ABSTRACT

The recent discovery of CSCs as responsible for tumor development and progression has considerable therapeutic implications, since current cytotoxic drugs have a limited efficacy on CSCs and need to be replaced by target therapies. For this reason, much research effort has been addressed to identify and isolate CSCs as well as to improve understanding of the molecular mechanisms that control oncogenic process and chemotherapy resistance.

Thyroid carcinomas are the most frequently diagnosed endocrine malignancy with a global increasing incidence. Data obtained during PhD course in Immunopharmacology showed that thyroid cancer tissues derived from follicular, papillary or anaplastic subtypes contain a small population of $ALDH^{high}$ cells that can undergo self-renewal and be expanded indefinitely *in vitro* as thyrospheres. When orthotopically injected into the thyroid gland of immunocompromised mice, these $ALDH^{high}$ cells are tumorigenic and reproduce the phenotypic characteristics of the original tumors, including the aggressive features of undifferentiated thyroid carcinoma (UTC).

The metastatic behavior of UTC is sustained by constitutive activation of Met and Akt. Consistently, the functional blockade of these molecules determined a complete abrogation of UTC spheres metastatic potential both *in vitro* and *in vivo,* indicating a promising effect for the treatment of aggressive thyroid cancer.

In this context, a better knowledge of the genetic alterations involved in thyroid cancer pathogenesis may provide new insights for development and preclinical

validation of novel targeted therapies.

Although *RET/PTC1, BRAF(V600E)* and mutant *p53* affect thyroid cancers, the role of these genetic alterations in tumor initiation and progression is not well known.

The aim of this study was to understand whether *RET/PTC1, BRAF(V600E)* and mutant *p53* may be responsible for cancer initiation. For this reason, thyroid SCs (TSCs) have been isolated from goiters in order to transform them with lentiviral vectors coding for *RET/PTC1, BRAF(V600E)* or *p53* mutant. It was noticed that normal cells, after exogenous expression of these oncogenes, showed an high degree of self-renewal and migration capacity. Moreover, *in vivo* results demonstrated that the same cells acquire the ability to initiate tumor in NOD/SCID mice and that the resultant xenografts possess the pattern of CK19 and Tg expression of PTC and UTC xenogratfs.

Overall, these findings define a good model to identify the specific genetic alterations involved into thyroid carcinogenesis.

INTRODUCTION

1.1 Thyroid carcinomas

Thyroid cancer is the most frequent endocrine malignancy. Despite its prevalence, the mortality rate remains low (about 0,5 per 100,000 patients) thanks to the availability of safe and effective therapies for the well-differentiated histological variants. On the contrary, the undifferentiated carcinoma ranks among the most deadly of all human cancers, due to the high invasiveness and insensitivity to radioiodine or chemotherapeutic treatments [1].

On the basis of histopathological characteristics, thyroid cancer is divided into four subtypes: papillary, follicular, anaplastic and medullary [2].

Papillary Thyroid Carcinoma (PTC) is the most common thyroid malignancy, representing approximately 60-80% of all thyroid cancers [3]. Its pathologic diagnosis is based on classic nuclear features, i.e. elongated nuclei with inconspicuous eccentric nucleoli and crinkled nuclear membranes, chromatin clearing, intranuclear grooves and holes [4].

Genetic alterations found in PTC involve genes coding for the receptor tyrosine kinases, RET and NTRK1, and two intracellular effectors of the MAPK pathway, GTP-binding protein RAS and a serine-threonine kinase BRAF [5-7].

In the thyroid gland, RET is expressed at high levels in parafollicular C-cells but not in follicular cells, in which it can be activated by chromosomal rearrangement with multiple partner genes known as "*ptcs"*[8].

Although more than 15 RET/PTC rearrangements have been described in sporadic and radiation-associated PTCs [9], RET/PTC1, formed by fusion with the *H4* gene, and RET/PTC3 by fusion with the NCOA4 gene account for the most common rearrangements [10].

The RET/PTC-induced effects involve ERK phosphorylation, inhibition of thyroid-specific gene expression, and increased cell proliferation [11].

Recent studies suggest that RET/PTC is tumorigenic in thyroid follicular cells since it transforms thyroid cells in culture [12] and develop thyroid carcinoma in trasngenic mice. The resultant xenografts displayed the histological aspect of PTCs, particularly the nuclear cytologic features and the presence of local invasion [13, 14]. Moreover, it was found that in rat thyroid epithelial cell line PC CI 3 (PC) the expression of RET/PTC1 oncogene alters the activity of TTF-1 and PAX-8 thyroid transcription factors [15].

In 2000, Davies et al. indicated a high prevalence of BRAF mutations in melanoma, colorectal cancer, and other types of tumors. The BRAF-activating point mutation is almost exclusively a thymine-to-adenine transversion at position 1799 in exon 15, which leads to a valine-to-glutamate substitution at residue 600 and a subsequent activation of BRAF kinase. [16].

Many studies consistently demonstrated that *BRAF* is mutated in 29-69% of PTC, especially in aggressive subtypes such as the tall cell variant of PTC and in those with extra-thyroidal extension and lymph node or distant metastases, which are associated with radioactive iodine refractoriness and tumor recurrence [17].

BRAF(V600E) plays an important role in tumor invasion and progression *via* up-regulation of some ECM remodeling genes (i.e. TSP-1, fibronectin, cathepsins) and ECM receptors (i.e. CD44 and integrin α3, α6, and β1) [16, 18].

The involvement of BRAF mutation in tumor initiation as well as its correlation with more aggressive tumor characteristics has also been supported by studies of transgenic mice with thyroid-specific expression of BRAF(V600E). These animals developed PTCs whose microscopic features closely recapitulated those seen in human papillary variants. Moreover, they revealed invasion of blood vessels and thyroid capsule, as well as multifocal progression to poorly differentiated carcinoma [19].

A second proto-oncogene, *NTRK1,* also known as *TRK,* has been found to be rearranged in 5-13 % of PTCs. It encodes the transmembrane tyrosine-kinase receptor for nerve growth factor, whose expression is normally restricted to neurons of the sensory spinal and cranial ganglia of neural-crest origin [20]. The partner genes, including *TPM3, TPR* and *TFG,* seem to constitutively activate the receptor tyrosine kinase, which initiates several signal-transduction pathways, such as Raf/MEK/ERK and PI3K/Akt ones [21].

Ras mutations have been found in PTC with rates of less than 20% [22]. In particular, mutations involving codon 61 of *NRAS* are the most common genetic alterations in the follicular variant of PTCs [23].

Follicular Thyroid Carcinoma (FTC) is the second most common thyroid malignancy and represents about 15-18% of thyroid cancers [3]. Like PTC, it arises from the

thyroxine (T4)- and thyroglobulin-producing follicular cells of the thyroid, which are TSH sensitive as well taking up iodine. This feature has both diagnostic and therapeutic value for managing residual disease and recurrences after surgical excision.

In FTC, the follicular cells do not have nuclear characteristic features like papillary carcinoma cells. Overall, they can have a solid, trabecular, or follicular growth pattern that usually produces microfollicles [4].

A chromosomal translocation between the transcription factor PAX8 and the peroxisome proliferator-activated receptor-γ (PPARγ) occurs in 25-63% of FTCs [24]. The *PAX8* gene encodes a transcription factor which regulates the thyroid specific gene expression. The PPARγ is a member of the steroid nuclear-hormone-receptor superfamily that drives heterodimerization with the retinoid X receptor and subsequent transcription of target genes. This genomic function controls immune response, as well as lipid and glucose metabolism. On the contrary, cytoplasmic PPAR-γ, by interacting with MEK1 and β-catenin as well as activating transmembrane proteinases, modulates EGF-R transactivation, calcium influx, and PI3K/Akt, IKK/NFκB and MAPKs [25].

The fusion oncoprotein contributes to malignant transformation during FTC oncogenesis by acting on several cellular pathways, at least some of which are normally regulated by PPARγ [24].

FTCs have been known to harbor activating-point mutations of the *RAS* genes [22]. Three *RAS* genes, *H-RAS*, *K-RAS*, and *N-RAS*, encode a family of 21-kDa proteins

that play a central role in the transduction of signals arising from tyrosine kinase and G protein-coupled receptors. Oncogenic *RAS* activation results from point mutations, affecting the GTP-binding domain (codons 12,13) in exon 1 or GTPase domain (codon 61) in exon2, which fix the protein in the activated state [26]. In particular, missense mutations in 61 of one of the three *RAS* genes have been found in 18–52% of FTCs [27].

Anaplastic Thyroid Carcinoma (UTC) is the most aggressive and lethal form of thyroid cancer, which accounts for only 3% to 10% [3]. Dedifferentiation, a hallmark of UTC, has revealed by loss of the biological features of follicular cells, such as uptake of iodine and synthesis of thyroglobulin. The histological patterns of UTC include giant-cell, spindle-cell and squamoid-cell tumors; these subtypes frequently coexist and are not predictive of patients' outcome[28].

It has a rapid course and early dissemination into the surrounding tissues, such as fat, trachea, muscle, esophagus, and larynx. Systemic metastases occur in up to 75% of patients, with lung as the most common site (80%), followed by bone (6% to 15%) and brain (5% to 13%). Despite the high rate of synchronous metastases, death is usually related to extensive local disease with ultimate airway obstruction [29].

