Primary cell cultures for the personalized therapy in aggressive thyroid cancer of follicular origin.

Poupak Fallahi [1], Silvia Martina Ferrari [2], Giusy Elia [2], Francesca Ragusa [2], Armando Patrizio [2], Sabrina Rosaria Paparo [2], Gianni Marone [3-6], Maria Rosaria Galdiero [4-7], Giovanni Guglielmi [1], Rudy Foddis [1], Alfonso Cristaudo [1], Alessandro Antonelli [2]

[1] Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Pisa, Italy; [2] Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy; [3] Department of Translational Medical Sciences, University of Naples Federico II, 80131 Naples, Italy; [4] Center for Basic and Clinical Immunology Research, University of Naples Federico II, 80131 Naples, Italy; [5] World Allergy Organization Center of Excellence, University of Naples Federico II, 80131 Naples, Italy; [6] Institute of Experimental Endocrinology and Oncology "Gaetano Salvatore," National Research Council, 80131 Naples, Italy; [7] Department of Translational Medical Sciences, University of Naples Federico II, 80131 Naples, Italy.

[*]***Corresponding Author***
Alessandro Antonelli, MD
Director: Immuno-Endocrine Section of Internal Medicine
Professor of Medicine, Endocrinology, Clinical Pathology
Head, Laboratory of Primary Human Cells
Department of Clinical and Experimental Medicine
University of Pisa, School of Medicine
Via Savi, 10, I-56126, Pisa, Italy
Phone: +39-050-992318
Fax: +39-050-993472 or +39-050-500841
e-mail: alessandro.antonelli@med.unipi.it

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Abstract

Thyroid cancer (TC) is the most prevalent endocrine malignancy. More than 90% of TC is represented by differentiated TC (DTC) arising from the follicular thyroid cells. DTC includes papillary TC (PTC), follicular TC (FTC), and Hürthle cell TC. Anaplastic TC (ATC) accounts for 1% of TC, and it represents 15-40% of TC death. Current treatment strategies are not completely effective against aggressive DTC or ATC, and mortality is one of the most important challenges.

Recently, progresses have been obtained in the understanding of the molecular/genetic basis of TC progression, and new drugs have been introduced [i.e. tyrosine kinase inhibitors (TKIs)], able to block the oncogenic or signaling kinases, associated with cellular growth.

Thyroid cell lines, obtained from tumoral cells and chosen for high proliferation *in vitro*, have been used as preclinical models. Actually, these cells lose the characteristic features of the primary tumor, because they adapt to *in vitro* growth conditions. For these reasons, the use of these cell lines has important limitations, and more recently human primary cell cultures have been established as monolayer cultures, and investigated for their biological behavior. Moreover, in the past, primary TC cells could be collected only through surgical biopsies, while recently human primary cell cultures can be established also from samples of fine-needle aspiration citology from aggressive dedifferentiated DTC or ATC. Testing *in vitro* different TKIs in each patient can help to develop new personalized treatments, without using ineffective drugs.

In conclusion, personalized medicine and precise oncology, which consider both patients and their disease features, represent the future of the treatment approach, and further progress is needed in this direction.

Keywords: aggressive thyroid cancer; thyroid primary cell cultures; targeted therapy; tyrosine kinase inhibitors; precision medicine

1. Introduction

Thyroid cancer (TC) accounts for 3–4% of cancers and it is one of the most common endocrine tumor [1,2]. Its occurrence has risen in the last 10 years, because of widespread application of neck ultrasound (**US**) and fine-needle aspiration (FNA), which allow to detect smaller cancer than before. Its prevalence is more elevated in women than in men [3].

More than 90% of all TC is represented by differentiated thyroid cancer (DTC) [4], that derives from follicular cells. Papillary TC (PTC), follicular TC (FTC), and Hürthle cells TC are DTC [5]. Medullary TC (MTC) derives from the parafollicular C cells of the thyroid, it occurs sporadically, but has also a hereditary component for about 25% of cases [6]. Conversely, anaplastic thyroid cancer (ATC) is rare and accounts for ~1% of TC, but it is more aggressive leading to 15-40% of TC death [7-11].

The overall survival (OS) (5- and 10-year) for DTC patients is generally outstanding but it depends on several factors such as the age at the diagnosis and the cancer subtype [2,4]. PTC, FTC, or MTC have, respectively, a 10-year-OS rate of 98%, 92%, or 80% [12], while in ATC patients median survival is approximately 6 months [7-11].

PTC and FTC have slow progression rates with normally a good prognosis [4]. ATC, that is an undifferentiated tumor of the thyroid follicular epithelium, shows a much more aggressive behaviour compared to PTC and FTC, although they share the same histological origin [13]. Almost 50% of ATC, indeed, occurs after a past history of thyroid nodules, PTC or FTC [14].

The gold standard therapy for DTC is surgery (lobectomy or total thyroidectomy), while aggressive PTC and FTC need subsequent Radioactive Iodine (RAI) ablation of

the remnants [15,16]. The monitoring strategies are based on the basal and rTSH (recombinant thyroid-stimulating hormone) stimulated thyroglobulin (Tg) determinations, and on neck US [15,17]. Following surgery, RAI, and TSH suppressive therapy, the 5-year OS rate of DTC patients is of around 98%, even though at 10 years locoregional recurrences are present in up to 10-20% of cases and distant metastases in about 5-10% [16]. About 2/3 of these patients with aggressive DTC will become RAI-refractory and they will never be cured with RAI therapy, with a 3-year OS rate lower than 50% [16].

Thus, an early characterization of thyroid nodules is mandatory. Neck US and FNA-cytology (FNAC) are the best tools to discriminate between benign and malignant thyroid lesions [18].

Because of the ATC poor prognosis, multiple therapeutic strategies are usually attempted including debulking, external beam radiotherapy (EBRT), and chemotherapy (cisplatin or doxorubicin), reaching 10 months of median survival [19-22]. ATA guidelines indicate the efficacy of docetaxel or paclitaxel, doxorubicin, and also carboplatin, or cisplatin, in the treatments of ATC, but none of them have shown to improve OS [23].

In recent years, progresses have been made in the understanding of the molecular basis of TC progression, and new drugs have been introduced, acting by blocking oncogenic kinases ($BRAF^{V600E}$, RET/PTC) or signaling kinases as Vascular Endothelial Growth Factor Receptor (VEGFR) and Platelet-Derived Growth Factor Receptor (PDGFR) associated with cell growth [16,24-26].

Most of these molecular alterations can be considered new diagnostic and prognostic molecular markers and therapeutic targets for TC, and novel targeted therapies for

progressive, aggressive refractory cancers are now present, as Tyrosine Kinase Inhibitors (TKIs) [16,24,26].

In this review, we report the conducted *in vitro* studies and the importance of primary cell cultures for the personalization of targeted therapy, in aggressive TC of follicular origin.

2. Genome-wide association studies (GWAS) in aggressive TC of follicular origin

In the last 20 years, GWAS help to understand genes implication in many diseases [27], as autoimmune thyroid disorders, thyroid physiology, and TC, and to distinguish susceptibility genes for thyroid related phenotypes [28].

GWAS permit to detect high-incidence single nucleotide polymorphisms (SNP) and copy number variations (CNV). Specific SNP showed susceptibility to DTC (especially PTC and FTC) with different strength [1].

Recently, GWAS demonstrate in large TC patient cohorts the association between DTC and the most common variants, on 14q13.3 (NK2 homeobox 1) and 9q22.33 (FOXE1), and a link among “disrupted in renal carcinoma 3” (DIRC3), FOXE1, “MBIP/NK2 homeobox 1”, and Neuregulin 1 (NRG1) [29-32]. Other studies confirmed those data [33-41].

Furthermore, FOXE1 is implicated in radiation-induced TC [42,43], and its variants are related to the size of PTC, as well as with its tumor stage, lymphocytic infiltration, and extrathyroidal extension, which determine clinical behavior and aggressiveness of the tumor [44].

