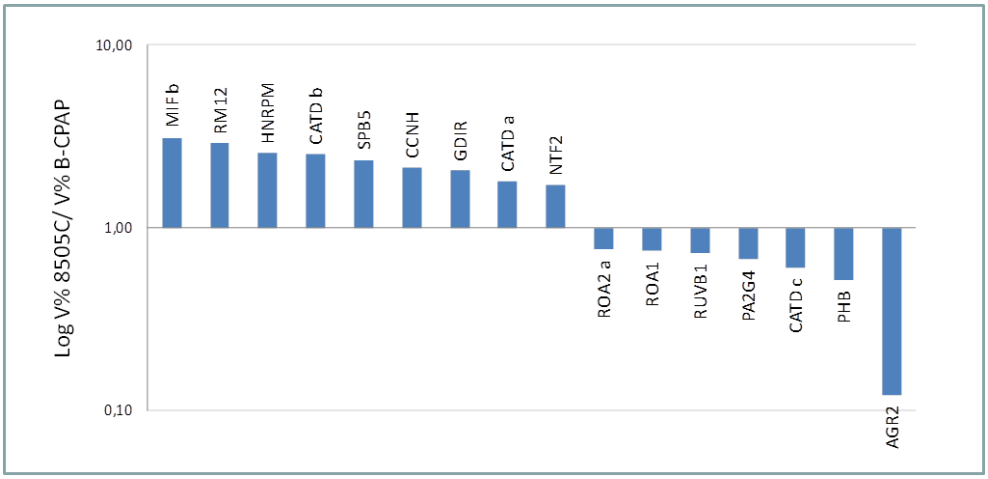


Figure. Diagram shows the distribution of cell growth and proliferation regulators among B-CPAP and 8505C cell lines.

The proteins of this category, implicated in different diseases, are briefly described below.
Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine involved in the innate immune response to bacterial pathogens. The expression of MIF at sites of inflammation suggests a role as mediator in regulating the function of macrophages in host defence. MIF is also thought to favour cancer growth or to render microenvironment favourable to cancer progression;

Cathepsin-D (CATD), an aspartyl lysosomal protease expressed in all tissues, may plays a role in antigen processing, cell proliferation, tissue renewal and activation of different pro hormones. CATD expression level is used also as a biological marker for prognosis of breast cancer (25). Interestingly, its high expression (two isoforms) in 8505C cells could be to indicate a possible involvement also in thyroid cancer;

39S ribosomal protein L12 (RM12) is involved in protein synthesis and the expression levels of RM12 were found altered in prostate cancer (Lexander et al., 2005);

Heterogeneous nuclear ribonucleoprotein M (HNRPM) belongs to family of heterogeneous nuclear ribonucleoproteins (hnRNP) and research has shown that these proteins have central roles in DNA repair, telomere biogenesis, cell signaling and in regulating gene expression at both transcriptional and translational levels. Through these key cellular functions, individual hnRNPs have a variety of potential roles in tumour development and progression including the inhibition of apoptosis, angiogenesis and cell invasion (Carpenter B 2005).

Serpin B5 (SPB5) was originally described as a breast tumour suppressor, a gene which was active in normal breast epithelial cells and which was down-regulated progressively towards malignancy. Several works suggest that SPB5 reduces angiogenesis, tumour invasiveness, growth and metastasis. Moreover, it seems that SPB5 expression in thyroid carcinomas is closely associated with a lack of differentiation of the tumour cells (Ogasawara S. 2004). Decreased expression level of this protein in 8505C cell line therefore may be correlated with a dedifferentiation from PTC to ATC;

Cyclin-H (CCNH) plays a key role in cell cycle regulation by modulating the activity of cyclin dependent kinase 7 (CDK7) which phosphorylates CDK1, 2, 4 and 6. In addition, it has been demostrated that CCNH expression is increased in gastrointestinal stromal tumours with very-high risk of malignancy. High expression level of this protein in 8505C cells thus may be associated to malignancy and poor prognosis;

Rho GDP-dissociation inhibitor 1 (GDIR) regulates the GDP/GTP exchange reaction of the Rho proteins by inhibiting the dissociation of GDP from them, and the subsequent binding of GTP to them. This protein plays an essential role in many cellular functions through its interactions with Rho family GTPases. RhoGDI is frequently overexpressed in human tumors and chemoresistant cancer cell lines, raising the possibility that RhoGDI might play a role in the development of drug resistance in cancer cells (Zhang B et al. 2005);

Nuclear transport factor 2 (NTF2) is implicated in nuclear import RanGDP. Ran protein belongs to RAS family and it is involved in RNA and protein trafficking within the nucleus and in mitotic spindle formation (Stewart et al., 2000);