Unlike its differentiated counterparts, UTC is highly unlikely to be curable by radioactive iodine treatment since it does not express the iodine symporter. Palliative treatment consists of chemotherapy. Monotherapy with doxorubicin demonstrated a response rate of approximately 20% with no evidence of a complete response and combination therapy with cisplatin or bleomycin demonstrated little improvement in

clinical response. Recently, it was found that the addition of paclitaxel allowed some improvements in response but did not alter the fatal outcome of disease [30].

The molecular pathogenesis of UTC includes mutations in *BRAF*, *RAS*, β-catenin, *PIK3CA*, p*53*, *AXIN1*, *PTEN*, and *APC* genes, and chromosomal abnormalities are common [31]. However, *p53* mutations are the most common genetic alterations accounting for 67-88% of UTCs [32-34].

The tumor suppressor gene *p53* regulates the expression of genes involved in the cell cycle arrest (p21), apoptosis (PIG3 and FDXR), and the p53 negative regulator (MDM2) [35]. As such, *p53* gene is mutated in more than 50% of all human cancers. The most common *p53* mutations are missense mutations in the DNA-binding domain (DBD), producing a full-length protein unable to transactivate its target genes [36]. There exist two classes of p53 DBD mutants, conformational mutants and contact site mutants. The first ones, including, V143A, R249S, and R175H, alter the protein structure, while the second ones, for example, R248W and R273H, have an altered residue at contact DNA site [37].

These mutant p53 proteins are often expressed at very high levels in cancer cells, and increasing studies support additional gain-of-function roles in the context of tumorigenesis [38]. Interestingly, the p53 mutant-R175H has been previously shown to promote the invasive phenotype of endometrial cancer cells by activation of EGFR/PI3K/Akt pathway [39] and of immortalized prostate epithelial cells by inducing Twist1 expression [40].

The first documentation of p53 mutations spectra in thyroid carcinomas comes from Fagin's research group. The results clearly indicated that all anaplastic carcinoma tissues examinated as well as the anaplastic carcinoma cell line ARO showed a CGT to CAT transition at codon 273, leading to an Arg to His substitution [41]. Anyway, this *p53* mutation was determined within a low number of tumor specimens and cell lines and little is known about its mutant phenotype both *in vitro* and *in vivo.*

Interestingly, the mutant 175 was found to be 3-10-fold more efficient that the mutant for residue 273 in cooperating with *RAS* to transfor primary rat cell culture [42].

The BRAF(V600E) protein was also found in 25-35% of UTC. The coexistence of *BRAF* and *p53* mutations in undifferentiated variants, containing areas resembling PTC morphological structures, suggests that UTC can develop from a *BRAF*-mutated PTC, after the acquisition of a further *p53* mutation [43, 44].

In addition to those derived from follicular cell differentiation, there exists another group of thyroid tumors developes by parafollicular C transformation. This minority of tumors (5%) [3], referred to as medullary thyroid carcinoma (MTC) and frequently caused by mutations in *RET,* has a much lower cure rate than the "well differentiated" thyroid cancers showing local or distant metastasis in the liver, lung, bone, brain and tumor recurrence after surgery [45].

1.2 Cellular origin of thyroid carcinomas

The cellular origin of thyroid carcinomas has been explained by two different models.

The multistep carcinogenesis model predicts that well-differentiated thyroid cancer cells can transform into undifferentiated thyroid cancer cells *via* multiple genetic mutations and dedifferentiation process. In particular, thyrocytes could give rise to PTC by *RAS* and *BRAF* mutations or *RET/ PTC* and *Trk* rearrangements and to FTC by point mutations of the *RAS* gene and *PAX8/ PPARγ* rearrangement. Moreover, UTC is derived from PTC and FTC, after the acquisition of a further *p53* mutation [46, 47].

The existence of common genetic changes between the well-differentiated carcinomas and the undifferentiated ones was questioned by Tallini et al. (1998), since they detected that specific genetic alterations associated with PTC and FTC, such as *RET/PTC* rearrangements and the *PAX8/PPARγ* gene fusion, are rarely found in UTC [9]. Moreover, the low turnover rate of mature thyroid follicular cells (fewer than 10 renewals per lifetime) limits the accumulation of multiple mutations [48].

Figure 1.

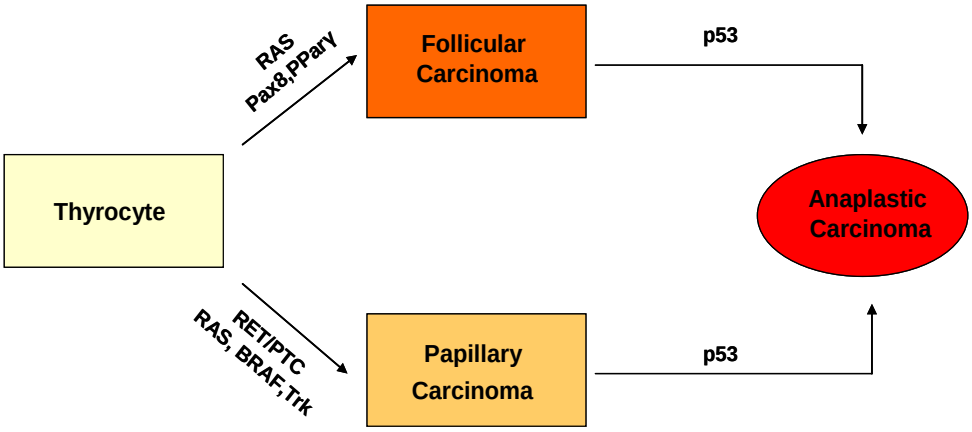

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Figure 1. The multistep carcinogenesis model.

The fetal cell carcinogenesis model suggests that thyroid cancer cells derive from remnants of fetal thyroid cell rather than from mature thyroid follicular cells.

On the basis of gene expression profiling data, this model emphasizes that a pre-existing hierarchy of fetal thyroid cells generate different variants of cancer cells. As a consequence, the thyroid cancer cells have a gene expression profile similar to that of fetal thyroid cells.

This model proposes the existence of three different types of fetal thyroid cells that could give rise to thyroid cancer cells. In particular, fetal thyroid stem cells, expressing the fetal protein oncofetal fibronectin but none of the markers characteristic of differentiated thyroid cells, are proposed to be the cellular origin of

UTC. Thyroblasts, characterized by the expression of Oncofetal Fibronectin (OF) and the differentiation marker Thyroglobulin (Tg), generate PTC. By contrast, prothyrocytes, the more differentiated cell type expressing thyroglobulin, give rise to FTC. Genetic changes, including *RET/ PTC* and *PAX8-PPARγ* rearrangements and mutation in *BRAF* and *RAS* genes, should prevent fetal thyroid cells from differentiating and confer proliferation advantages. **Figure 2.**

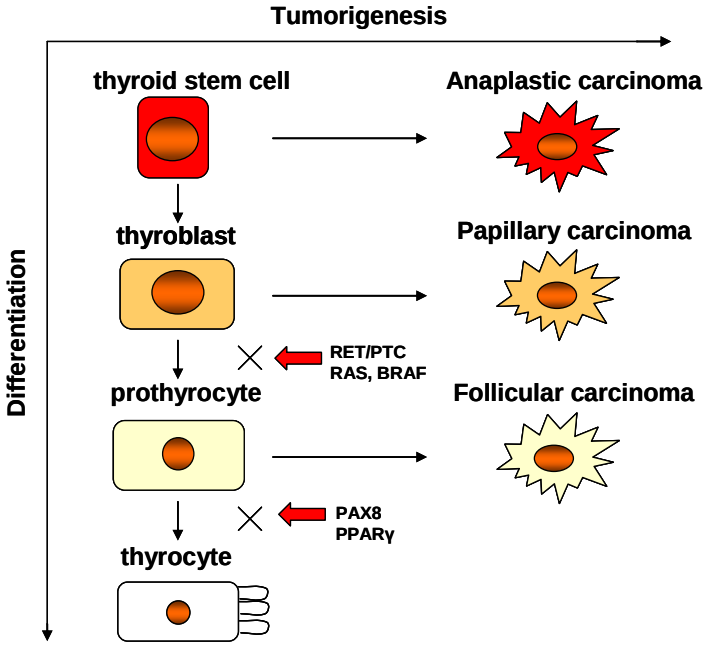

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Figure 2. The fetal cell carcinogenesis model.

Interestingly, when thyroid SCs themselves proliferate, the resulting tumor acts as an undifferentiated carcinoma; otherwise, when they give rise to thyroblasts and prothyrocytes, the proliferating tumors act as differentiated variants [46].

Several evidences support this model. First, radioactive iodine released after the Chernobyl nuclear accident provoked the development of PTCs among infants and young children but not among adults, similarly exposed [49]. Second, *RET/PTC1* transgenic mice, lacking normal thyroid follicular cells, develop PTCs and congenital hypothyroidism since the oncoprotein encoded by transgene could induce thyroid follicular cells to dedifferentiate [13].

1.3 The CSC model and its clinical implications

The classical carcinogenesis model posits that every cell within the organism is equally susceptible to acquire a combination of genetic and epigenetic alterations resulting in an unlimited proliferative potential. Interestingly, these subclones with different (epi) genetic profiles are responsible for phenotypic differences observed in tumor cell population.