Another GWAS, in 1085 DTC patients and 8884 control subjects, demonstrated 15 variants of 11 loci linked to DTC, with the most robust signals in the gene encoding NRG1 [45], acting on the erb-b2 receptor TK (ERBB) family of TK receptors [46].

The dysregulation of NRG1 is also related to “mitogen-activated protein kinase” (MAPK) and Phosphoinositide-3 kinase/protein kinase-B (PI3K/Akt) pathway via ERBB [47].

“Small nuclear RNA activating complex polypeptide 4” (SNAPC4), “binner mitochondrial membrane peptidase subunit 2” (IMMP2L), “basic leucine zipper ATF-like transcription factor” (BATF), “DEAH-box helicase 35” (DHX35), “WD Repeat Domain 11 antisense RNA 1”, “5-hydroxytryptamine receptor 1B”, FOXA2, and “retinoic acid receptor responder 1” (RARRES1) have been described only by some papers as associated with DTC [29,30,48,49].

Forty-five SNP selected from the GWAS were investigated in an Italian population, and those with an evidence of association were investigated in the Polish and Spanish cohorts [29]. The highest correlation was shown for rs10136427 localized in BATF and rs7267944 in DHX35, providing evidence for new DTC susceptibility variants [29].

Another GWAS reported an association among mutations in non-coding RNA genes RARRES1, DIRC3, IMMP2L, SNAPC4, and an elevated DTC risk, in an Italian population (constituted by 690 patients and 497 control subjects) and proved these data in 3 low-incidence populations (with 2 958 patients and 3 727 controls) [30].

Looking for risky sequence variants of non-medullary TC, 22 SNP were analysed, that are associated with significant serum levels of TSH, in 27 758 Icelanders [31]. The SNP rs965513 was previously associated with TC, and the other 21 SNP were genotyped in 561 Icelandic patients with TC and 40013 controls. Variants associated with TC were genotyped in other 595 non-Icelandic patients and 2604 controls. Three variants were significantly associated: rs966423 in non-coding RNA-DIRC3,

rs2439302 membrane glycoprotein implicated in cell signaling NRG1, and rs116909374 in thyroid-specific transcription factor NKX2–1 [31].

The ThyroSeq microarray panel is broadly used and permits to test contemporaneously >1000 hotspots in 14 TC related genes and >40 fusions [50-52].

Three thousand and one patients and 287550 controls of different countries (Island, Germany, Spain and America) entered in a follow-up study, reporting 5 new loci: 1q42.2 [rs12129938 in pecanex-like 2 (PCNXL2)], 3q26.2 [rs6793295 a missense mutation in Leucin rich repeat containing 34 (LRCC34) near telomerase RNA component (TERC)], 5q22.1 [rs73227498 between "neuronal regeneration related protein" (NREP) and "erythrocyte membrane protein band 4.1 like 4A" (EPB41L4A)], 10q24.33 [rs7902587 near "oligosaccharide-binding folds containing 1" (OBFC1)], and two independently associated variants at 15q22.33 [rs2289261 and rs56062135; both in SMAD family member 3 (SMAD3)] [53].

Considering that the DNA repair genes of base (BER) or nucleotide (NER) excision repair pathways could be implicated in TC carcinogenesis, known SNP were genotyped in an Italian study in which 450 case-control paired DTC were enrolled. A raised risk of DTC was associated with the TT variant of BER gene NEIL3, that encodes for a DNA glycosylase [54].

A study was conducted in East Asian, or European, patients, showing common genetic loci [DIRC3, NRG1, FOXE1, PCNXL2, and MAP3K12 binding inhibitory protein 1 (MBIP)-NK2 homeobox 1 (NKX2-1)], whilst some of them were present only in one of the two populations [28]. Furthermore, the risk for common SNPs allele is different by race, and the DTC risk by genotype changes across ethnicities (i.e. the risk allele frequencies of variants on FOXE1 were 0.08 to 0.13 in Asians, and 0.14 to 0.34 in Europeans). These studies endorse ethnic differences in allele

frequencies, but also a little role of FOXE1 in DTC onset in East Asia individuals [28]. Moreover, recurrent variants on FOXE1 were related to an increased DTC risk (odds ratio of 1.35 in East Asian people, and 1.80 in Europeans) [55].

3. Molecular pathways involved in TC of follicular origin

Gene fusions are commonly present in TC and correlate with its histological subtype. Fusions of TK activating the MAPK pathway can be found in PTC. On the contrary, FTC, follicular adenomas, and follicular variant of PTC (FVPTC) frequently harbor PAX8/peroxisome proliferator-activated receptor (PPAR)γ rearrangements and THADA gene fusions [56].

Oncogenic fusions can be found in 6–46% of sporadic PTC patients [57-60]. The genes frequently rearranged in PTC are RET, BRAF, NTRK1, NTRK3, ALK, PPARG, and THADA [56].

The proto-oncogene RET (REarranged during Transfection) is located on 10q11.2, and it encodes for a transmembrane protein bearing intracellularly a TK. In human cancers, activating RET mutations and rearrangements are well-known [61]. Approximately 40% of adult sporadic PTC patients show RET/PTC rearrangements [62], that are observed also in microcarcinomas and benign thyroid lesions, suggesting that these rearrangements have a crucial role in the initial phase of carcinogenesis, but not in the subsequent natural history of the neoplasia [63-67]. The most frequent RET/PTC fusions are CCDC6-RET (RET/PTC1) and NCOA4-RET (RET/PTC3), comprising up to 90% of all RET/PTC rearrangements. RET/PTC and PAX8/PPARγ chromosomal rearrangements were evaluated in post-Chernobyl TCs [68-70].

PAX8/PPARγ rearrangements prevail in encapsulated FVPTC [71], and they are found in FTC (30-40%), in Hurthle cell tumors (5%) [72,73], and in follicular adenomas (2-10%) [74], whereas are present in only 0-1% of PTC [73].

ALK fusions are present in about 1–3% of PTC [75-77], and STRN-ALK is the most prevalent. The TCGA study, conducted in 400 patients with PTC, reported the following prevalence: ALK in 0.8%, NTRK1 in 0.8%, NTRK3 in 1.2%, BRAF in 2.7%, RET in 6.8% of tumors [57].

Gene fusions have been found in few cases of ATC and poorly differentiated TC (PDTC). In PDTC, rearrangements were found in 10–14% of patients and include NTRK3, RET/PTC, ALK, PAX8/PPARγ, and BRAF [78], with no correlation with the clinicopathologic features of TC.

In ATC, the reported fusions in about 3–5% of cases include RET/PTC3 and STRN-ALK [78]. Three ATC patients had RET/PTC rearrangements [79], probably for the presence of ATC and PTC in the same tissue.

RAF family proteins include the BRAF kinase [67]. These kinases are activated after RAS binding to the cell membrane; then they are responsible of MEK phosphorylation and activation, that in turn activate ERK and the effectors of the MAPK pathway [62]. About 45% of PTC (overall in classic papillary and tall cell variants) show a BRAF mutation [substitution from valine to glutamate at residue 600 (V600E)], and ~25% of ATC [67,80].

Mutations in the RAS gene (upstream of BRAF) are the second most frequent [3].

RAS gene family include N-RAS, H-RAS, and K-RAS; NRAS and HRAS genes are the most mutated in TC, and activate constitutively the MAPK and PI3K/AKT pathways [57]. About 40-50% of FTC and 15% of PTC (especially FVPTC) have RAS mutations (characterized by a more aggressive TC behaviour), and 50% of ATC

[81,82]. The detection of RAS mutations occurs in 20–40% of benign follicular adenomas, suggesting that RAS-positive adenomas could be precursor lesions for FTC or FVPTC [83].

Angiogenesis is an important process in tumor progression and an attractive target for therapy [84]. An increased angiogenesis correlates with TC aggressiveness [85], and TC clinical features are associated with the expression of PDGF/PDGFR, VEGF/VEGF receptor (VEGFR), hepatocyte growth factor (HGF)/c-Met, epidermal growth factor (EGF)/EGFR, and fibroblast growth factor (FGF)/FGFR [86]. Both VEGF and VEGFR-2 are implicated in tumor progression and invasiveness, resulting overexpressed in DTC [87].