Heterogeneous nuclear ribonucleoproteins A1 and A2/B1 (ROA1 and ROA2) are RNA binding proteins involved in a variety of functions, including regulation of transcription, mRNA metabolism and translation. Alterated expression of heterogeneous nuclear ribonucleoproteins has been reported in several types of cancers. ROA1 is overexpressed in oligodendrogliomas (Xu X. 2001) and chronic myelogenous leukemia (Iervolino A 2002). ROA1 expression is also increased in myeloid progenitor cells expressing the BRC/ABL oncoprotein. In these cells, an alteration of ROA1 normal functioning results in a decrease of colony formation and tumorigenesis of these transformed cells, in part due to a downregulation of the antiapoptotic factor Bcl-xL (Iervolino A 2002). ROA2 is overexpressed in lung, breast, pancreatic and esophageal cancer (Zhou J. 2001 and Fielding P 1999). In contrast, loss of ROA2 expression was observed in thyroid neoplasms and this event seems to be a characteristic feature of thyroid malignant lesions (Bidot P. 2001). Indeed, we observed ROA2 expression decrease in 8505C respect B-CPAP cells and thus the detection of this protein may be useful to distinguish different thyroid tumor histotypes;

RuvB-like 1 (RUVB1) is a component of the NuA4 histone acetyltransferase complex which is involved in transcriptional activation of select genes principally by acetylation of nucleosomal histones H4 and H2A. This modification promotes interaction of the modified histones with other proteins which positively regulate transcription. This complex may be required for the activation of transcriptional programs associated with oncogene and proto-oncogene mediated growth induction, tumor suppressor mediated growth arrest and replicative senescence, apoptosis, and DNA repair;

Proliferation-associated protein 2G4 (PA2G4) is involved in ribosome assembly and rRNA processing regulation. PA2G4 can interact also with the cytoplasmic domain of ErbB3 receptor and may contribute to growth through the signals transduction. Indeed, this protein has been implicated in growth inhibition and the induction of differentiation of breast cancer cells (Xia X. 2001);

Prohibitin (PHB) has a role in regulating cell proliferation; it may be also localized in the mitochondria where it might have a role in the maintenance of mitochondrial function and protection against senescence. PHB has potential roles of tumor suppressor, through an anti-proliferative function or as a regulator of cell-cycle progression and apoptosis.

Anterior gradient protein 2 homolog (AGR2) is a secretory protein that may play a role in cell migration, cell differentiation and cell growth. This protein is highly expressed in adenocarcinomas of the esophagus, pancreas, breast, and prostate (Zheng Wang. 2008). In particular, AGR2 may be used as a means of detecting the outcome of hormonal therapy in patients with breast cancer. Although currently there aren't AGR2 experimental evidences for thyroid cancer, the increased expression level was detected in B-CPAP cell line and thus AGR2 may represent a marker associated with PTC.

Membrane-associated proteins with multiple activities

In the category of membrane-associated proteins with multiple activities (11 protein spots) high levels were showed for 1 isoform of ANXA1, 2 isoforms of ANXA2, 2 isoforms of VDAC1, LEG1 and 1 isoform of LEG3, while low levels for 1 short form of ANXA1, 1 short form of ANXA2, 1 isoform of LEG3 and ANXA4. Figure displays the diagram of protein fold variation between B-CPAP and 8505C cell lines belonging to molecular membrane-associated proteins cluster and expressed in a logarithm scale.

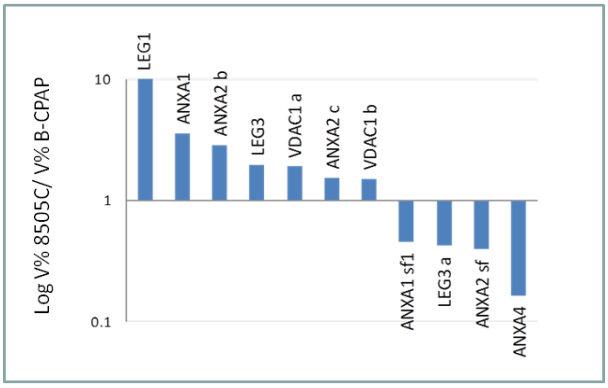


Figure. Diagram shows the distribution of membrane-associated proteins with multiple activities among B-CPAP and 8505C cell lines.

The proteins of this category, implicated in different diseases, are briefly described below.

Annexins (ANXA1, ANXA2 and ANXA4) are a group of structurally related calcium-binding proteins that have a domain that binds to phospholipids and an amino terminal domain that determines specificity (10-12). The annexins are involved in regulation of membrane trafficking, cellular adhesion, and possible tumorigenesis.