In the last decade, emerging evidences suggest that tumors are organized in a hierarchy of different tumor cells and only a subset of cancer cells called Cancer Stem Cells (CSCs) possess the ability to self-renew and produce progenitor cells that can reconstitute and sustain tumor growth.

The CSC model was first demonstrated in acute myeloid leukemia. Bonnet and Dick

isolated a subset of leukemic cells, expressing the known stem-cell marker CD34, but not the leukocyte differentiation marker CD38, which was able to induce leukemia when transplanted into immunodeficient mouse [50]. Recently, the same research group showed that this cell fraction was the result of $CD38^+$ cells depletion caused by the anti-CD38 antibody and that the $CD34^+CD38^+$ subpopulation was equally able to initiate leukemia in immunodeficient mice [51]**.**

The first characterization of a cancer-initiating population in tumors comes from breast cancer by Al-Haji et al. They demonstrated that 200 cells expressing the cell surface markers $CD44^+$/ $CD24^{-/low}$/lineage (lin^-) showed tumorigenic activity upon injection into the cleared mammary fat pad of NOD/SCID mice, respect to 20.000 cells lacking this phenotype [52]. Later on, CSCs have been isolated from other tumors, such as brain [53], prostate [54], colon [55],[56], melanoma [57] by the use of cell-surface markers and the exploitation of *in vitro* and *in vivo* stem-cell assays.

Several *in vitro* strategies are used to evaluate CSCs potential in a tissue culture dish.

Dissociating spheres into single cells, plating them to limiting diluition and subjecting them to serial culture represent the best-studied assay to determine the clonality and their long-term proliferation potential. Moreover, given that CSCs promote tumor initiation and its recurrence after chemotherapy, researches have investigated whether putative CSCs are resistant to chemotherapy by establishing *in vitro* cytotoxicity assays.

The most definitive way to assess the long-term tumorigenic potential of CSCs is to inject them into animal models lacking the major elements of immune system.

Putative CSCs are isolated from tumor samples, dissociated into single-cell suspensions, immunolabeled with specific antibodies carrying a specific fluorophore, and then subjected to FACS. Sorted cells are orthotopically transplanted into immunodeficient mice obtaining that only CSCs form tumors. The cellular composition of tumors derived from secondary and tertiary xenografts should be examined to verify whether these tumors re-establish the phenotypic and histologic heterogeneity of parental ones [2].

The discovery of CSCs in a variety of tumors has changed the view of carcinogenesis and therapeutic strategies. Tumors can become resistant to therapeutic drugs through the evolution of multiple mechanisms including up-regulation of ATP-binding cassette (ABC) transporters [58], active DNA-repair capacity [59] and over-expression of anti-apoptotic molecules [60-63]. Such mechanisms could be even more pronounced in CSCs, which have been shown to be particularly resistant to conventional chemotherapy [60, 63]. Moreover, the presumed slow division of CSCs could represent another advantage against chemotherapy, which preferentially targets rapidly proliferating cells [64, 65]. With this in mind, the identification and characterization of the tumorigenic population is a crucial step to develop effective therapies able to eradicate tumors and prevent their recurrence. In particular, the increasing knowledge of thyroid tumors pathogenesis may contribute to an improved management of the treatment-resistant thyroid variants. **Figure 3.**

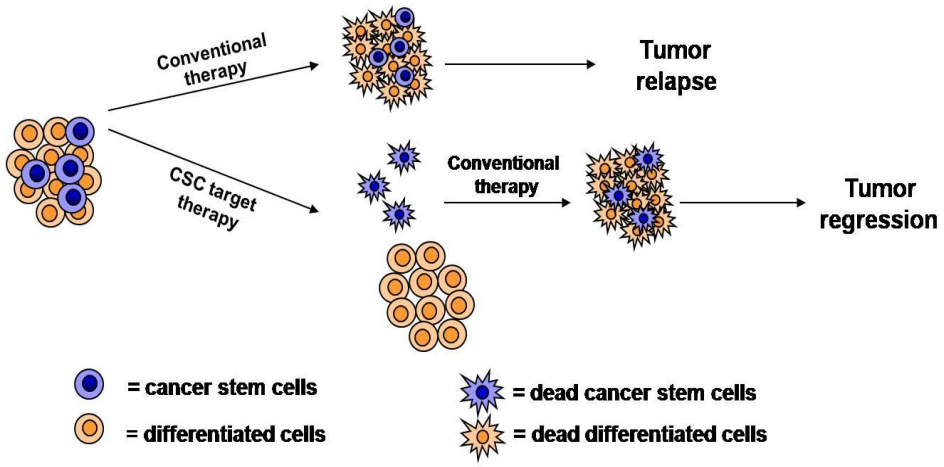

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Figure 3. Clinical Implications of CSCs.

It has been previously reported that in thyroid cancer the resistance to ligand- and chemotherapy-induced cell death correlates with autocrine production of interleukin (IL)-4 and IL-10, through the up-regulation of anti-apoptotic molecules such as cFLIP, Bcl-xL and PED [66, 67]. Next, it has been demonstrated that in UTC, the propagation of the survival signalling and the related refractoriness to death is likely to depend on impaired expression of suppressors cytokine signalling (SOCS) molecules that control the expression levels of Bcl-2 family members through Akt down-modulation. Exogenous expression of SOCS-3 and SOCS-5 genes reduces tumor growth and potently enhances the efficacy of chemotherapy both *in vitro* and *in vivo*, by altering the balance of pro-apoptotic and anti-apoptotic molecules. These

results clearly indicated that SOCS regulation of cytokine-prosurvival programs might be a new strategy to overcome the resistance to chemotherapy in the most aggressive variant of thyroid cancer [68].

Moreover, deregulation of Wnt/β-catenin pathway in UTC appears to be associated with antineoplastic effects of the selective tyrosine kinase inhibitor imatinib mesylate (Gleevec), which is not effective as monotherapy, despite it is able to target β-catenin and reduce invasiveness and proliferation of thyroid cancer cells [69].

Several agents have been promising in the experimental systems. They include specific tyrosinkinase inhibitors, e.g. the pyrozolopyrimided PP2 for RET/PTC or RET signalling [70] and imatinib for Wnt/β-catenin signalling [71] as well as thiazolididione for stimulation of PPARγ signalling [72]. Nowadays, efforts have focused on the development of BRAF inhibitors. Among the several known multikinase inhibitors, the AY43-9006 compound is able to block the growth of all thyroid cell lines carrying the mutant BRAF by inducing cell arrest in G1 phase [73].

In the management of aggressive MTCs, recent data demonstrate the therapeutic efficacy of specific agents against RET and FGF receptors to inhibit tumor growth *in vitro* and *in vivo,* thus suggesting the combined genetic and pharmacologic approaches as effective forms of therapy [45]. However, orthotopic models could be exploited for experimental testing and preclinical validation of these new treatments, studied at least in experimental systems.

1.4 Thyroid cancer stem cell biomarkers

Recent findings support the concept that a subpopulation of thyroid cancer cells displays properties characteristic of stem cells, the so called thyroid cancer stem cells (TCSCs). Much research effort has been addressed to correctly identify this subpopulation of undifferentiated cells though the phenotype of stem and/or progenitor cells of thyroid gland has not been identified yet. Several biomarkers, currently regarded as prospective CSCs markers in different tumor types, have been identified to distinguish thyroid CSCs from non-cancer stem cells.

The cell-surface marker CD133 was used to isolate subsets of cells, enriched for the presence of CSCs, from many types of solid tumors [53-56, 74-78].

In thyroid, the first attempt to isolate TCSCs by CD133 expression was reported in 2009 when Lin et al. demonstrated that as few as 1.000 ARO cells, strongly positive for CD133, form tumors in NOD/SCID mice, in contrast to 100.000 with little or no expression of CD133. However, the identity of CD133-negative putative TCSCs has been questioned, since this cell line employed in the thyroid research was found to be cross-contaminated with the human colon cancer cell line HT-29 and does not recapitulate all aspects of primary tumors [79]. Therefore, it should be necessary to use freshly isolated human thyroid cancer cells in order to determine whether these CD133-positive cells possess the ability to promote *in vivo* tumor formation.

Moreover, Todaro's data pointed that CD133 is not present in the putative stem cell population of epithelial thyroid cancer specimens [30].

In 2007, researchers used flow cytometry to sort Hoechst-33342-excluding cells in normal mouse thyroid. This so called “side population” was found to enrich in stem cells and progenitors [80]. Later on, adult thyroid stem cells were isolated from nodular goiters of patients as a small side population accounted for 0,1% of the total. They displayed a primitive morphology, with a high nuclear-to-cytoplasmic ratio, and expressed the stem-cell marker Oct-4. Upon exposure to TSH, they differentiated into thyroid follicular cells with the ability to take up radioactive iodine [81].

A similar technique was used to isolate the side population from papillary, anaplastic and follicular thyroid cancer cell lines. They were found to possess stem-cell properties, such as the ability to undergo thyrosphere formation and the expression of so-called ‘stemness’ genes, including those in the Notch (*HES1*, *JAG1*) and Wnt (*MYC*, *JUN*, and *FZD5*) signaling pathways. However, CSCs are not identical to, or restricted to, side-population cells since both side-population cells and non-side-population cells were tumorigenic when injected subcutaneously into immunodeficient mice [82].