The incidence of local and distant metastasis was also associated with an increased VEGF expression, in PTC [88].

The EGFR cell-surface protein (ErbB-1; HER1 in humans) belongs to the ErbB receptor family [89], constituted by 4 strictly related receptor TKs: EGFR (ErbB-1), HER2/c-neu (ErbB-2), Her 3 (ErbB-3) and Her 4 (ErbB-4). EGFR misregulations, amplifications, or mutations are involved in ~30% of epithelial cancers [89]. EGFR is related to TC progression and invasiveness [90], and it is overexpressed in ATC.

Neoplastic cells frequently show an impairment of histone deacetylase (HDAC) or histone acetyltransferase. A raised gene transcription rate is favored by an open chromatin configuration, whereas a closed configuration of the chromatin is linked to transcriptional repression [91]. Most ATC present an overexpression of HDACs, and a minor histone acetylation leads to an altered expression of the proteins that control cell cycle and growth [92]. Overall, an overexpression of HDAC is related to the dysregulation of PI3K-AKT and ERK1/2-MEK1/2 pathways [92].

Thioredoxin-interacting protein (TXNIP) is a tumor suppressor in TC cells, whose levels are higher in DTC with respect to ATC, due to the tumor suppressor's negative regulator PPAR-γ, and it is down-regulated in the transition from DTC to ATC [93]. ATC have a raised expression level of PPAR-γ, while it is lower in DTC [93].

In ATC, the mutation of the tumor suppressor gene p53 is common (from 70% to 88%), instead it is not frequent in FTC and PTC [94].

About 5–15% of ATC have the mutation of PTEN, a tumor suppressor. The methylated PTEN may lead to the transformation of benign thyroid adenoma into aggressive ATC [95]. Moreover, PTEN expression stimulates other genetic mutations involved in PI3K/Akt/mammalian target of rapamycin (mTOR) pathway, as RAS and PIK3CA [95].

PIK3CA encodes the p-110α catalytic subunit of phosphoinositide 3-kinase, able to activate the PI3K/Akt/mTOR signaling that modulates cell cycle, adhesion, and motility [95]. About 10–20% of ATC show PIK3CA mutations [96].

The oncogene AKT1 is overexpressed and is relevant in thyroidal carcinogenesis [97]. It is activated in approximately 5–10% of ATCs, and it is responsible of radiation therapy/standard chemotherapy resistance.

Telomerase reverse transcriptase (TERT) is a telomerase catalytic subunit, whose persistent activation is necessary to immortalize cancer cells [98]. TERT mutations are more common in ATC (42.6%) than in PTC (11.3%) [99]. TC harbouring TERT promoter mutations have worse prognosis and are more aggressive [100,101]. TERT mutations are the most frequent in Tall Cell Variant PTC.

4. Treatment strategies for TC

As stated above, surgery is considered the first line treatment in the vast majority of all TC patients, with the exception of ATC cases. During lobectomy, a lobe of the thyroid is removed, while thyroidectomy implicates the surgical removal of all or part of the gland [102]. After thyroidectomy, the subsequent treatment plan can be RAI remnant ablation with 131I, which is absorbed only by thyrocyte. RAI is administered by fluid or tablet and it is taken up and concentrated by thyroid cells, where it causes acute death of both normal and malignant thyroid cell, with no damage to the rest of the body. As consequence, the patient needs exogenous thyroid hormone supplementation, and levothyroxine needs to be administered [102].

Most patients with well-differentiated TC have resectable tumors, and the results from surgery, radioiodine, and thyroxine therapy are excellent. EBRT is used infrequently in the management of DTC, and it is predominantly indicated for palliation of locally advanced unresectable or recurrent/metastatic disease in patients whose tumors do not concentrate radioiodine, and in ATC [102].

Chemotherapy is a systemic therapy, during which cytotoxic agents are given by mouth or by injection to kill cancer cells, and its application have historically been limited for most types of TCs. To date, chemotherapy is occasionally used with EBRT therapy for ATC or occasionally it is used in patients with progressive symptomatic TC that is unresponsive to other treatment strategies [102].

These treatment strategies are not completely effective against aggressive DTC or ATC, and mortality is one of the most important challenges. For this reason, recently new medications have been developed, targeting the critical molecules responsible for tumor formation (known as “target therapy”) [103]. These novel approaches based on targeted chemotherapies are emerging as effective alternatives for progressive disease

and, conversely to conventional cytotoxic agents, they do not kill normal fast developing cells, as those of bone marrow, gut and skin.

Novel targeted therapies for progressive, aggressive refractory cancers have been developed, as TKIs [16,24,26]. TKIs are compounds with low molecular weights able to modulate TK-dependent oncogenic pathways, and to compete with the ATP-binding site of their catalytic domain. The occupation of the ATP-binding site reduces autophosphorylation and TK activation, inhibiting the following activation of intracellular signaling pathways. TKIs can be specific to one or several TKs; in fact, a single TKI may target multiple TKs [16,24,26].

TKIs are currently used as therapies of aggressive DTC, and ATC, thanks to their capability of inducing clinical responses and stabilization of disease. TKIs improve median progression-free survival (PFS); however major side effects are common. Preclinical studies, as well as clinical trials, evaluated TKIs role, and their interaction with the most important signaling cascades implicated in the pathogenesis of TC of follicular origin. New attempts are made for the personalization of the therapy in each TC patient, by identifying novel, more effective and safe agents.

Considering aggressive TC of follicular origin, the approved TKIs are: lenvatinib (Lenvima®) and sorafenib (Nexavar®) for treating radioiodine-refractory DTC [16,104]; the combination of dabrafenib (Tafinlar®) plus trametinib (Mekinist®) for ATC with $BRAF^{V600E}$ mutation [105].

In the last decades, thyroid cell lines, derived from tumoral cells and selected for strong proliferation *in vitro*, have been considered preclinical models for research purposes [106]. Actually**,** immortalized or continuous cell lines can proliferate indefinitely. Cell lines are used for convenience because they are easy to handle, but they may have lost some of their thyroid specific characteristics compared to the

beginning. The cells adapt to the *in vitro* growth conditions, and in some cases they are not able to maintain important features that are determinant in normal thyroid physiology and signaling pathways. Furthermore, genetic analysis, conducted by short tandem repeats and SNP, has demonstrated that several thyroid cell lines are misidentified or cross-contaminated with other cells [107], and they do not mimic closely the *in vivo* environment. For these reasons, the use of these cell lines for research on targeted therapies in thyroid cells has important limitations [108]. In the last years, thyroid primary cells have been studied as monolayer cultures, and their biological behavior have been investigated [106].

Primary human cells have some advantages than continuous cell lines in the study of the antineoplastic action of different drugs, in fact:

a) primary cells are more differentiated than continuous cell lines;

b) have phenotypic features that are quite similar to those of the primary tumor;

c) can be used for chemosensitivity test in the single patient.

Even though primary cell cultures usually have a limited lifespan, they permit researchers to investigate, not only cells, but also donors, as various factors (i.e. medical history, age, sex, race, etc.) can be considered during the evaluation of the experimental model, while continuous cell lines do not reproduce the true variability of a living tissue.

5. *In vitro* studies

5.1 VEGF Pathway (Table 1)

5.1.1 Sunitinib

Sunitinib is an oral multi-target TKI, that received the approval to treat imatinib-resistant gastrointestinal stromal tumor, renal carcinoma, and pancreatic

neuroendocrine tumors. It is able to simultaneously inhibits several pathways, [i.e. PDGF-Rs, c-KIT, VEGFRs, RET and fms-related tyrosine kinase 3 (FLT3)], therefore reducing tumor vascularization and inducing cancer cell apoptosis, and tumor shrinkage [24].