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). It has been reported that ANXA1 is up-regulated in human breast cancer [17], hepatocellular carcinoma [18], and pituitary adenoma [19] and down-regulated in human esophageal squamous cell carcinoma [20], prostate cancer [21], and endometrial carcinoma [22]. A number of studies have also shown that can ANXA1 promote cell death but also increase resistance to apoptosis. The underlying causes for these differences are not known and may be dependent on cell type or due to differences in the differentiation status of individual cell types.

ANXA2 is involved in cell proliferation and membrane physiology (Gerke et al, 2005). It was also observed that ANXA2 expression in renal cell carcinomas was associated with metastasis and poor prognosis. The ANXA2 might play an important role in the development of metastasis and might be a useful marker for formulating individualised follow-up protocols as well as for identifying patients suitable for adjuvant therapy (Y Ohno 2009).

ANXA4 plays a role in paclitaxel resistance in lung and colon cancer cell lines and it is among one of the earliest proteins that is induced in cells in response to cytotoxic stress such as antimitotic drug treatment (E Kyu-Ho Han 2000).

Voltage-dependent anion-selective channel protein 1 (VDAC1) is a component of the mitochondrial permeability transition pore, regulates mitochondrial ATP/ADP exchange suggesting that its over-expression could be associated with energy dependent processes including increased proliferation and invasiveness (Abu-Hamad S. 2006). Gene expression analysis has identified VDAC1 gene expression as a predictor of poor outcome in NSCLC (non-small cell lung cancer) (Claire Grills 2011). Therefore, levels increased of VDAC1 in 8585C cell line may be associated with aggressive tumour behaviour.

Galectin 1 and 3 (LEG1 and LEG3) are lectin-binding proteins expressed ubiquitously in mammalian organism. Since its discovery, the galectins were shown to participate in many cellular processes. The most extensively studied galectins are LEG1 and LEG3, which have molecular weights of 14.5 kD and 29 to 35 kD, respectively.

Several works indicate that LEG1 induces apoptosis of activated human T cells and this process represents a new mechanism regulating the immune response (21). Moreover, the participation in angiogenesis suggest that protein could be involved in tumor progression.

Recently, LEG1 expression was reported to be low in normal rat thyroid cell lines and very high in oncogene-transformed cell lines (25). Thus, cellular transformation in cultured thyroid cells was accompanied by a loss of differentiated functions and by a dramatic increase in LEG1 mRNA expression. Furthermore, the level of LEG1 mRNA was higher in human thyroid cancer than in normal thyroid tissue (25). These results suggest that LEG1 could serve as marker for differentiating between benign and malignant thyroid neoplasms. Because a greater differential expression of LEG1 was observed between B-CPAP and 8505C cell line, this protein can also be used as marker associated to cancer thyroid histotype and malignancy grade.

LEG3 expression has also been linked to cell proliferation, because it was enhanced within a short time after mitogenic stimulation of quiescent mouse fibroblasts (45). Increased expression of LEG3 was also seen in thyroid malignancies.

Detoxification and redox proteins

Detoxification and redox proteins are related enzymes performing important roles in the cell catabolism and protection against metabolic stresses. In this group (11 protein spot) high levels were observed for 1 isoform of GSTO1, 1 isoform of THIO, 1 isoform of SODM, PRDX6 and CAH1, whereas low levels for 1 isoform of GSTO1, 1 isoform of PRDX2, 1 short form of SODC, 1 isoform of SODM, 1 isoform of AK1BA and AK1C3. Figure displays the diagram of protein fold variation between B-CPAP and 8505C cell lines belonging to detoxification and redox proteins cluster and expressed in a logarithm scale.

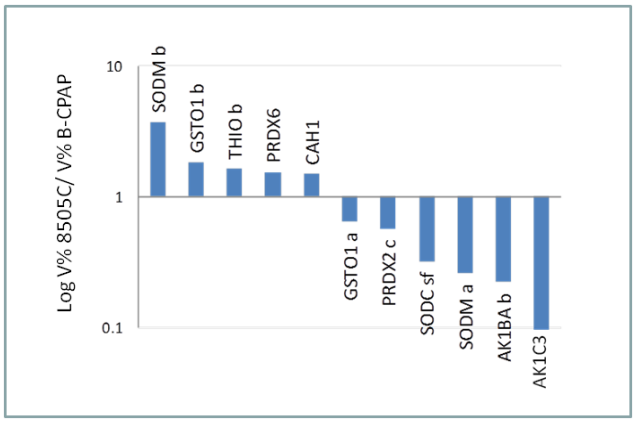


Figure. Diagram shows the distribution of detoxification redox proteins cluster among B-CPAP and 8505C cell lines.

The proteins of this category, implicated in different diseases, are briefly described below.