Recently, much attention has been given to the aldehyde dehydrogenase 1 (ALDH1), since experimental evidences support the role of ALDH1 in early stem cell differentiation by conversion of retinol to retinoic acid [83]. Moreover, increased ALDH activity has been described in cells with stem/progenitor features of murine and human haematopoietic compartment [84, 85] and murine brain [86]. ALDH activity has also been found in primitive cells from other tissues, including multiple myeloma [87], acute myeloid leukaemia [88], pancreatic [89], breast [90] and lung [91] carcinomas.

Our data suggest that thyroid cancer tissues derived from follicular, papillary or anaplastic subtypes contain a small population of $ALDH^{high}$ cells that can undergo self-renewal and be expanded indefinitely *in vitro* as thyrospheres. When orthotopically injected into the thyroid gland of immunocompromised mice, these $ALDH^{high}$ cells are tumorigenic and reproduce the phenotypic characteristics of the original tumor, including the aggressive features of UTC to invade local tissues and generate metastatic lesions.

Moreover, the *in vitro* migration abilities of $ALDH^{high}$ cells derived from UTC exceed those of $ALDH^{high}$ cells derived from PTC and FTC. We showed that this enhanced migratory ability is associated with increased expression of c-Met and Akt, suggesting a promising effect of these molecules for a broad clinical applications[30]. Overall, these findings show that a high ALDH activity characterizes the tumorigenic thyroid population.

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EXPERIMENTAL PROCEDURES

Specimens

Thyroid cancer tissues were obtained at the time of thyroidectomy from patients affected by PTC, FTC and UTC in accordance with the ethical standards of the institutional committee responsible for human experimentation. Normal thyroid tissues were obtained from the uninvolved, controlateral lobes of tumor glands. Diagnosis was based on the histological analysis of thyroid specimens determining size, tumor progression and the involvement of regional lymph nodes. Staging was established according to the UICC TNM classification of malignant tumor (ICD- O C73) (UICC TNM. *Classification of Malignant Tumors; 7th Edition.* New York: Wiley Liss 2002).

Cell culture, clonogenic and invasion assay

Normal and tumoral thyroid tissues were dissociated for 1 hours with collagenase (1.5 mg/ml) (GIBCO) and hyaluronidase (20 μg/ml) (Sigma Chemical) in DMEM [66].

To obtain thyroid spheres, cells were re-suspended in medium containing bFGF (20 ng/ml, Sigma) and EGF (10 ng/ml, Sigma) [56, 63] and plated on ultra low-adhesion 96-well plates at a concentration of a single cell per well. Wells containing either none or more than one cell were excluded for the analysis. Migration of TCSCs *in vitro* was measured by the migration through growth factor-depleted Matrigel–coated

(BD Biosciences) transwell inserts (Costar). Dissociated sphere cells ($1.5x10^3$) in 200 µl serum-free medium (described above) were plated onto Matrigel-coated (25 µl of 2.5 mg/ml) transwell with 8 µm pore size. DMEM supplemented with 5% of Human Serum was plated in the lower compartment of transwell. After plating, number of migrated cells was counted up to 72 hours and stained for markers involved in the migrating capacity. Triplicates were counted in each experiment.

Istochemistry, Immunohistochemistry and Immunofluorescence

Histochemical and immunohistochemical analyses were performed on 5-µm-thick paraffin-embedded sections of thyroid specimens. For H&E staining slides were stained for 1 min in hematoxilin, washed in water and then exposed for 30 sec to eosin. Stained sections were dehydrated and mounted in synthetic resin.

For immunohistochemical analysis, slides were heated for antigen retrieval in 10 mM sodium citrate (pH 6.0). Sections were subsequently exposed to specific antibodies for thyroglobulin (Tg, DAK-Tg6, mouse IgG1κ, Dako), TTF1 (SPT24, mouse IgG1κ, Novocastra), cytokeratin 19 (CK19, RCK108, mouse IgG1κ, Dako), ALDH1 (ALDH1, 44, mouse IgG1, BD Biosciences) or isotype-matched controls at appropriate dilutions. Then, sections were incubated with biotinylated anti-mouse immunoglobulins for 30 min at RT and treated with streptavidin-peroxidase following manufacturers' instructions (LSAB2 Kit, Dako). Stainings were revealed using 3-amino-9- ethylcarbazole (AEC) substrate and counter-stained with aqueous hematoxylin.

For immunofluorescence, cytospins of thyroid spheres were fixed with 2% paraformaldehyde. Later on, cells were permeabilized with 0.1% Triton X-100, washed in PBS and successively exposed to antibodies against, β-catenin (17C2, mouse IgG_{2a}, Novocastra) pMet (AF2480, anti-phospho-HGFR, rabbit polyclonal, RD), pAkt (9271, ser 473, rabbit polyclonal, CST) and E-cadherin (4065, rabbit polyclonal, CST) diluted in PBS plus 3% BSA and 0.05% Tween 20 (PBS-T). After two rinses in PBS-T, cells were then incubated with Rhodamine Red-conjugated anti-mouse or anti-rabbit antibodies (Molecular Probes, Invitrogen), plus RNAse (200 μg/ml, Sigma). Counterstaining was performed using Toto3 iodide (642/660, Molecular Probes, Invitrogen).

Flow Cytometry and ALDEFLUOR assay

Freshly isolated cells from the three histological variants of thyroid cancer were analysed for ALDH1 expression by flow cytometry. Detection of intracellular antigens stainings were performed by using a permeabilizing kit following manufacturers' instructions (Cytofix/Cytoperm Kit, BD). Cells were exposed to antibody specific for ALDH1 (BD Biosciences), or the corresponding isotype control. Then, cells were rinsed and incubated with FITC-labelled anti-mouse secondary antibodies. Analysis was performed using a FACSCalibur flow cytometer (BD).

Purification of thyroid cells with a high ALDH enzymatic activity was performed using the ALDEFLUOR kit (Stem Cell Technologies). Cells obtained from freshly

dissociated cancer thyroid tissues were re-suspended in the ALDEFLUOR assay buffer containing the ALDH substrate BODIPY®- aminoacetaldehyde (BAAA, 1μmol/Lx10^6 cells) and incubated for 40 min at 37°C. As negative control, an aliquot of cells of each sample was treated with 50 mmol/L of diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor. Intracellular fluorescent product was measured by flow cytometry and sorting gates were established using the negative control.

Immunoblotting validation

Lysates were fractioned on SDS-polyacrylamide gels and blotted to nitrocellulose. Membranes were blocked for 1 h with non fat dry milk in TBS containing 0.05% Tween-20 and successively incubated with antibodies specific for Met (sc-161, C20, rabbit polyclonal, Santa Cruz), pMet (D26, Tyr 1234/1235, rabbit IgG CST), AKT (9272, rabbit polyclonal, CST), pAkt (9271, ser473, rabbit polyclonal, CST), E-cadherin (4065, rabbit polyclonal, CST) and β-actin (Ab-1 mouse IgM, Calbiochem). Membranes were then washed, incubated for 1 hour with horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulins (Amersham) and visualized by ChemiDoc-IT® 410 Imaging System (UVP).

Production of lentiviral particles and infection

BRAF(V600E) cDNA with 5'- and 3' cohesive ends was subcloned at XhoI and EcoRV sites, while RET/PTC1 cDNA at XbaI and EcoRV. Mutant p53 (pcDNA3-

p53R175H) was subcloned at XhoI site. Gene transfer was performed using a TWEEN lentiviral vector containing green fluorescent protein (GFP) as reporter gene. Transfection of packaging human embryonic kidney cell line HEK-293T was assessed using FuGENE 6 Reagent (Roche) and following the manufacturer's instructions. Lentiviral supernatants were collected following 48 hrs transfection and filtered with a 45 μm mesh. Then, 10^5 normal thyroid stem cells were exposed to 1 mL of viral supernatant of BRAF(V600E), RET/PTC1 or p53(R175H) for 40 min by centrifugation at 800 rpm, in the presence of 8μg/μL of polybrene to improve infection efficiency. Infection was repeated twice and efficiency of gene delivery was verified monitoring GFP expression.

Stable shRNA expressing UTC cells were obtained by infection with pLK01 lentiviral plasmid containing the interfering sequence of puromycin resistant Akt (Sigma). UTC cells were selected with puromycin drug (2 ng/ml, Sigma) for 15 days to enrich for positive transfectants. ShMet (pCCLsin.PPT.hPGK.GFP.Wpre) [92] was kindly provided by Prof. S. Giordano (Institute for Cancer Research and Treatment, Torino).

Animal Model

To evaluate the tumorigenicity and metastatic potential 100 dissociated PTC, FTC and UTC sphere cells were orthotopically injected into 6 weeks-old NOD/SCID mice. The injection procedure was done with the support of a dissecting microscope. After anesthetization, a midline cervical incision was made. Retracted the

submandibular glands and midline strap muscles, the central compartment of the neck was visualized with the thyroid gland adjacent to the trachea. Direct injection of the right thyroid gland was then done using a 25 μL Hamilton syringe (Hamilton Company, Reno, NV) and 32-gauge needle. The injection volume was 5 μL. The submandibular glands were returned to the original position. After 4 weeks mice were sacrificed and lungs, thyroid glands in continuity with the larynx and trachea and lymph nodes, were removed. In order to localize and quantify dynamically the optical signal-bioluminescence in a non invasive localization of the luciferase marked cell population, 150 mg/kg D-luciferin (Promega) was administrated i.p. at time 0 and up to 12 weeks, 5 min before the bioluminescence analysis. Bioluminescence was detected by Photon imager *in vivo* imaging system (Biospace Lab), assisted by the M3 Vision analysis software. For photon flux counting, animals were scanned for 10 min. The quantitation of signal intensity was calculated as the sum of all detected photon flux counts within a uniform region of interest, manually selected during data post processing.