An *in vitro* study evaluated the efficacy of the kinase inhibitors SU11248, SU5416, and SU6668 in inhibition of RET/PTC [109]. SU11248 suppressed phosphorylation of the synthetic TK substrate peptide E4Y by RET/PTC3 dose-dependently. SU11248 induced a morphological reversion of transformed NIH-RET/PTC3 cells and stopped the proliferation of TPC-1 cells (a human PTC cell line) with an endogenous RET/PTC1. The reported data suggested sunitinib could be effective in DTC [109].

Another study investigated the action of sunitinib on gene expression of iodide-metabolizing proteins and on signal transduction pathways in PTC harbouring the RET/PTC1 rearrangement. In RET/PTC1 cell lines, sunitinib targeted MEK/ERK and SAPK/JNK pathways, and blocking these pathways it could inhibit cell proliferation and stimulate the sodium/iodide symporter (NIS) gene expression [110].

Using B-cPAP, SNU-790, and TPC-1M cell lines, a further study evaluated the effect of sunitinib on PTC cells with BRAF mutation and RET/PTC rearrangement. Sunitinib inhibited significantly cells bearing RET/PTC rearrangement, and not those BRAF mutated; in fact, low doses of this drug were sufficient to inhibit significantly the cell growth of PTC cells having a RET/PTC rearrangement, whereas higher doses were necessary for BRAF mutated cells [111].

Moreover, the effectiveness of sunitinib was investigated in TPC-1 cells and human ATC cell lines obtained (C643, CAL-62, and 8505C). Sunitinib (at nanomolar concentrations) reduced significantly the viability only of TPC-1 cells, and it reduced cyclin D1 levels, and the phosphorylation of ERK and Akt. On the contrary,

micromolar concentrations of sunitinib reduced in a significant manner the growth of the three ATC cell lines, through a necrotic, rather than apoptotic, death. The data suggested that sunitinib is not effective in the treatment of ATC, because of its absent or low effect on ATC growth or differentiation [112].

Another study evaluated the responsiveness to sunitinib of different TC cell lines bearing wild-type KRAS and BRAF genes, the G12R KRAS, the RET/PTC1 rearrangement, or the $BRAF^{V600E}$ mutation. Constitutive RAS/RAF/ERK pathway activation in TC cells appeared to favor the resistance to sunitinib [113].

Moreover, the effect of sunitinib was studied *in vitro* and *in vivo* in H-ras- or BRAF-mutated ATC cells (FB3 and 8305C, respectively) and microvascular endothelial cells, and the molecular mechanism underlying its effect. Cells were incubated with sunitinib for 72 hours, and its antiproliferative and proapoptotic effect was shown in both types of cells. *In vivo*, sunitinib inhibited significantly tumor growth. The data suggested that sunitinib is effective *in vivo* and *in vitro* in endothelial and ATC cells, by inhibiting the phosphorylation of Akt and ERK1/2, and by down-regulating cyclin-D1 [114].

Recently the effect of irinotecan plus sunitinib was investigated *in vitro* on ATC cell growth, as well as their antitumor effect *in vivo* [115]. SN-38, the active metabolite of irinotecan, and sunitinib synergistically act on ATC cells, causing an increase of the intracellular concentrations of SN-38, and a decrease of the gene expression and of the protein levels of VEGF, colony stimulating factor 1 and ATP-binding cassette transporter G2. In ATC xenografts *in vivo*, these drugs together had a more potent antitumor effect than monotherapy. It was concluded that this combination had a synergistic, significant ATC antitumor activity, *in vitro* and *in vivo* [115].

5.1.2 Sorafenib

Sorafenib (BAY 43-9006) is a multikinase inhibitor (mKI), which acts against VEGFR-2, VEGFR-3, PDGFR, RAF, c-KIT and RET kinases [104,116]. In preclinical studies in various cancer xenograft models (i.e. colon, breast and non-small-cell lung cancer), sorafenib has revealed a large spectrum antitumor activity [116].

Another study examined sorafenib activity against oncogenic RET *in vitro*. Sorafenib inhibited the growth of TPC-1 and TT (from human MTC) cell lines, bearing respectively the RET/PTC1 and C634W RET mutation, suggesting that it could inhibit TC growth by anti-proliferative and anti-angiogenic mechanisms [117].

Considering the promising data from *in vitro* (and *in vivo* trials) and the need of novel treatment options for RAI recurrent metastatic TC, additional researches are needed.

More recently, a study compared sorafenib and forskolin, an adenylyl cyclase activator, in *in vitro* models of 3 human DTC cell lines (TPC-1, KTC1, and WRO) [118].

Autophagy can be induced during different cancer therapies, but its role in sorafenib treatment of TC has not been completely clarified. Chloroquine (CQ) is an autophagy inhibitor that increases the sensitivity to different cancer treatments. TC xenograft model mice [obtained by the injection in nude BALB/c mice of the human TC cell lines 8505C and FTC133 (a human FTC cell line)] were treated with sorafenib, CQ, or their combination. CQ or sorafenib suppressed tumor growth, whereas mice treated with their combination had a significantly higher decreased tumor growth than those receiving sorafenib or CQ alone. It was shown in TC that sorafenib inhibited the MAPK and AKT/mTOR pathways, and autophagy was activated by sorafenib, both *in vitro* and *in vivo* (that is due in part to the suppression of the AKT/mTOR pathway).

The combined treatment with CQ could inhibit the sorafenib-induced autophagic flux [119].

5.1.3 Pyrazolo[3,4-d]pyrimidine (PP) derivatives

The pyrazolo[3,4-d]pyrimidine (PP) heterocyclic core is one of the most investigated chemical template in TC as a useful scaffold to obtain effective TKIs, and derivatives of this structural class have a broad spectrum of activity [120].

The PP derivatives, CLM3 and CLM29, inhibit VEGFR, EGFR, RET TK, and BRAF, and have antiangiogenic activity [121]. CLM3 and CLM29 inhibited significantly the proliferation of primary dedifferentiated PTC (DePTC) cells, and the migration, increasing also the apoptosis. In the human TC cell line AL, obtained from primary DePTC cells, with $BRAF^{V600E}$ mutation, CLM3 and CLM29 enhanced thrombospondin-1 expression. CLM3 was used to treat xenografts, obtained upon the injection of the AL cell line in CD nu/nu mice, and it reduced tumor growth and weight, with no toxicity [121].

The antiproliferative and pro-apoptotic action of CLM3 was reported also in 8305C cells, derived from ATC, and TT cells (synergistically enhanced by SN-38), inhibiting the phosphorylation of VEGFR-2, EGFR and RET TKs and the related signaling pathways [122].

CLM3 [123], and CLM94 [124] (a cyclic amide) exert also an antineoplastic activity in primary ATC cells. CLM3 inhibited in a significant manner the proliferation of 8305C and AF cells (obtained from primary ATC cells), as well as the proliferation of primary human ATC cells, inducing the apoptosis, and inhibiting migration and invasion. CLM3 inhibited the tumor growth also in xenografts, obtained upon the injection of the AF cells in CD nu/nu mice, reducing VEGF-A expression and

microvessel density [123]. CLM94 inhibited the proliferation of continuous 8305C and primary human ATC cells [124]. It increased the number of apoptotic ATC cells, and suppressed migration and invasion. CLM94 reduced significantly VEGF-A gene expression in AF cells and the VEGF-A protein and microvessel density in AF tumor tissues, obtained in CD nu/nu mice, once injected with AF [124].

The antitumor activity of CLM29, and CLM24 (another pyrazolopyrimidine derivative) has been demonstrated *in vitro* in ATC, independently from the presence of the $BRAF^{V600E}$ mutation [125]. CLM29, and CLM24 significantly inhibited the proliferation of 8305C cells; in addition, they reduced the proliferation of primary human ATC cells, and stimulated the apoptosis. CLM29 inhibited migration and invasion of primary ATC cells, but not CLM24 [125].