Glutathione transferase omega-1 (GSTO1) plays an important role in detoxification by catalysing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione. Aberrant expression of GSTO1 is associated with carcinogenesis and development of multidrug resistance. Moreover, it is interesting to note that isoforms of GSTO1 in 8505C cells are characterized by opposite expression, probably due to a different post-translational regulation of the native protein, which require different skills for each isoform;

Thioredoxin (THIO) is a small redox-regulating protein, which plays crucial roles in maintaining cellular redox homeostasis and cell survival and is highly expressed in many cancers.

Several studies implicate over-expression of THIO as one of the enhancers of cancer cell growth, either through the direct stimulation of cancer cell growth or through the inhibition of cancer cell apoptosis. Moreover, high levels of THIO expression have also been correlated with highly invasive and metastatic tumor activity both in vitro and in vivo (Ceccarelli 2008 and Lincoln 2003). An in vitro study using a neuroblastoma cell line revealed a possible mechanism by which THIO can enhance the metastasis of cancer cells. THIO was shown to stimulate cell invasion in these cells and to promote overall matrix metalloproteinase (MMP) activity by preferentially inhibiting the MMP inhibitors (Farina 2001). Therefore, it is possible that this mechanism is triggered in the ATC since an increased expression of THIO and MMPs was detected in 8505C cells.

Superoxide dismutase [Cu-Zn] and [Mn] mitochondrial (SODC and SODM) destroy radicals which are normally produced within the cells and which are toxic to biological systems.

Peroxiredoxin 2 and 6 (PRDX2 and PRDX6) are family of antioxidant enzymes, which reduce hydrogen peroxide and alkyl hydroperoxides. The encoded proteins may play an antioxidant protective role in cells, and may contribute to the antiviral activity of CD8(+) T-cells. PRDX2 may have a proliferative effect and play a role in cancer development or progression, while PRDX6 may be involved in the regulation of phospholipid turnover.

Carbonic anhydrase 1 (CAH1) is a member of a family of isoenzymes that catalyse the reversible hydration of carbon dioxide and participate in various biological processes like

electrolyte transport and maintenance of pH. Moreover, CAH1 is highly expressed in intestinal mucosa and takes care of the maintenance of the intracolonic and cellular pH. In colorectal tumours CAH1 is significantly less expressed than in normal mucosa (Kivela A. J. 2001).

Aldo-keto reductase family 1 member A1 and C3 (AK1BA and AK1C3) belong to aldo/keto reductase family and are involved in the reduction of biogenic and xenobiotic aldehydes.

Calcium binding proteins

One other group of interest is the calcium binding proteins (8 protein spots), including some protein forms of the S100 protein family and calmodulin. Increased levels were detected for: 1 isoform of S100-A6 and 1 isoform of S100-A7, while decreased levels were found for: CALM, SEGN, 1 isoform of S100-A7, 2 isoforms of S100-P and S100-A13. Figure displays the diagram of protein fold variation between B-CPAP and 8505C cell lines belonging to calcium binding proteins cluster and expressed in a logarithm scale.

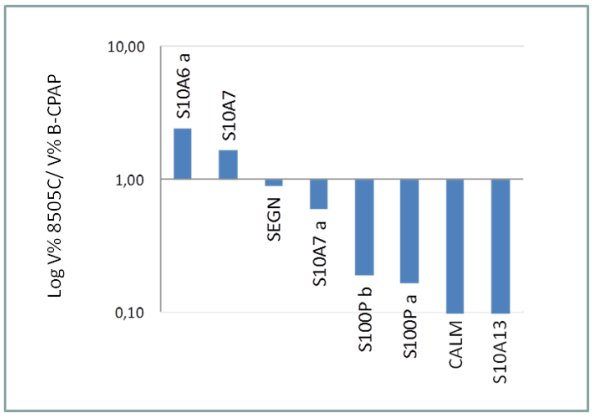


Figure. Diagram shows the distribution of calcium binding proteins cluster among B-CPAP and 8505C cell lines.

The proteins of this category, implicated in different diseases, are briefly described below.

Proteins S100 (S100) are small, acidic- Ca2 binding proteins, found exclusively in vertebrates and are involved in several biological processes, such as cell cycle regulation, cell growth, cell differentiation and motility through a broad range of intracellular and extracellular activities (22-23). Moreover, dramatic changes in S100 proteins expression are exhibit in of

different forms of cancer. The S100-RAGE signalling pathway plays an important role in linking inflammation and cancer and in tumour cell survival and malignant progression (RAGE-deficient tumours are characterized by accelerated apoptosis, reduced activation of NFκB and significantly impaired proliferation).
In particular, our attention has focused on:
S100A6 was detected in melanoma, pancreatic and colorectal cancers and the expression of this protein may be to correlate with tumor growth and metastatic progression suggesting a potential role for S100A6 in the development of malignancy (Lesniak et al., 2009; Salama et al., 2008);

S100A7 is up-regulated in ductal carcinoma in situ and ER negative invasive breast cancer and expression correlates with aggressive phenotype and patient survival (Emberley et al., 2004);

S100P is often in association with tumor progression, and may be considerated a potential tumor biomarker and therapeutic target. However, the S100P role in tumor progression is not completely understood;

S100A13 is widely expressed in many types of tissues, with particularly high expression in the thyroid gland. Therefore, decreased expression of S100A13 in 8505C cell line may indicate low differentiated level typical of ATC.