Dissociated normal thyrospheres as well as those transduced with lentiviral vectors coding for *RET/PTC1, BRA(V600E), p53(R175H)* ($5x10^5$) were injected subcutaneously with Matrigel GF reduced (BD Biosciences, Erembodegem, Belgium) at a 1:2 ratio in a total volume of 100 μL. Tumor size was calculated once a week up for to 10 weeks according to the following formula: (π/6) x larger diameter x (smaller diameter).

Statistical Analysis

Data were expressed as percentage ± standard deviation of the mean. Statistical significance was determined by Analysis of Variance (one-way or two-way) with Bonferroni post-test. Results were considered significant when p values were less than 0.05. * indicates $P<0.05$, ** indicate $P<0.01$ and *** indicate $P<0.001$.

RESULTS

Thyroid cancer tissues obtained at the time of thyrodectomy from patients with papillary, follicular or anaplastic histological variants have been analyzed for the presence of putative cancer stem/progenitor cells. Immunohistochemistry analyses of differentiation/stemness markers showed that the terminal thyroid differentiation marker Tg and the transcription factor TTF1 were expressed in PTC and FTC, whereas their expression was reduced in UTC. CK19 was detected in PTC and FTC, but not in UTC, confirming the absent of thyroid-specific functions. Moreover, CD133 was not found while CD44 was constitutively expressed in normal and tumoral tissues. Interestingly, ALDH1 expression, restricted to a rare population in normal tissues, increases progressively in the more aggressive UTC (**Figure 4).**

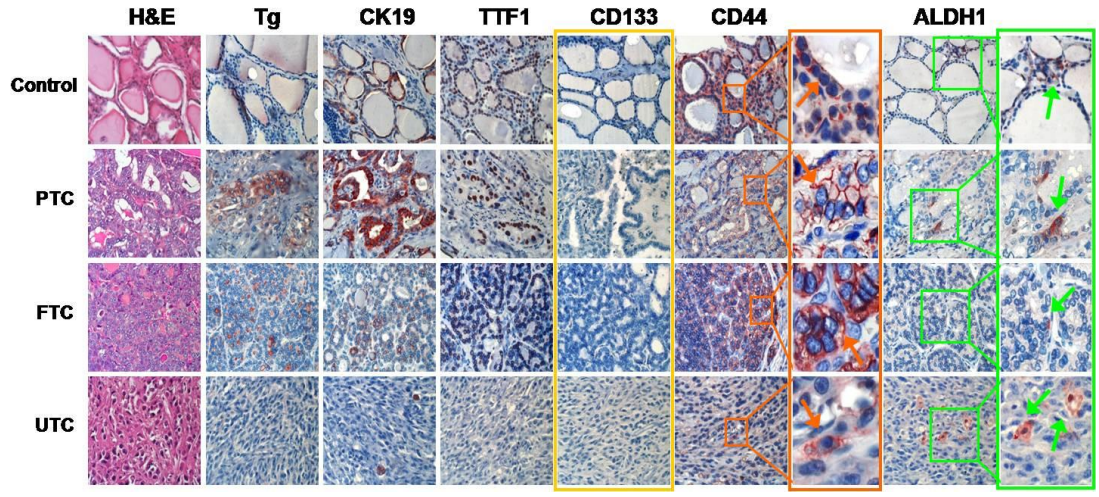

Figure 4. H&E staining analysis for indicated antigens on paraffin-embedded sections of human thyroid gland (Control), PTC, FTC and UTC.

To evaluate whether ALDH activity could be used as a prospective marker of TCSCs, ALDH^{high} cells have been isolated from the three histological variants by using the ALDERFLUOR assay, a strategy previously exploited for the identification of hematopoietic [87, 88] and breast [90] cells with stem/progenitor properties. In each experiment, thyroid cancer cells were exposed to ALDERFLUOR substrate BAAA which, specifically metabolized by cells harbouring ALDH1, creates a fluorescent dye facilitating positive selection by FACS. As shown in Figure 5, cells with ALDH^{high} activity were detected in all histological variants with the highest percentage in freshly dissociated UTC tissues (on average, 14±3% of positive cells).

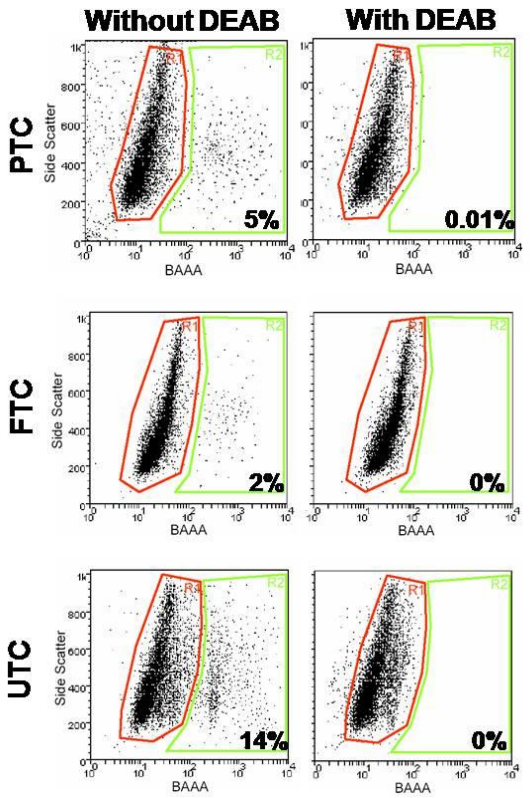

Figure 5. Representative FACS analysis of PTC, FTC and UTC cells using ALDEFLUOR assay. Thyroid cancer cells exposed to ALDEFLUOR substrate (BAAA) and specific inhibitor of ALDH (DEAB) were used to define populations with ALDH^{high} activity (R2, green region) and with ALDH^{low} activity (R1, red region).

PTC and FTC revealed a smaller ALDH^{high} fraction accounting for 5±2% and 2±1.2% of the whole-cell pool, respectively.

A prerequisite of putative CSCs is the ability to initiate tumor development in recipient animals and reproduce the phenotypic characteristics of the original tumor. Whole-body *in vivo* imaging technique and macroscopic analysis revealed that orthotopic injection of 100 ALDH^{high} cells from dissociated PTC, FTC and UTC spheres retain tumor-initiating capacity and recapitulate the clinical and pathological behaviour of their respective parental tumors. Consistently, PTC spheres determined a local tumor growth, while FTC spheres resulted in thyroid gland infiltration and compression of adjacent structures, such as larynx and trachea. Likewise, ALDH^{high} UTC spheres formed tumors that aggressively invaded local tissues and metastasized to distant locations, including cervical lymph nodes metastasis and lung **(Figure 6).**

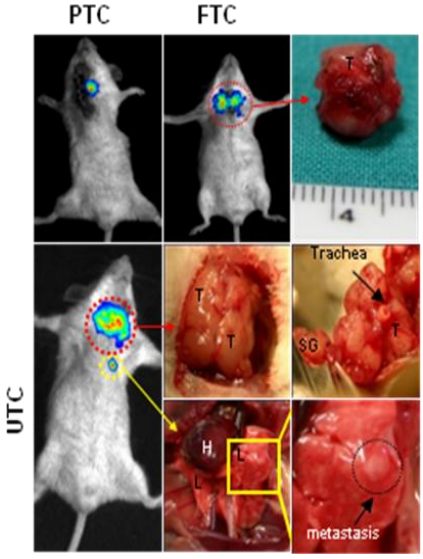

Figure 6. Whole-body *in vivo* imaging and macroscopic analysis of primary and metastatic thyroid tumors after injection of dissociated PTC, FTC (upper panels) and UTC (lower panels) spheres into the mouse thyroid gland. Letters indicate salivary glands(SG), thyroid tumor (T), lung (L) and heart (H).