Other PPs, such as PP1 [126], PP2 [127], were evaluated in TC. PP1 inhibited RET TK [126], and PP2 lowered RET/PTC1-mediated MAPK signaling and inhibited growth and invasion of human TC cells with RET/PTC1 rearrangements [127]. PP2 suppressed c-Src and related kinases, thus it was not selective for RET [128].

5.1.4 Vandetanib

The mKI Vandetanib has receveid the approval from Food and Drug Administration (FDA) and European Medicines Agency (EMA) to treat aggressive MTC. Its targets are RET TK, EGFR, and VEGFR-2 and -3, and it has anti-angiogenic activity [129].

A study compared the effect of 4 TKIs (vandetanib, axitinib, XL184, and sunitinib) on cell proliferation, RET expression and autophosphorylation, and ERK activation in different cell lines expressing a MEN2A (TT cells), a MEN2B (MZ-CRC-1, a MTC cell line with a M918T RET mutation), and a RET/PTC rearrangement (TPC-1 cells) [130]. XL184 and vandetanib inhibited more strongly cell proliferation, RET

autophosphorylation and reduced RET expression, and ERK phosphorylation in TT and MZ-CRC-1 cells, respectively. XL184 reduced RET autophosphorylation in TPC-1 cells, but no effects were observed on ERK activation. These data indicated that mutation specific therapies are more effective in treating MTC and PTC [130].

A study reported the case of a 42-year old male patient who had undergone total thyroidectomy for ATC, and central and lateral neck dissection [131]. A primary cell culture was obtained from the tumor tissue. The patient received a combined radio-chemotherapy, and cell viability tests were carried out treating cells with vandetanib, sorafenib, and the aurora kinase inhibitor MLN8054. The 3 compounds inhibited cell viability time- and dose-dependently, and sorafenib had the higher effect. Then, he received a daily treatment with 400mg of sorafenib for 75days, and no evidence of the disease was noticed after 43 months from the diagnosis. The reported data demonstrate that *in vitro* drug evaluation of individual tumoral cells in each patient could permit to improve the fatal prognosis of ATC patients [131].

Furthermore, vandetanib had an anti-tumor and anti-angiogenic effect in primary human ATC cells, by inducing apoptosis and reducing significantly cell growth dose-dependently, and by inhibiting invasion and migration [7]. It reduced cyclin D1 and blocked the phosphorylation of ERK1/2, AKT and EGFR. Vandetanib reduced significantly the proliferation of the continuous cell lines 8305C and AF, and it induced apoptosis. Xenografts, obtained after the subcutaneous injection of the 8305C cells in CD nu/nu mice, received vandetanib (25 mg/kg/day), that showed its efficacy in inhibiting in a significant manner the tumor growth, the VEGF-A expression and microvessel density [7].

5.1.5 Lenvatinib

The oral mKI lenvatinib acts against FGFR-1, -2, -3, -4, VEGFR-1, -2, -3, PDGFRb, RET and c-KIT, approved by FDA and EMA to treat advanced radioiodine–refractory DTC [16,82].

A study explored lenvatinib in RET gene fusion-driven preclinical tumor models. Lenvatinib reduced auto-phosphorylation of NcoA4-RET, CCDC6-RET, and KIF5B-RET, inhibited the growth of CCDC6-RET human TC and lung cancer cell lines, and suppressed tumorigenicity of RET gene fusion-transformed NIH3T3 cells, suggesting its antineoplastic activity against RET gene fusion-driven tumor models [132].

Another study investigated the antitumor activity of lenvatinib in human TC xenograft models in nude mice [133]. Lenvatinib was administered orally in xenograft models of 5 DTC, 5 ATC, and 1 MTC, and it had an antitumor activity. Moreover, lenvatinib showed an antiangiogenetic effect in 5 DTC and 5 ATC xenografts, while *in vitro* the antiproliferative action has been described only in 2/11 TC cell lines [i.e. RO82-W-1 (a human FTC cell line) and TT cells]. Lenvatinib inhibited also the RET phosphorylation in TT cells bearing the activated mutation C634W [133].

More recently, a study focused on the effects of HDAC, lenvatinib and sorafenib, both alone and in combination [134]. Cells were obtained from patients with ATC and PTC, and these cells were treated with lenvatinib or sorafenib, individually or combined with HDAC. The treatment with both drugs induced a significant tumor shrinkage, especially combining HDAC and lenvatinib, the FGFR signaling pathway, important for epithelial-mesenchymal transition (EMT), was blocked. The combination with HDAC and lenvatinib was more effective than the one with sorafenib and HDAC or agent alone. These data help to overcome drug resistance in cancer stem cells, that could be responsible of poor outcome of these anticancer drugs [134].

Another paper assessed *in vitro* and *in vivo* the antitumor effectiveness of the combined treatment with lenvatinib and the microtubule inhibitor paclitaxel in ATC [135]. Lenvatinib alone had less impact than paclitaxel in ATC cell lines C643, 8305C and 8505C and in xenografts of female athymic nude mice. In nude mice, the combination of lenvatinib and paclitaxel inhibited synergistically colony formation and tumor growth, and induced G2/M phase cell cycle arrest and apoptosis with respect to lenvatinib or paclitaxel alone. The reported data suggested that the combination of these 2 drugs could be an encouraging new therapy for ATC [135].

Lenvatinib has been evaluated in primary human ATC cells, in the human cell line 8305C and in AF cells [8]. Lenvatinib reduced in a significant manner ATC cell proliferation, and increased the percentage of apoptotic ATC cells. Moreover, lenvatinib inhibited migration and invasion in ATC, and it inhibited EGFR, AKT and ERK1/2 phosphorylation, and downregulated cyclin D1 in the ATC cells. In the continuous cell lines, it inhibited the proliferation, increasing apoptosis. Once inoculated AF cells in CD nu/nu mice, tumor masses were observed. Tumor growth was significantly inhibited by lenvatinib, such as the microvessel density and the expression of VEGF-A [8].

5.1.6 Apatinib

The novel TKI apatinib inhibits multiple tumor related kinases, such as VEGFR-2, RET, PDGFR-β, v-Src sarcoma viral oncogene homolog (c-Src), and stem cell factor receptor (c-Kit) [136,137]. Apatinib compete for the ATP binding site of VEGFR-2, thus it blocks downstream signaling pathways inhibiting tumor angiogenesis [138].

A study evaluated the role of apatinib on tumor progression and angiogenesis in continuous ATC cells *in vitro*, and *in vivo* [137]. Apatinib suppressed the growth of

ATC cells (especially CAL-62 and BHT-101) dose- and time-dependently through the induction of apoptosis and blocking cell cycle at G0/G1 phase. Furthermore, apatinib inhibited angiogenesis of ATC cells *in vitro* and *in vivo* (in 4-week old male BALB/c nude mice xenografts) through the suppression of Akt/GSK3β/ANG signaling pathway, suggesting its potential therapeutic effectiveness in the treatment of ATC [137].

Apatinib is able to induce autophagy and apoptosis in human ATC cells, downregulating p-AKT and p-mTOR signals through the AKT/mTOR pathway. Furthermore, combining apatinib and CQ, an additional tumor suppression was shown *in vitro* (in the human ATC cell lines KHM-5M and C643) and *in vivo* (in 4-week old male BALB/c nude mice xenografts) [139].

5.2 EGFR Pathway (Table 1)

5.2.1 Docetaxel

Docetaxel is a microtubule stabilizer, and a common chemotherapeutic agent for the treatment of different metastatic cancers.

Flavonoid baicalein or docetaxel were added on the continuous ATC cell line 8505c, alone or in combination [140]. Baicalein improves the anticancer effect of docetaxel in ATC, in fact proliferation was reduced substantially and apoptosis rate was increased more than with monotherapy [140].

5.3 Targeting BRAF (Table 1)

5.3.1 Vemurafenib

FDA approved vemurafenib to treat patients affected by metastatic melanomas with $BRAF^{V600E}$ mutation, because it is able to inhibit the enzymatic activity of BRAF, and block the MAPK pathway.