Calmodulin (CALM) mediates the control of a large number of enzymes, ion channels and other proteins by Ca2+. Recent evidence suggests that calmodulin may regulate cellular proliferation and that its function may be altered in malignancy. The discovery of drugs wich antagonize the action of calmodulin led to the study of these antagonists against tumor cells;

Secretagogin (SEGN) is considered a marker for neuroendocrine differentiation expressed at high levels in the pancreatic islets of Langerhans. The SEGN function is unknown,but it has been suggested that this protein influences the calcium-influx, insulin secretion and proliferation. Previous microarray-based studies have demonstrated that this protein is highly expressed in normal colon mucosa compared to basal expression in colon adenocarcinomas (Birkenkamp-Demtröder K. 2005).

Proteins with binding function

In the category of proteins with binding function (8 protein spots) we observed the increased levels of SH3L1, FABP5 and U3IP2, whereas decrease levels of ACBP, RABP2 and COPE. Figure displays the diagram of protein fold variation between B-CPAP and 8505C cell lines belonging to proteins with binding function cluster and expressed in a logarithm scale.

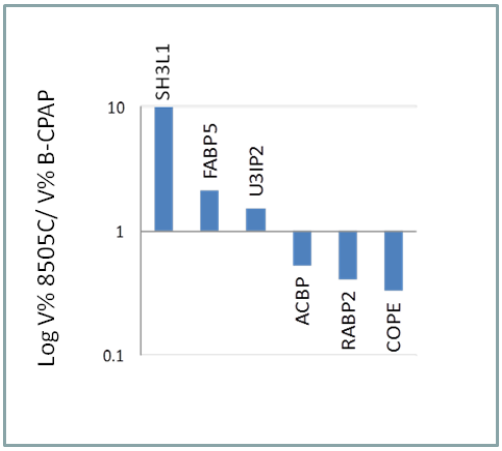


Figure. Diagram shows the distribution of proteins with binding function cluster among B-CPAP and 8505C cell lines.

The proteins of this category, implicated in different diseases, are briefly described below.

SH3 domain-binding glutamic acid-rich-like protein (SH3L1) is up-regulated in many cancers such as lung and pancreatic and implicated in increased resistance of cancer cells to free-radicals. There is little current evidence which directly links SH3L1 with survival in cancer cells, however the protein has recently been identified as up-regulated in glioblastoma multiforme compared to normal cerebral tissue on proteomic analysis (Khalil AA 2007). Moreover, this protein is potentially involved in resistance of cells to the apoptosis-inducing affect of TNF-α.(Xu C 2005);

Fatty acid-binding protein epidermal (FABP5) belongs to the conserved multigene family of the intracellular lipid-binding proteins (iLBPs). Various functions have been proposed for these proteins, including the promotion of cellular uptake and transport of fatty acids, the targeting of fatty acids to specific metabolic pathways, and the participation in the regulation

of gene expression and cell growth (H Haunerland 2004). In particular, FABP5 may be involved in mechanisms leading to chemoresistance in pancreatic adenocarcinoma;

U3 small nucleolar RNA-interacting protein 2 (U3IP2) is a component of a nucleolar small nuclear ribonucleoprotein particle (snoRNP) and likely this protein plays a role in the processing and modification of pre-ribosomal RNA;

Acyl-CoA-binding protein (ACBP) is involved in the regulation of multiple biological processes such as neuronal Cl^- influx, steroidogenesis, acyl-CoA metabolism, and glucose-mediated insulin secretion. In addition, the high expression of ACBP in brain tumors might play a role in the neoplastic growth of glial cells via the mitochondrial benzodiazepine receptor, or it may be involved in the regulation of the high energy consumption of these tumors via acyl-CoA metabolism (H AIho 1995);

Cellular retinoic acid-binding protein 2 (RABP2) is an intracellular lipid-binding protein and it is involved in the process of retinoic acid transport to the nucleus. A research have demonstrated that RABP2 mRNA and protein are down-regulated in prostate cancer (Okuducu AF 2005).

Coatomer subunit epsilon (COPE) is involved in the regulation of intracellular protein trafficking between the endoplasmic reticulum and the Golgi complex (24). 8505C cells express lower levels of this protein than B-CPAP cells and it may correlate with the tendency of B-CPAP cells to maintain cells function in vesicular trafficking typical of thyroid cells.