H&E staining and immunohistochemical analysis showed that spheres-derived tumor xenografts reproduce the morphology and antigen expression of parental tumors. In particular, PTC and FTC xenografts expressed Tg and CK19 differentiation markers similarly to the well-differentiated variants from which they were purified as well as the highly tumorigenic $ALDH^{high}$ UTC cells generated CK19 and Tg negative undifferentiated tumors. The pattern of ALDH1 expression indicated a significant enrichment of the putative tumorigenic $ALDH1^{+}$ cells in lung metastases as compared with the thyroid tumor (**Figure 7).**

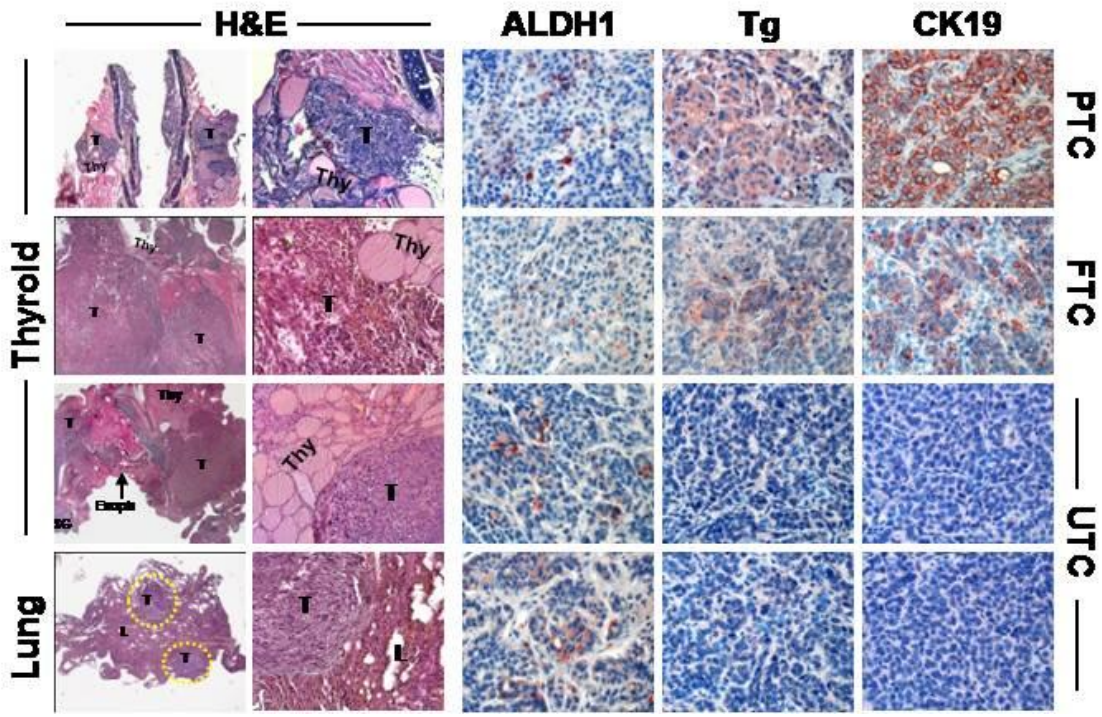

Figure 7. H&E staining and immunohistochemical analysis of ALDH1, Tg or CK19 revealed by AEC (red staining) on paraffin-embedded sections of mouse thyroid gland invaded by human PTC, FTC and UTC cells, and mouse lung metastasis by human UTC cells. Nuclei were revealed by haematoxylin (blue staining). Letters Thy indicate mouse thyroid gland.

The study of transcriptional program awarding tumorigenic and metastatic potential of TCSCs indicated an important relationship between Met/Akt pathway and their invasive behaviour. In particular, immunoblot analyses demonstrated the activation of Akt, Met in sphere cells from UTC, as compared with those from normal thyroid (Control), PTC and FTC **(Figure 8. a).** Moreover, UTC sphere cells showed a higher percentage of β-catenin nuclear accumulation indicating a more aggressive phenotype **(Figure 8. b)**.

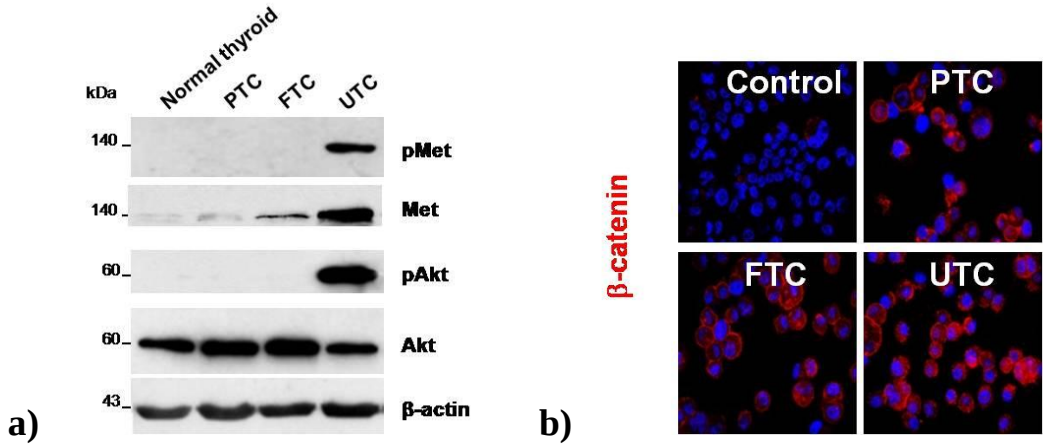

Figure 8. a) Representative immunoblot for pMet, Met, pAkt, Akt in normal thyroid (Control), PTC, FTC, UTC sphere cells. Loading control was performed by using β-actin. **b)** Immunofluorescence analysis of β-catenin nuclear accumulation in normal thyroid (Control), PTC, FTC, UTC sphere cells.

Similarly, in the invasion assay analysis UTC stem cells demonstrated a higher migration capacity than the stem cells derived from the other tumor histotypes **(Figure 9).**

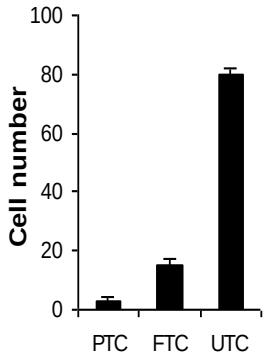

Figure 9. Number of migrated PTC, FTC and UTC sphere cells after 48 hr.

Interestingly, migrated UTC stem cells showed pMet and pAkt upregulation, nuclear accumulation of β-catenin, and complete loss of E-cadherin expression in accordance with their metastatic potential **(Figure 10).**

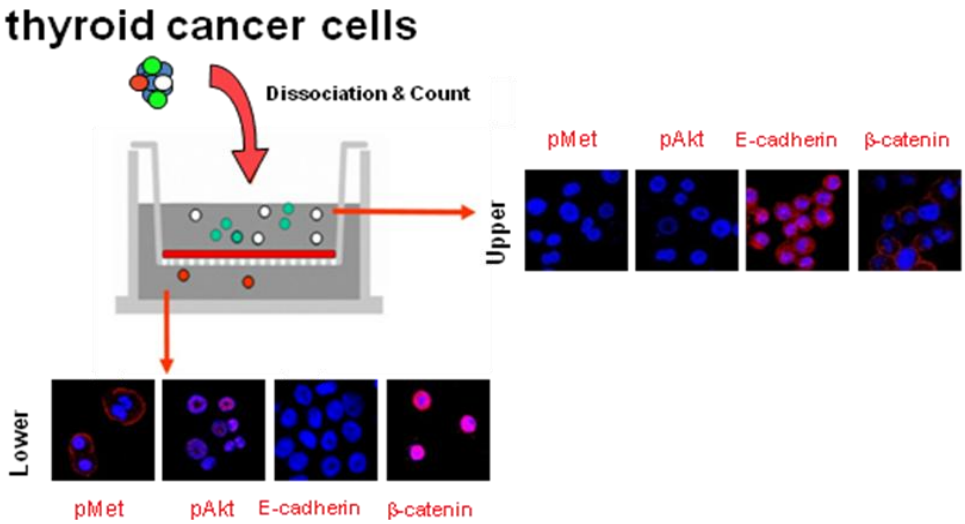

Figure 10. Immunofluorescence analysis of pMet, pAkt, β-catenin nuclear accumulation, E-cadherin in migrated UTC stem cells.

In order to evaluate whether Met and Akt activation are involved in the regulation of tumorigenic and metastatic activity of TCSCs, it was induced the functional blockade of these molecules. Immunoblot analysis of UTC sphere cells transduced with lentiviral vectors encoding Met and Akt shRNA sequences showed an efficient knockdown of their activated forms **(Figure 11.a)**. Moreover, down-regulation of expression levels dramatically reduced the migration activity of UTC spheres, as shown in **Figure 11. b**.

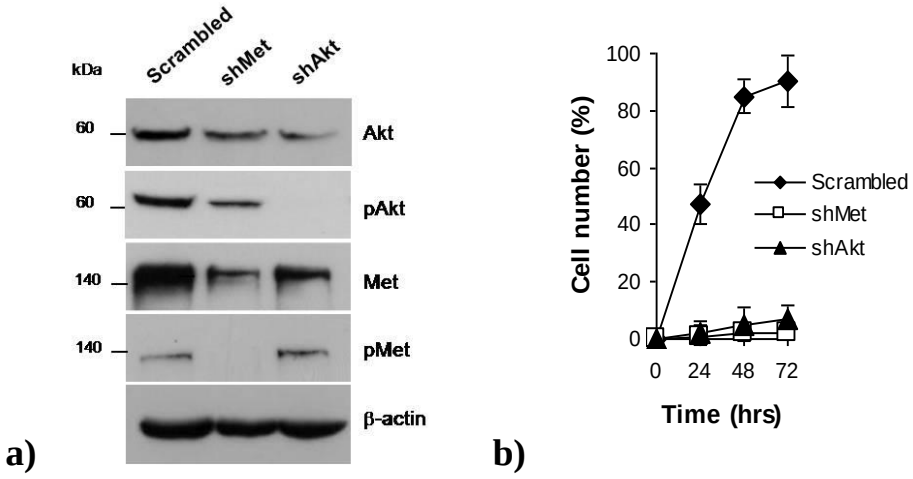

Figure 11. a) Representative Western Blot analysis of Met, pMet, Akt and pAkt in UTC sphere cells transduced with control shRNA (Scrambled), Met shRNA (shMet) or shRNA Akt (shAkt). Loading control was performed by using β-actin. **b)** Migration analysis of UTC sphere cells transduced with Scrambled, shMet or shAkt.