Since single targeted therapy with vemurafenib will flow into drug resistance and disease progression in the long term, association with $BRAF^{V600E}$ inhibitors could be considered for $BRAF^{V600E}$-positive TC [141]. A study assessed the combined treatment with vemurafenib plus the mTOR inhibitors, metformin and rapamycin, in BCPAP (a PTC cell line) and 8505c cells [141]. Cell viability was reduced and apoptosis was increased in both cell lines (also in vemurafenib-resistant BCPAP cells). Variations in expression of signaling molecules (i.e. reduced mTOR expression in BCPAP and enhanced inhibition of phospho-MAPK in resistant BCPAP and 8505c) were reported. Vemurafenib plus rapamycin increased cell death in BCPAP cells. The Authors concluded that combination of $BRAF^{V600E}$ and mTOR inhibition might provide clinical benefits to $BRAF^{V600E}$-positive advanced TC patients, and it should be investigated in *in vivo* models [141].

The reason of the resistance of BRAF-mutant TC to vemurafenib is still unclear. A study investigated whether autophagy is activated in vemurafenib-treated BRAF-mutant TC cells, and if the treatment effectiveness of vemurafenib can be improved by the inhibition of autophagy [142]. Vemurafenib induced an elevated level of autophagy in BRAF-mutant TC cells [BCPAP, 8505c, and FRO (an ATC cell line)]. Impairment of autophagy, by either hydroxychloroquine (HCQ) or interfering RNA knockdown of essential autophagy genes, increased the cell death induced by vemurafenib. Moreover, administration of vemurafenib with the autophagy inhibitor HCQ facilitated higher tumor suppression *in vivo* (in 5-7-week old male SCID mice

injected with BCPAP cells). The findings indicated that autophagy inhibition could be an effective approach to sensitize BRAF-mutant TC to vemurafenib [142].

Another study evaluated the outcomes in $BRAF^{V600E}$-bearing TC cells of vemurafenib, alone or combined with bortezomib (a proteasome inhibitor) [143]. Combining these drugs, synergistic effects were shown on cell growth inhibition, an enhanced G2-phase cell cycle arrest and apoptosis. In 6-week old inbred homozygous athymic BALB/C nude (nu/nu) male mice, a reduction of tumor size and of the expression of the markers of cell growth and proliferation was observed after the combined therapy with vemurafenib and bortezomib, with respect to monotherapy (i.e. Ki-67 and cyclin D1).

Moreover, bortezomib increased vemurafenib effect on TC cells through mitochondrial dysregulation and apoptosis, supporting their use in association for the treatment of this type of cancer [143].

S100A4, belonging to the S100 family of Ca2+-binding proteins, is markedly expressed in different metastatic tumor cells, and it regulates certain cell functions (i.e. proliferation, invasion and migration, and cell-cell communication) [144]. Most advanced TC and lymph node metastases overexpress S100A4, and this is associated with poor prognosis. For these reasons, targeting S100A4 could aid in the reduction of local invasion and metastasis in TC.

A study evaluated whether the combination of targeting S100A4 and BRAF increases the anti-tumor effect in ATC. S100A4 knockout stimulated apoptosis and decreased proliferation through the inhibition of pAKT and pERK signaling; moreover, it inhibited invasion and migration, inactivating *in vitro* pAKT and RhoA/ROCK1/2 in 8505C or Cal-62 cells (conversely, in SW1736 and KAT18 cells). Vemurafenib decreased proliferation of both 8505C and SW1736 cells, but did not have any effect

on apoptosis, and it favoured migration and invasion *in vitro*. Vemurafenib plus S100A4 knockdown decreased cell proliferation, migration and invasion *in vitro* vs. vemurafenib or the S100A4 knockdown, alone. The treatments used in combination caused a complete inhibition of the ERK1/2 and AKT activation in 48 h, compared to the treatment with vemurafenib alone that provoked only a transient inhibition of pERK and AKT.

In an *in vivo* model of 6-week female nude mice injected with SW1736 and 8505C, single vemurafenib therapy did not substantially suppress cancer growth, but inhibited tumoral growth in combined groups. These findings suggested that S100A4 knockout increases the effect of vemurafenib on tumor regression *in vitro* and *in vivo*, and that S100A4 knockout blocks permanently ERK1/2 and AKT activation after the treatment with vemurafenib, reversing its resistance [144].

5.3.2 Dabrafenib (and trametinib) (Table 1)

Dabrafenib is a new treatment for cancers bearing BRAF, even if BRAF-positive tumors become resistant to it in about 6-7 months. In order to circumvent this issue, dabrafenib was administered with the MEK inhibitor trametinib [145]. The combination of a dabrafenib plus trametinib therapy was approved by FDA for BRAF V600E/K-mutant metastatic melanoma in 2014 [146].

A study evaluated the effect of the inhibition of the activated RAS/RAF/MEK pathway in the human ATC cell lines OCUT-4, OCUT-6 ACT-1, and OCUT-2 [147]. Dabrafenib suppressed the viability in BRAF mutated cells, by G0/G1-arrest, and downregulated MEK/ERK phosphorylation. After the treatment with dabrafenib, upregulated phosphorylation of MEK was shown in RAS mutated cells with consequent VEGF overexpression. Trametinib, downregulating ERK phosphorylation,

inhibited cell viability. The data indicated that the dual blockade by both inhibitors had cytostatic effects in the 4 ATC continuous cells [147].

BRAF or RAS genes mutations aberrantly activate the MAPK pathway in PTC, leading to malignant transformation, dedifferentiation, and to a decrease in the expression of NIS, that causes resistance to RAI therapy [148]. Twenty-four human primary PTC cultures were established, and $BRAF^{V600E}$ mutation was identified in 18/24, one had a HRAS mutation, and the other 5 were BRAF and RAS wildtype. An increased NIS expression was obtained after treatment with dabrafenib and trametinib (MEK inhibitor), while no effect was observed with dabrafenib alone. The reported findings suggested that the combined treatment with BRAF and MEK inhibitors lead to an upregulation of the NIS expression, therefore increasing tumor iodine uptake (this effect was higher in younger patients) [148].

5.4 Targeting TERT (Table 2)

Recently, the targeting of telomerase complex has been shown effective in preclinical studies, in fact TERT gene promoter (TERTp) mutations (C228T and C250T) had high frequency in TC cell lines and tumor biopsies [149].

A drug library constituted by 51 epigenetic drugs (23 HDAC inhibitors; 14 Aurora kinase inhibitors; 5 sirtuin modifiers; 3 hypoxia-inducible factor inhibitors; 2 DNA methyltransferase inhibitors; 2 histone methyltransferase inhibitors, a histone demethylase inhibitor, and a bromodomain inhibitor) was investigated in 3 TC cell lines [WRO (wild-type TERTp), TPC-1, and KTC2]. PF-03814735, a small molecule inhibitor of Aurora kinase A, was the most potent on KTC2 cells, while CUDC-101, a multitarget inhibitor, was effective on KTC2 and WRO cells. In particular, PF-

03814735 was the most effective epigenetic drug on cell lines with the C228T mutation [149].

5.5 Targeting PPARγ (Table 2)

PPARγ are nuclear hormone receptors which trigger antineoplastic effects in tumoral cells [150]. PPARγ agonists: a) have antiproliferative effect in PTC cells, and induce apoptosis [151]; b) prevent distant metastasis of BHP18–21 tumors in nude mice [151]; c) induce redifferentiation of DeTC cells [152]. Human ATC cells overexpress PPARγ, and it inhibits proliferation and invasion, inducing also apoptosis [153-155].

Rosiglitazone (a PPARγ ligand) induced redifferentiation in ATC cells [9].

Human primary ATC cells were established and treated with increasing concentrations of pioglitazone or rosiglitazone or antiblastics (cisplatin, bleomycin, gemcitabine) [9]. Rosiglitazone and pioglitazone reduced the proliferation of primary cells, such as bleomycin, cisplatin and gemcitabine; the inhibition of proliferation was similar in tumors with/without $BRAF^{V600E}$ mutation, both for thiazolidinediones and antiblastics [9].