Protein-degradation

The protein-degradation machinery is a very important cellular design to maintain protein homeostasis and cellular health. In group of protein degradation (7 protein spots) high levels were found for UCHL1, PSME1 and PSA6, while low levels for UBIQ, PSA1, PSB4 and SUMO1. Figure displays the diagram of protein fold variation between B-CPAP and 8505C cell lines belonging protein-degradationcluster and expressed in a logarithm scale.

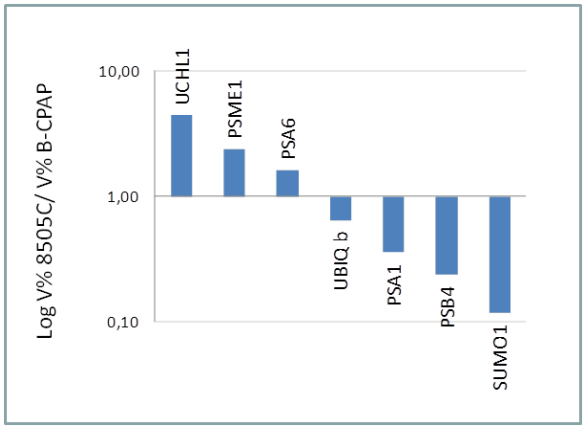


Figure. Diagram shows the distribution of protein degradation cluster among B-CPAP and 8505C cell lines.

The proteins of this category, implicated in different diseases, are briefly described below.

Ubiquitin Carboxyl-Terminal Hydrolase-L1 (UCHL1) is a deubiquitinating enzyme that is highly expressed throughout the central and peripheral nervous system and in cells of the diffuse neuroendocrine system. This protein exhibits a variable expression pattern in cancer, acting either as a tumour suppressor or promoter, depending on the type of cancer. In non-small cell lung carcinoma (NSCLC), UCH-L1 is highly expressed and is associated with an advanced tumour stage. This suggests UCH-L1 may be involved in oncogenic transformation and tumour invasion in NSCLC (Katy S Orr 2011). Therefore, high expression level of this protein in 8505C cells may be associated to an advanced stage of thyroid cancer.

Ubiquitin–proteasome system is involved in the regulation of various cellular processes; among them is the important role of targeting proteins for degradation. Nevertheless, ubiquitination also plays a significant role in DNA damage repair, cellular signaling, intracellular trafficking, and ribosomal biogenesis (Li and Ye, 2008). Moreover, ubiquitin–proteasome system alterations are been associated to the initiation and progression of cancer and this knowledge is beginning to be exploited for molecular diagnostics and the development of new strategies to fight cancer (Nature 2008). in particular, it has been demonstrated that Inhibition of the proteasome is effective in reducing cell growth and induce apoptosis of ATC in vitro and inhibit tumor growth and vascularization in vivo (Constantine S. 2006)

Proteins with multiple activities

Finally, in the category of *proteins with multiple activities* (2 protein spots) high levels were showed in SAHH, while low level in PNPO. Figure displays the diagram of protein fold variation between B-CPAP and 8505C cell lines belonging proteins with multiple activities cluster and expressed in a logarithm scale.

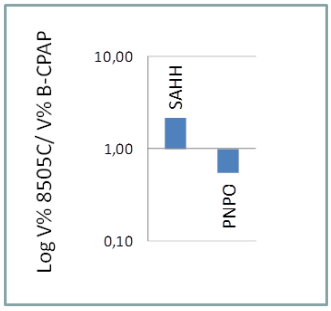
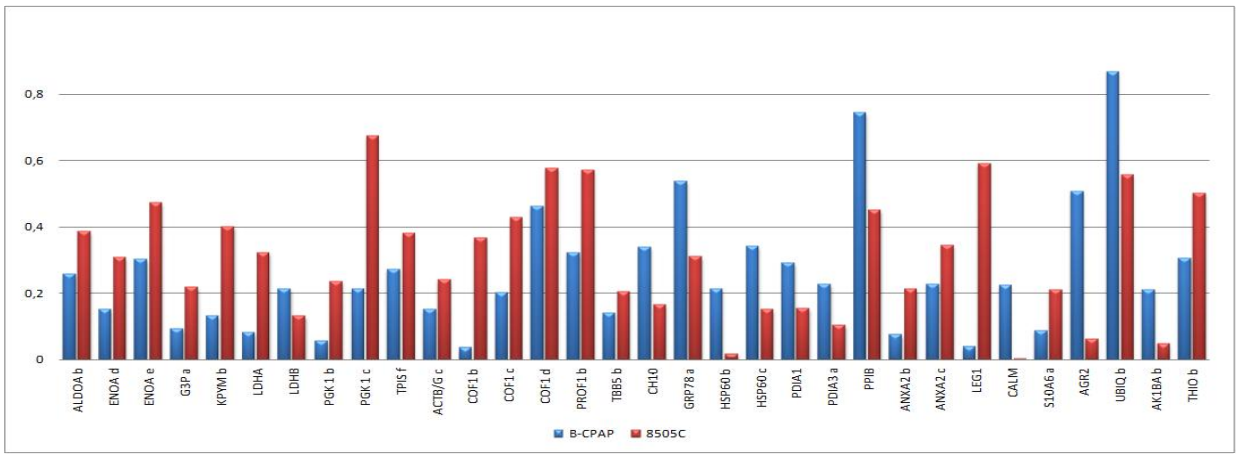