These promising effects of Akt and Met for the treatment of invasive thyroid cancer have been confirmed with *in vivo* results. UTC sphere cells transduced with ShMet or ShAkt were allowed to orthotopically grow into mouse thyroid gland. The bioluminescence analysis detected by Photon imaging system revealed that Met

knockdown delayed tumor outgrowth by about 9 weeks while ShAkt dramatically reduced the capacity to give rise to orthotopic tumor generated by UTC spheres **(Figure 12)**.

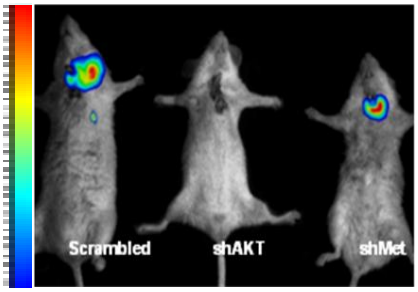

Figure 12. Whole-body *in vivo* imaging analysis of orthotopic tumor growth and lung metastasis generated by UTC sphere cells transduced with Scrambled, shMet or shAkt.

Both ShMet and ShAkt determined the complete abrogation of the UTC spheres metastatic potential, suggesting that these molecules could be candidate targets for a broad clinical application of invasive thyroid cancer **(Figure 13)**.

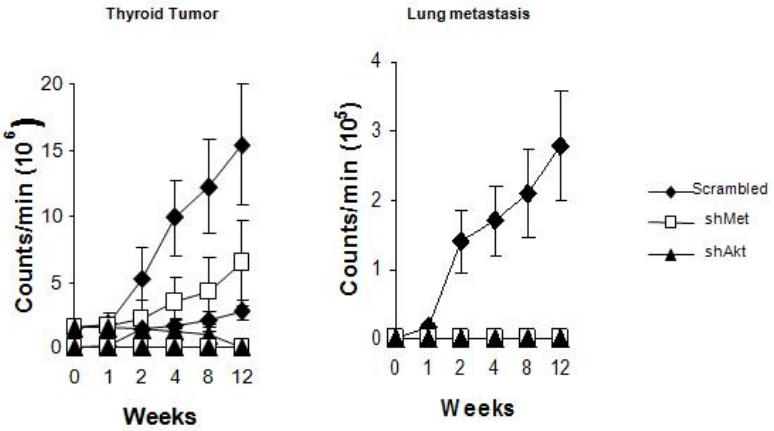

Figure 13. Representative *in vivo* imaging analysis of growth at 12 weeks of tumor generated by UTC sphere cells transduced with Scrambled, shMet or shAkt .

In order to understand whether the self-renewal ability is directly influenced by the most common genetic alterations, thyroid SCs (TSCs) isolated from goiters have been transduced with lentiviral vectors coding for *RET/PTC1*, *BRAF(V600E)* and mutant *p53* **(Figure 13).**

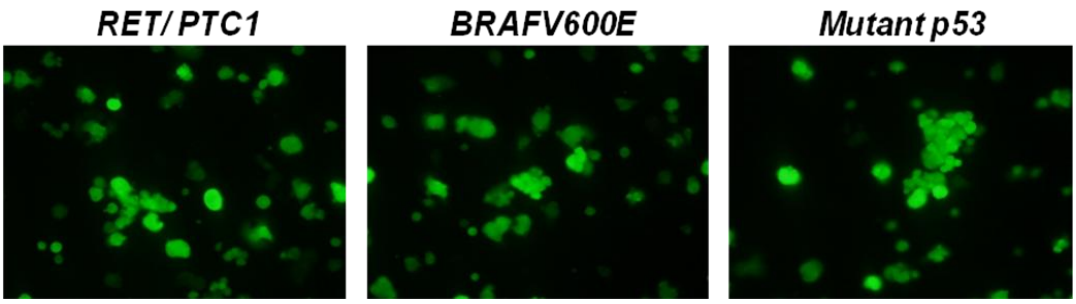

Figure 14. Representative images of TSCs transduced with lentiviral vectors above mentioned.

It was found that the exogenous expression of *BRAF(V600E)* was associated with a lower clonogenicity, while *RET/PTC1* rearrangement and mutant *p53* massively promoted the self-renewal activity **(Figure 15).**

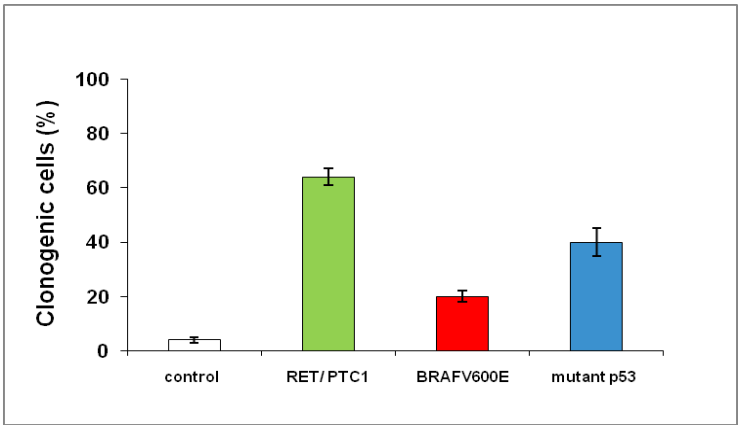

Figure 15. Clonogenic percentage of TSCs (control) and those transduced with the oncogenes before mentioned.

In the invasion assay analysis, TSCs transduced with the above mentioned lentiviral vectors demonstrated a higher migration capacity than the control, in particular the more invasive cells were those transduced with *RET/PTC1* rearrangement and mutant *p53*, in accordance with their higher clonogenenic activity **(Figure 16).**

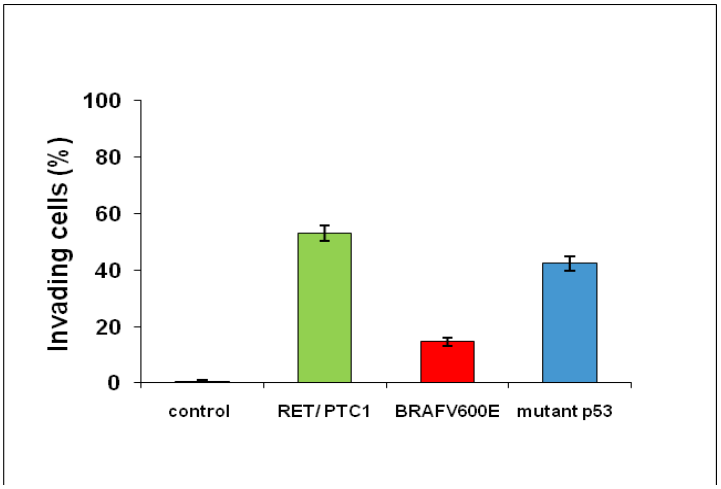

Figure 16. Number of migrated TSCs transduced with *RET/PTC1*, *BRAFV600E* or mutant *p53*.

Furthermore, it has been investigated the *in vivo* tumorigenic capacity of the same cells by subcutaneous injection in NOD/SCID mice. Although normal thyroid cells, both wild type and transduced with *BRAFV600E*, failed to generate tumors following xeno-transplantation, those transduced with *RET/PTC1* rearrangement and mutant *p53* gave rise to palpable tumors in about 6-7 weeks. Macroscopic examination of xenografts removed after 10 weeks showed that *RET/PTC1* rearrangement generated a tumor with higher size than that determined by mutant *p53* **(Figures 17, 18).**

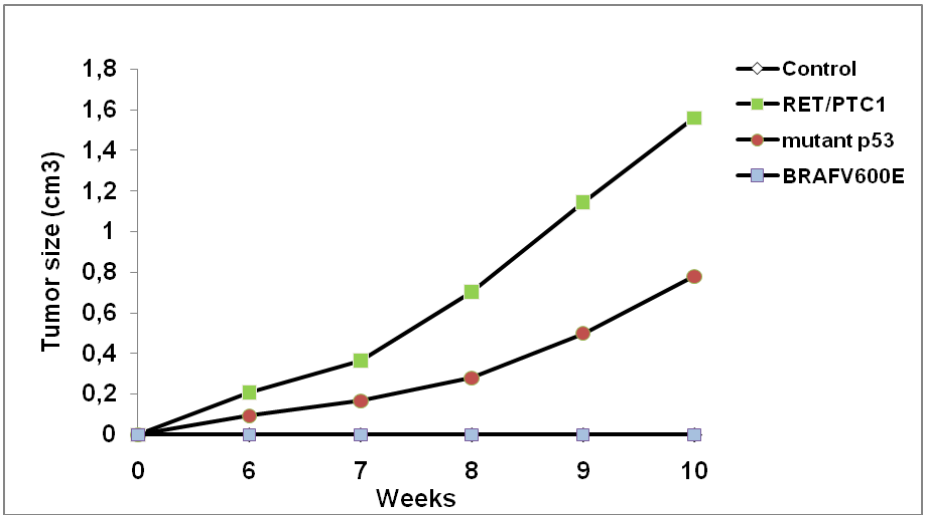

Figure 16. Size of *RET/PTC1* rearrangement- and mutant *p53*-derived tumor xenografts calculated once a week up for to 10 weeks according to the following formula: π/6) x larger diameter x (smaller diameter).