Other two studies evaluated the effects of chemotherapeutic agents and/or thiazolidinediones in human primary cultures of ATC, obtained from surgical biopsies and from FNA [10,11], with no significant different outcomes of the chemosensitivity tests between these two types of samples [10,11].

5.6 Targeting HDAC (Table 2)

Vorinostat is an oral HDAC inhibitor, approved by the USA FDA for the treatment of cutaneous T-cell lymphoma [156], that can arrest TC cell growth and induce apoptosis *in vitro* [157].

A study investigated the effect of the HDAC inhibitor PXD101, alone and combined with conventional chemotherapy (doxorubicin, paclitaxel and docetaxel), in 8 TC cell lines: 8305C, 8505C, KAT18, KAT4C (anaplastic); BHP7-13 (papillary); WRO82-1 (follicular); FRO81-2 (follicular undifferentiated); TT (medullary). PXD101 inhibited TC proliferation, and acted synergistically with doxorubicin and paclitaxel for the treatment of ATC, supporting further clinical trials using PXD101 in these patients [94].

Another paper investigated the first-in-class dual inhibitor of EGFR, HER2 and HDAC, CUDC-101, in ATC. The CUDC-101 anticancer effect was related to raised expression of p21 and E-cadherin, and decreased expression of survivin, XIAP, β-catenin, N-cadherin, and Vimentin. In an *in vivo* mouse model of metastatic ATC, CUDC-101 inhibited tumor progression and metastases, and significantly prolonged survival, providing a preclinical basis to investigate CUDC-101 therapy in this type of cancer [158].

Moreover, in a recent study $BRAF^{V600E}$-mutant (BCPAP and K1) and BRAF-wild-type (BHP 2-7) cells were cultured in presence of the HDAC inhibitor panobinostat and a MAPK inhibitor (dabrafenib or selumetinib), alone or combined, to test the expression of iodine- and glucose-metabolizing genes, RAI uptake and efflux, and toxicity [159]. Panobinostat alone enhanced iodine-metabolizing gene expression, stimulated RAI uptake and toxicity, and blocked GLUT1 expression in all cells, while dabrafenib or selumetinib had these effects only in $BRAF^{V600E}$-mutant cells. Combining panobinostat and dabrafenib or selumetinib, a stronger $BRAF^{V600E}$-dependent redifferentiation was achieved (vs. panobinostat alone). These data showed that MAPK inhibitors increase HDAC inhibitor-induced redifferentiation in PTC cells bearing the $BRAF^{V600E}$ mutation [159].

6. Personalized targeted therapies

Personalized medicine and precise oncology, which consider both patients and their disease features, represent the future of the treatment approach, but their wide clinical application needs further progress [160,161].

The *in vitro* screening of targeted drugs by using human primary tumoral cells from each patient [162] can hint an *in vivo* non-responsivity (with a 90% negative predictive value), and a 60% positive predictive value of clinical response [162,163]. By this methodology, it is possible not to administer ineffective and even potentially harmful drugs [164].

In the past, primary TC cells could be collected only through surgical biopsies. Recently, however, it has been demonstrated that FNAC from aggressive dedifferentiated TC or ATC can also represent a reliable source of primary cells on which various therapies can be tested *in vitro*, in order to avoid ineffective drugs administration [9-11].

In recent times, the anti-cancer properties of lenvatinib and vandetanib have been described also in human primary cells, obtained from biopsy or FNAC, from 6 ATC patients [165]. In these types of primary cells (with no significant difference in sensitivity to lenvatinib and vandetanib), both drugs reduced significantly the proliferation, vs. control, and increased dose-dependently the percentage of apoptosis. It could be hypothesized that, since FNA collects material from a restricted area of the tumor, it could be the expression of a selected cellular population, not representative of the whole tumor. The results with primary cells, obtained from biopsies in the same conditions, were rather similar to those from FNAC, excluding the above reported hypothesis.

Testing *in vitro* the sensitivity to different TKIs in each patient could open the way to personalized treatments**,** and the use of primary cells from FNAC could avoid worthless surgical procedures [165].

7. Conclusion

Thyroid cancer (TC) is one of the most common endocrine tumor [1,2]. More than 90% of all TC is represented by DTC [4] (FTC, PTC, and Hürthle cells TC) [5]. ATC accounts for 1% of TC [7-11].

The gold standard therapy for DTC is surgery (lobectomy or total thyroidectomy), while aggressive PTC and FTC need subsequent RAI remnant ablation with 131I [15,16]. About 2/3 of these patients will become RAI-refractory and they will never be cured with RAI therapy, with a 3-year OS rate lower than 50 % [16].

Because of the ATC poor prognosis, multiple therapeutic strategies are usually attempted including debulking, EBRT, and chemotherapy (cisplatin or doxorubicin), with the achievement of a 10-month median survival [19-22].

Most patients with well-differentiated TC have resectable tumors, and the results from surgery, radioiodine, and thyroxine therapy are excellent. EBRT is indicated for palliation of locally advanced unresectable or recurrent/metastatic disease in patients whose tumors do not concentrate radioiodine, and in ATC [102]. To date, chemotherapy is used with EBRT therapy for ATC or it is used in patients with progressive symptomatic TC that is unresponsive to other treatment strategies [102].

These treatment strategies are not completely effective against aggressive DTC or ATC, and mortality is one of the most important challenges.

In recent years, progresses have been made in the understanding of the molecular basis of TC progression, and new drugs have been introduced, able to inhibit

oncogenic kinases (BRAF^{V600E}, RET/PTC) or signaling kinases as VEGFR and PDGFR associated with cellular growth and proliferation [16,24-26].

TKIs are compounds with low molecular weights able to modulate TK-dependent oncogenic pathways, competing with the ATP-binding site of their catalytic domain. These drugs prolong median PFS; however major side effects are common.

In the last decades, thyroid cell lines from neoplastic cells have been used as preclinical models, thanks to their convenience in long-term culture [106]. Actually, these cells adapt to the *in vitro* growth environment, and they are not able to maintain certain features that are determinant in normal thyroid function and signaling pathways. Furthermore, genetic analysis has demonstrated that several thyroid cell lines are misidentified or cross-contaminated with other cells [107]. In the last years, human primary cell cultures have been established as monolayer cultures, and investigated for their biological behavior [106].

The *in vitro* screening of targeted drugs by using human primary tumoral cells from each patient [162] can hint an *in vivo* non-responsivity (with a 90% negative predictive value), and a 60% positive predictive value of clinical response [162,163], avoiding to administer ineffective and even potentially harmful drugs [164].

Moreover, in the past, primary TC cells could be collected only through surgical biopsies, while recently it has been demonstrated that FNAC from aggressive dedifferentiated TC or ATC can also represent a reliable source of primary cells [9-11]. The chance to test in each patient the sensitivity to multiple TKIs *in vitro* could continue the road to the new era of personalized treatments [165].

In conclusion, personalized medicine and precise oncology, which consider both patients and their disease features, represent the future of the treatment approach, and further progress is needed in this direction.