Figure. Diagram shows the distribution of proteins with multiple activities cluster among B-CPAP and 8505C cell lines.

In particular, our attention has been focused on highly expressed proteins (cut - off <2) in B-CPAP and 8505C cells. This screening has highlighted 32 proteins and most of these belong to the group of metabolic enzymes, cytoskeleton and associated proteins and molecular chaperones / heat shock proteins. These results highlight the crucial role of glycolysis, cell motility and protein folding in thyroid tumorigenesis . Moreover, the differential expression of these proteins in B-CPAP and 8505C cells could provide the opportunity to identify potential markers associated with PTC and ATC .



Cell motility (Scratch Assay)

Cell motility is a complex phenomenon primarily driven by the actin network and it is essential to a variety of biological processes such as morphogenesis, wound healing, immune response and cancer metastasis.

We studied the cell motility of B-CPAP and 8505C cell lines using scratch assay to assess the differences in the migratory and invasive abilities associated with aggressive phenotype. We found that wound healing was observable between 6 and 24 hrs after wounding with a pipette (Fig.). Significant cell migration was seen at 6 hrs (Fig. A-B) with formation of lamella, protrusions and intercellular contacts at the wound margin in 8505C cells. After 24 hrs (Fig. C-D) wound was completely healed in 8505C unlike the B-CPAP, where it wasn't observed the wound healing. Cell motility results showed that the 8505C cells display a very aggressive in vitro phenotype respect to B-CPAP.