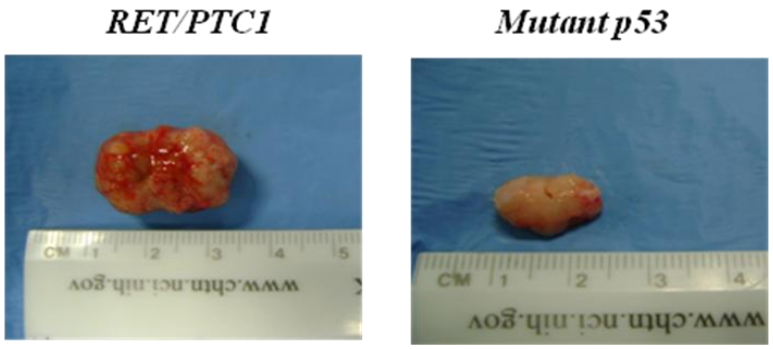

Figure 17. Images of *RET/PTC1* rearrangement- and mutant *p53*-derived tumor xenografts removed after 10 weeks.

Interestingly, mutant *p53*-derived tumor xenograft was more vascularized and showed infiltrating margins in the peritoneum.

The resultant subcutaneous xenografts recapitulated the antigen expression of PTC and UTC orthotopic xenografts, as suggested by immunohistochemical analyses

shown in Figure 18. *RET/PTC1* rearrangement-derived tumor xenograft expressed Tg and CK19 differentiation markers as observed in PTC orthotopic xenograft, while mutant *p53* generated CK19 and Tg negative undifferentiated tumors.

Moreover, *RET/PTC1* rearrangement- and mutant p*53*-derived tumor xenografts demonstrated a significant loss of the thyroid transcription factor (TTF-1) in comparison with the control.

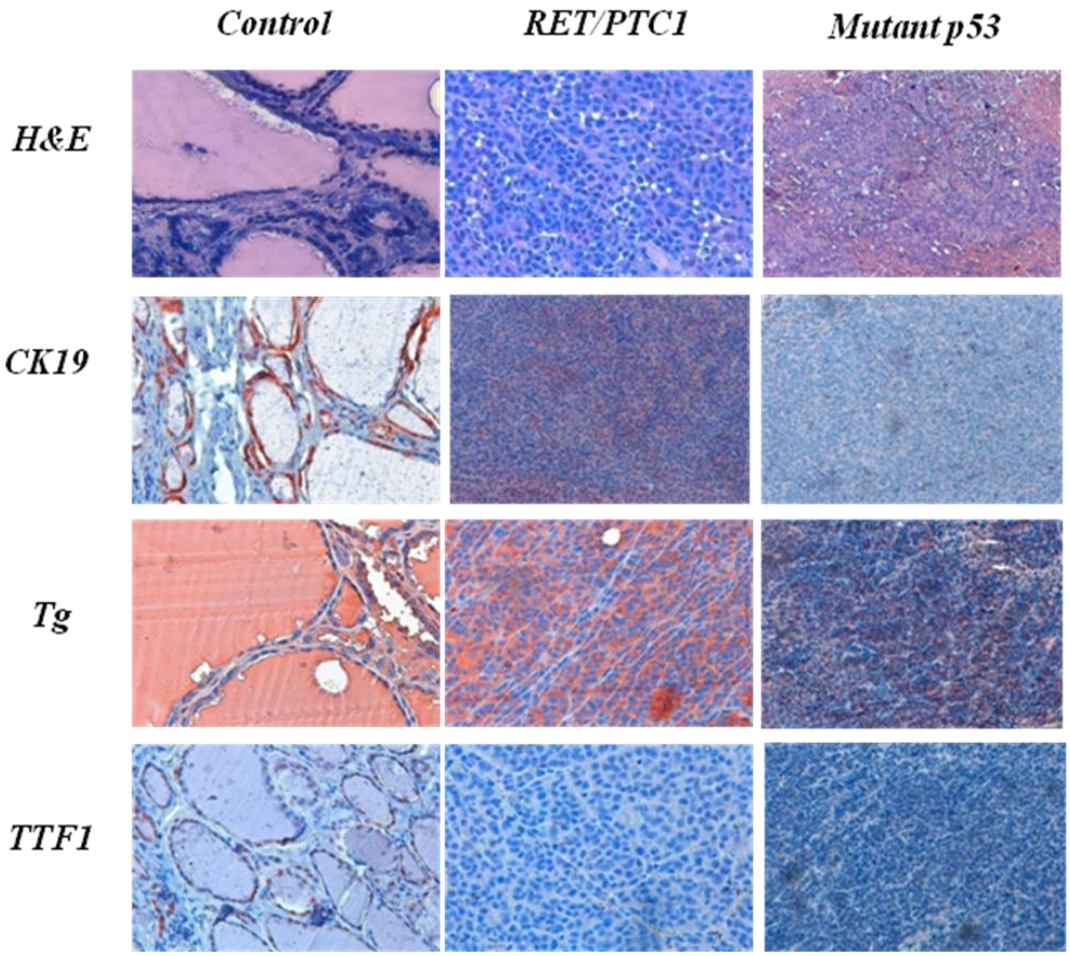

Figure 18. Representative H&E staining and immunohistochemical analysis of TTF1, Tg or CK19 on paraffin-embedded sections of control compared to *RET/PTC1* rearrangement- and mutant *p53*-derived tumor xenografts.

Overall, these results clearly indicate that *RET/PTC1* rearrangement and *p53* mutant are the specific genetic alterations involved in thyroid transformation since they confer to thyroid SCs the ability to initiate tumors in NOD/SCID mice and the resultant subcutaneous xenografts showed similar antigen expression of PTC and UTC orthotopic xenografts.

DISCUSSION

Increasing advances in stem cell research have opened new chances for the treatment of many types of cancers, since the identification of CSCs might lead to the development of more effective therapeutic approaches than those currently available. For this reason, much research effort has been addressed to identify and isolate CSCs in different tumor types as well as to advance understanding of the molecular mechanisms that control oncogenic process and chemotherapy resistance. Moreover, such knowledge could potentially lead to the creation of animal models useful to understand the strengths and limitations of new individualized treatment regiments, especially against UTC.

Although the phenotype of thyroid stem/progenitor cells has not been identified, several biomarkers have been proposed to distinguish TCSCs from non-cancer stem cells.

Data obtained during PhD course in Immunopharmacology showed that self-renew and tumorigenic capacity in thyroid cancer are confined in a small population of tumor cells with high ALDH activity. Expanded indefinitely *in vitro* as thyroid spheres, these $ALDH^{high}$ cells are able to generate serial tumor xenografts in immunocompromised mice.

Sphere-derived tumor xenografts reproduce the original tumor, both in terms of morphology and antigen expression. The pattern of ALDH1, CK19 and Tg expression as well as the percentage of tumorigenic cells did not change between

parental tumors and respective primary xenografts in all histological variants examined, suggesting that TCSCs possess a functional hierarchy and heterogeneity.

Moreover, the identification of thyroid cancer-initiating cells and the availability of an orthotopic xenograft model may provide considerable informations about the molecular mechanisms that regulate oncogenic process and powerful tools for development and preclinical validation of novel targeted therapies.

Previous studies on malignant cancer development supported a role for Met activation to induce tumor growth and spreading, including thyroid cancer [93]. With this in mind, it has been investigated the existence of a possible relationship between Met/Akt pathway and the aggressive phenotype of UTC, obtaining that Met and Akt activation promotes invasive behaviour of UTC stem cells. Consistently, the functional blockade of these molecules determined a complete abrogation of UTC spheres tumorigenic potential both *in vitro* and *in vivo.* These results clearly indicate that Met and Akt could be candidate targets for a broad clinical applications of invasive thyroid cancers.

In this context, a better knowledge of the genetic alterations associated with initiation and progression of thyroid cancer may provide unique signatures for the development of more effective therapeutic strategies.

Although *RET/PTC1, BRAF(V600E)* and *p53* mutant affect thyroid cancers, the role of these genetic alterations in tumor initiation and progression is not well known.

For this reason, the aim of study was to understand whether *RET/PTC1,*

BRAF(V600E) and mutant *p53* may be responsible for cancer initiation.

Thyroid SCs (TSCs) have been isolated from goiters in order to transform them with lentiviral vectors coding for *RET/PTC1*, *BRAF(V600E)* or mutant *p53*. It was noticed that normal cells, after exogenous expression of these oncogenes, showed an high degree of self-renewal and migration capacity.

Interestingly, *in vivo* results demonstrated that the same cells acquire the ability to initiate tumor in NOD/SCID mice and the resultant subcutaneous xenografts possess the pattern of CK19 and Tg expression of PTC and UTC orthotopic xenogratfs. Moreover, immunohistochemical analysis of TTF-1 revealed that *RET/PTC1* rearrangement- and *p53* mutant-derived tumor xenografts lost the expression levels of this key transcription factor compared to control. These results are similar with those reported by Shibru et al., according to which a gradual loss of TTF-1 is found during thyroid cell transformation from the well-differentiated benign follicular adenoma (FA), to FTC and PTC, with the lowest expression in UTC [94].

Overall, these findings define a good model to identify the genetic alterations involved in thyroid carcinogenesis as well as outline prospective therapeutic strategies.

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