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Table 1. ***In vitro*** **studies on drugs targeting BRAF, VEGFR and EGFR pathways.**

	VEFG Pathway	EGFR Pathway	BRAF
Sunitinib	-It was effective in DTC, showed by evaluating the kinase inhibitors SU11248, SU5416, and SU6668 in inhibition of RET/PTC [109] -It targeted MEK/ERK and SAPK/JNK pathways in RET/PTC1 cell lines, inhibiting cell proliferation and stimulating NIS gene expression [110] -It inhibited significantly cells bearing RET/PTC rearrangement, and not those BRAF mutated [111] -It reduced significantly the viability of TPC-1 cells at nM concentrations, reduced cyclin D1 levels, and the phosphorylation of ERK and Akt. At μM concentrations it reduced significantly the growth of 3 ATC cell lines [112] -Constitutive RAS/RAF/ERK pathway activation in TC cells appeared to favor the resistance to sunitinib [113] -It is effective in vivo and in vitro in endothelial and ATC cells, by inhibiting the phosphorylation of Akt and ERK1/2, and by the down-regulation of cyclin-D1 [114] -Combined with irinotecan, it had a significant, synergistic ATC antitumor activity, in vitro and in vivo [115]		
Sorafenib	-It inhibited the growth of TPC-1 and TT cell lines, bearing the RET/PTC1 and C634W RET mutation, respectively [117] -The combined treatment with CQ could inhibit the autophagic flux induced by sorafenib [119]		
Pyrazolo [3,4-d] pyrimidine (PP) derivatives	-CLM3 and CLM29 inhibited significantly the proliferation of primary DePTC cells, and the migration, increasing also apoptosis [121] -The antiproliferative and pro-apoptotic action of CLM3 was reported in 8305C cells, derived from ATC, and TT cells (synergistically enhanced by SN-38) [122] -CLM3 and CLM94 exert an antineoplastic activity in primary ATC cells [123,124] - CLM29 and CLM24 significantly inhibited the proliferation of 8305C cells, reduced the proliferation of primary human ATC cells, and stimulated apoptosis. CLM29 inhibited migration and invasion of primary ATC cells, but not CLM24 [125] -PP1 inhibited RET TK [126], and PP2 lowered RET/PTC1-mediated MAPK signaling and inhibited the invasive phenotype and the growth of human TC cells with RET/PTC1 rearrangements [127]. -PP2 suppressed c-Src and related kinases, thus it was not selective for RET [128]		
Vandetanib	-XL184 and vandetanib inhibited more strongly cell proliferation, RET autophosphorylation and reduced RET expression, and ERK phosphorylation in TT and MZ-CRC-1 cells, respectively. XL184 reduced RET autophosphorylation in TPC-1 cells [130] -Antitumor and antiangiogenic effect in primary human ATC cells [7]		
Lenvatinib	-Antineoplastic activity against RET gene fusion-driven tumor models [132] -It showed an antiangiogenetic effect in 5 DTC and 5 ATC xenografts. It inhibited also the RET phosphorylation in TT cells bearing the activated mutation C634W [133] -Cells obtained from patients with ATC and PTC were treated with lenvatinib or sorafenib, individually or combined with HDAC. The treatment with both drugs induced a significant tumor shrinkage, especially combining HDAC and lenvatinib, the FGFR signaling pathway was blocked [134] -Combined with paclitaxel, it inhibited synergistically colony formation and tumor growth in nude mice, and induced G2/M phase cell cycle arrest and cell apoptosis vs. lenvatinib or paclitaxel alone [135] -It inhibited migration and invasion in ATC, inhibited EGFR, AKT and ERK1/2 phosphorylation, and downregulated cyclin D1 in the ATC cells. In continuous ATC cell lines, it inhibited the proliferation, increasing apoptosis [8]		
Apatinib	-It suppressed the growth of ATC cells time- and dose-dependently, inducing apoptosis and blocking cell cycle at G0/G1 phase. It inhibited angiogenesis of ATC cells in vitro and in vivo through the suppression of Akt/GSK3β/ANG signaling pathway, suggesting its potential therapeutic effectiveness in the treatment of ATC [137] -It induces autophagy and apoptosis in human ATC cells, downregulating p-AKT and p-mTOR signals through the AKT/mTOR pathway. Combining apatinib and CQ, an additional tumor suppression was shown in vitro (in the human ATC cell lines KHM-5M and C643) and in vivo (in 4-week old male BALB/c nude mice xenografts) [139]		

Docetaxel		Baicalein improves the anticancer effect of docetaxel in ATC: proliferation was reduced substantially and apoptosis rate was increased more than with monotherapy [140]	
Vemurafenib			-Combined with the mTOR inhibitors, metformin and rapamycin, in BCPAP and 8505c cells, it reduced cell viability and increased apoptosis in both cell lines. Vemurafenib plus rapamycin amplified cell death in BCPAP cells [141] -Vemurafenib plus HCQ facilitated higher tumor suppression in vivo [142] -Combined with bortezomib, synergistic effects were shown on cell growth inhibition, an enhanced G2-phase cell cycle arrest and apoptosis. In vivo a reduction of tumor size and of the expression of the markers of cell growth and proliferation was observed, vs. monotherapy (i.e. Ki-67 and cyclin D1) [143] -Combined with S100A4 knockdown, it decreased cell proliferation, migration and invasion in vitro vs. vemurafenib or the S100A4 knockdown, alone. In an in vivo model of 6-week female nude mice injected with SW1736 and 8505C, vemurafenib did not suppress cancer growth, but inhibited tumoral growth in combined groups [144]
Dabrafenib (and Trametinib)			-It suppressed the viability in BRAF mutated cells, through G0/G1-arrest, and downregulated MEK/ERK phosphorylation in 4 human ATC cell lines. Dabrafenib upregulated phosphorylation of MEK in RAS mutated cells, with consequent VEGF overexpression. Trametinib, downregulating ERK phosphorylation, inhibited cell viability. The dual blockade by both inhibitors had cytostatic effects in the 4 ATC continuous cells [147] -Twenty-four human primary PTC cultures were established, and BRAFV600E mutation was identified in 18/24, one had a HRAS mutation, and the other 5 were BRAF and RAS wildtype. An increased NIS expression was obtained after treatment with dabrafenib and trametinib, and no effect was observed with dabrafenib alone.

Anaplastic thyroid cancer (ATC), chloroquine (CQ), dedifferentiated PTC (DePTC), differentiated thyroid cancer (DTC), histone deacetylase (HDAC), hydroxychloroquine (HCQ), epithelial-mesenchymal transition (EMT), papillary thyroid cancer (PTC), sodium/iodide symporter (NIS).

Table 2. *In vitro* studies on drugs targeting TERT, PPARγ and HDAC.

	TERT	**PPARγ**	**HDAC**
Epigenetic drugs	-PF-03814735 was the most potent on KTC2 cells, while CUDC-101 was effective on both WRO and KTC2 cells. PF-03814735 was the most effective epigenetic drug on cell lines with the C228T mutation [149]		
Thiazolidinediones antiblastics		-Human primary ATC cells were treated with increasing concentrations of pioglitazone or rosiglitazone or antiblastics. Rosiglitazone and pioglitazone reduced the proliferation of primary cells, such as bleomycin, cisplatin and gemcitabine; the inhibition of proliferation was similar in tumors with/without BRAFV600E mutation, both for thiazolidinediones and antiblastics [9] -Evaluation of the effects of chemotherapeutic agents and/or thiazolidinediones in human primary cultures of ATC, obtained from surgical biopsies and from FNA, with no significant different outcomes of the chemosensitivity tests between these two types of samples [10,11]	
HDAC inhibitors			-A study investigated the effect of the HDAC inhibitor PXD101, alone and combined with conventional chemotherapy, in 8 TC cell lines. PXD101 inhibited TC proliferation, and acted synergistically with doxorubicin and paclitaxel in treating ATC [94] -Another paper investigated HER2 and CUDC-101 in ATC. The CUDC-101 anticancer effect was related to increased expression of p21 and E-cadherin, and reduced expression of survivin, XIAP, β-catenin, N-cadherin, and Vimentin. CUDC-101 inhibited tumor progression and metastases, and significantly prolonged survival, in an in vivo mouse model of metastatic ATC [158] -In a recent study $BRAF^{V600E}$-mutant and BRAF-wild-type cells were cultured in presence of the HDAC inhibitor panobinostat and a MAPK inhibitor, alone or combined. MAPK inhibitors increase HDAC inhibitor-induced redifferentiation in PTC cells bearing the $BRAF^{V600E}$ mutation [159]

Anaplastic thyroid cancer (ATC), fine-needle aspiration (FNA), histone deacetylase (HDAC), papillary thyroid cancer (PTC).