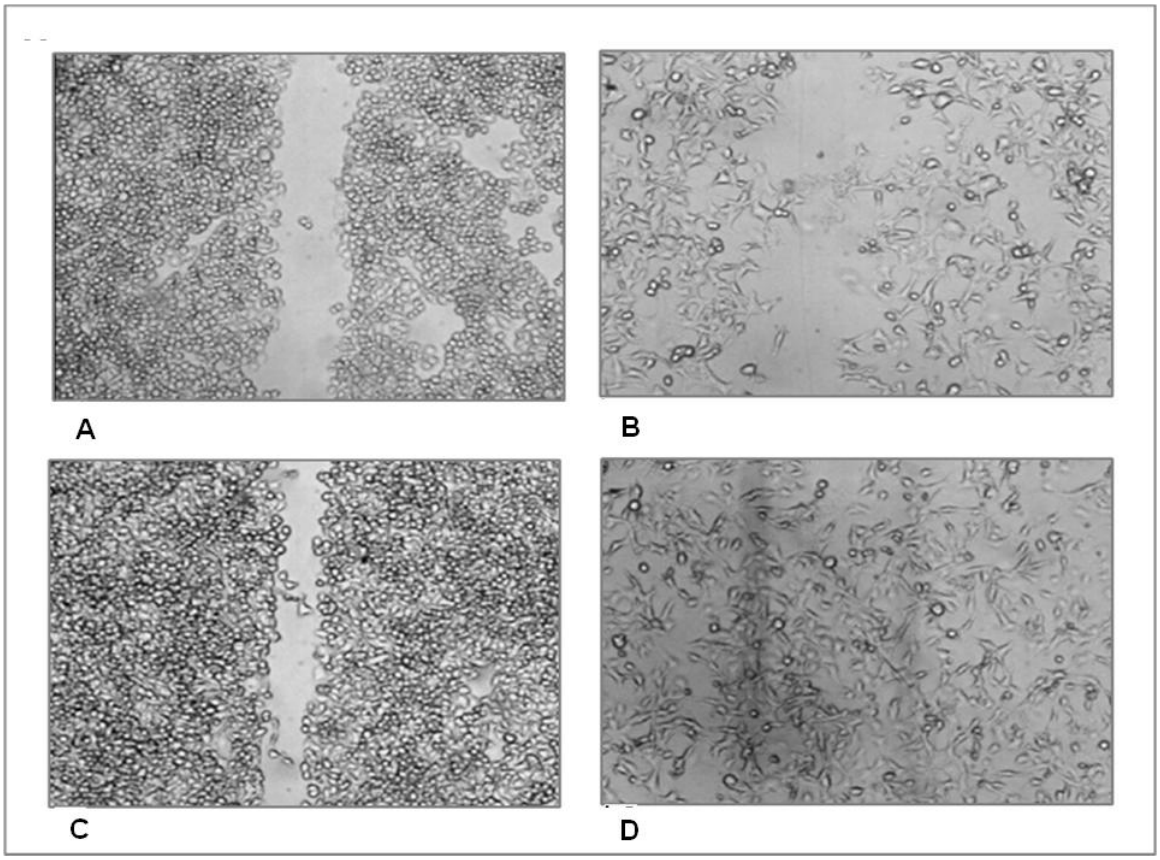


Figure. Representative optical micrographs of B-CPAP and 8505C cell lines at different time from scratch: 6h (**A** B-CPAP and **B** 8505C) and 24h (**C** B-CPAP and **D** 8505C);

Zymographic analysis and Western Blot validation

A deregulated proteolysis of ECM molecules in thyroid tumors may cause the alteration of follicular structure and the loss of cell polarity. These events, in turn, may induce neoplastic cells to elude cell–cell and cell–ECM adhesions, promoting cancer progression (27). In general, these alterations are correlated with poor prognosis in many tumor histotypes, where a positive relationship between the increase of MMPs and the malignancy grade has also been observed (28). Moreover, "in vitro" studies performed on thyroid cells suggest that BRAF V600E mutation promotes the invasion, by increasing the expression of certain metalloproteinases (MMP-3, MMP-9, MMP-13) (29-27). For these reasons we focused on MMP-2 and MMP-9 activity which are over-produced in several carcinomas. B-CPAP cell line express the MMP-2 and MMP9, with activity levels quite low compared with 8505C cell line characterized by high gelatinolytic activity.

Figure shows the results of a representative zymogram of B-CPAP and 8505C conditioned media (Fig. A) loaded at the same protein concentration (10 µg/lane). B-CPAP sample contained only two gelatinolytic bands corresponding to monomeric pro-MMP-9 (92 kDa) and pro-MMP-2 (72 kDa). Unlike, 8505C sample showed increased activity of proenzymatic forms (pro-MMP-9 and pro-MMP-2), but also displayed a band corresponding to MMP-9 (86 kDa). In order to validate our results, we performed immunoblot analyses on B-CPAP and 8505C conditioned media (20 µg), using a specific antibody to MMP-9 and MMP-2, as described in materials and methods. The antibodies have given a positive response for 8505C conditioned medium (Fig. B).

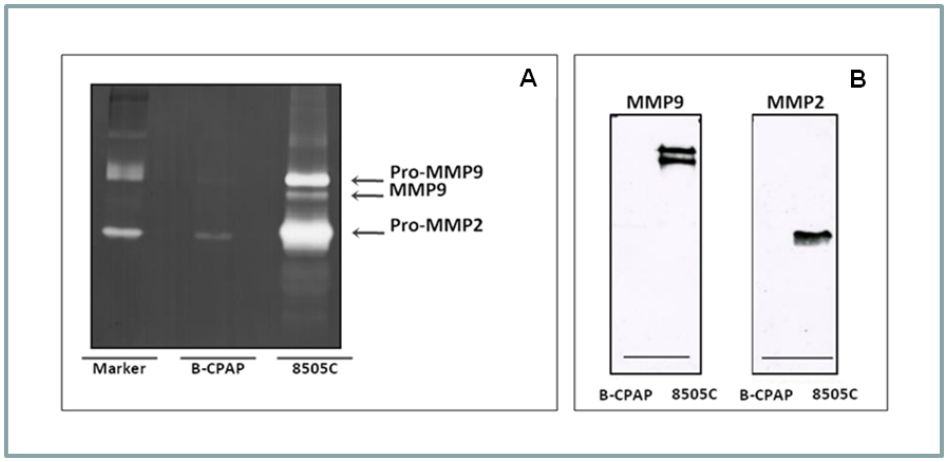


Figure .A) Gelatin zymogram (7.5% SDS-PAGE) of conditioned media from B-CPAP and 8505C cell lines and B) Western blot of conditioned media probed with anti-MMP 9 and anti-MMP 2 antibodies.

In particular, the antibody MMP9 recognized two bands at high molecular weight (180 kDa) corresponding to dimeric MMP-9 and pro-MMP9. The reaction to anti-MMP-2 localized in the 70 kDa region may correspond to pro-MMP-2. In contrast, in conditioned media of B-CPAP, the antibodies have given a negative reaction, reflecting the low levels of enzymatic expression in this cell line.

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