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Resistance to Kinase Inhibitors in Poorly Differentiated and Anaplastic Thyroid Cancer: Preclinical *In vitro* Evidences

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Abstract

Poorly differentiated thyroid cancer (PDTC) and anaplastic thyroid cancer (ATC) are rare but highly aggressive malignancies with an extremely short survival. Poor prognosis is due to their unlimited growth, invasion, migration and resistance to common anticancer therapies. Advances in understanding the molecular alterations in thyroid carcinomas led to development of new therapeutic strategies such as kinase inhibitors. Although several of these compounds have been approved by FDA and EMA for the treatment of radioactive-iodine refractory differentiated thyroid cancer (DTC) and medullary thyroid cancer (MTC), no significant clinical efficacy with targeted therapies have been observed in those patients.

Herein, we review and summarize the preclinical *in vitro* evidences of mechanisms of resistance to kinase inhibitors currently used in PDTC and ATC patients.

Keywords: Poorly differentiated thyroid cancer; Anaplastic thyroid cancer; Targeted therapy; Resistance; Preclinical *in vitro* studies.

Introduction

Thyroid cancer is the most common endocrine malignancy, and its incidence continues to rise worldwide. Is the fifth most common cancer in women in the United States with more than 62,000 new cases in 2015 (American Cancer Society, 2015). Many experts attribute this increasing incidence to a more intensive diagnostic procedure for thyroid nodules. The most common type (about 95% of cases) is differentiated thyroid carcinoma, which originates from thyroid follicular cells. Also anaplastic and poorly differentiated carcinomas arise also from thyroid follicular cells, and account for 2-3% and 3-5% of all thyroid cancers, respectively. Poorly differentiated thyroid carcinoma (PDTC) and anaplastic thyroid carcinoma (ATC) are the most aggressive form of thyroid cancer. Despite their low incidence, they account for a disproportionate number of thyroid cancer-related deaths because of their resistance to the most common therapeutic approaches.

The mean survival rate in patients with ATC is approximately 6 months after the diagnosis, with a disease-specific mortality close to 100%. Poor prognosis is attributed to its unlimited growth and invasiveness, and to resistance to anticancer therapies.

The standard treatment (surgery followed by either radioiodine or observation) is usually effective for the differentiated tumors, because cells maintain the ability of iodine uptake and thyroglobulin production in response to TSH stimulation. On the contrary, poorly differentiated thyroid carcinoma, anaplastic carcinoma and about 10% of the differentiated forms, fail to uptake radioiodine and therefore are refractory to this therapy.

In the last decade, several advances have been achieved in the understanding of molecular basis of thyroid tumorigenesis, leading to the development of new therapeutic options such as kinase inhibitors (KIs) for iodine-refractory differentiated, poorly differentiated and anaplastic thyroid carcinomas.

These targeted therapies, which has been approved for poorly differentiated thyroid and medullary thyroid carcinomas, increased disease-free survival, although not curative and reserved to patients with progressive or symptomatic disease.

Currently, the most effective treatment for ATC includes locally extensive surgery combined with postoperative adjuvant chemotherapy (doxorubicin and cisplatin) and external beam radiation therapy (EBRT). This multimodal approach is associated with prolonged median patient survival of 10 months. However, complete resection is often no feasible because most patients have advanced disease at diagnosis.

Resistance to targeted therapy of these forms has been observed in several reports, although mechanisms underlying this resistance have not been described in detail.

In this review, we describe findings obtained *in vitro* about the possible mechanisms of resistance to novel therapy of different types of thyroid cancer.

Classification of thyroid cancer

The thyroid gland gives rise to a variety of tumors that differ in morphology, molecular profile, tumorigenicity and invasiveness. Based upon the histo-pathological features thyroid cancer is divided into four main subtypes: papillary, follicular, anaplastic (follicular-derived thyroid cancer) and medullary (neuro-endocrine C-cell derived thyroid cancer). The review is focused on from follicular-derived tumours.

Differentiated thyroid cancer: Approximately 85-90% of all thyroid cancers are papillary, occurring in women between 30 and 50 yrs with

a dissemination in the thyroid gland, in the near lymph nodes and distant metastasis. The second most common subtype is follicular carcinoma, which represents 10-15% of all thyroid cancers and that usually occurs in women aged between 40 and 60 yrs. These two forms are well-differentiated and display the features of mature thyroid tissue and, if diagnosed early, have an excellent prognosis with almost 100% disease free survival at 5 yrs.

Poorly differentiated thyroid cancer: Poorly differentiated, often overlapping with PTC and FTC, has an intermediate aggressiveness between differentiated and undifferentiated thyroid cancers and there are several controversies in the definition of this tumor type. The usual therapy is surgery, radioiodine in selected cases, chemotherapy in more advanced cancer, radiotherapy and novel drugs. According to the Turin Proposal, the diagnosis of PDTC requires the presence of island model/solid/trabecular growth, the absence of nuclear features of papillary carcinoma, and one or more of the following three characteristics: convoluted nuclei, mitotic index ≥ 3/10 high power fields (HPFs), and/or tumor necrosis. On the other hand, the Memorial Sloan-Kettering Cancer Center criteria (MSKCC) for PDTC are less restrictive and define PDC based exclusively on high mitotic index (≥ 5/10 HPFs) and/or tumor necrosis regardless growth pattern and nuclear characteristics [1].

Anaplastic thyroid cancer: Anaplastic carcinoma is very rare among young individuals and has an onset peak around 65 yrs or older. At the time of diagnosis many patients have already an advanced disease including a large cervical expanding mass, with breathing problems and changes in voice tone. These tumors often require tracheostomy to maintain airway patency. In most cases the disease is diagnosed at an advanced stage, and with the presence of distant metastases.

Genetics of thyroid cancer

DNA sequencing studies have revealed the most relevant genetic alterations in the majority of thyroid carcinomas. Several of these alterations occur in genes with kinase activity, which are involved in the MAP kinase and/or the PI3K/Akt/mTOR signaling cascade. Activation of these pathways leads to neoplastic transformation and progression. These pathways transmit growth signals from the plasma membrane to the nucleus and play a central role in the regulation of cellular proliferation. Thus, kinase inhibitors targeting these pathways are very important in this field [2].

Differentiated thyroid cancer: The Cancer Genome Atlas program (TCGA) has recently completed the whole genomic analysis of about 500 papillary thyroid cancer (PTC), largely confirming previous studies about the frequency of mutations in the key molecular driver of this disease (all effectors in the MAPK signaling pathway): BRAF 57% RAS 12% and the fusion oncogene (*RET/PTC, NTRK1, BRAF*, others) 9%. All of these alterations are mutually exclusive.

The most frequent mutation in differentiated thyroid cancer is the BRAFT1799A mutation, resulting in a mutant $BRAF^{V600E}$ kinase, present in papillary thyroid cancer and papillary-derived anaplastic thyroid cancer. Mutations in the RAS family oncogenes are also common in thyroid cancer, more frequently in follicular thyroid carcinomas and follicular variant of papillary thyroid carcinomas. 30% of follicular carcinoma may harbour chromosomal translocations resulting in a fusion gene between the thyroid transcription factor *PAX8* gene and the *PPARγ* gene (PAX8- PPARγ). *RET* oncogene rearrangements are present in about 7% of papillary thyroid. Less common translocation partners include *BRAF* genes, the *NTRK* gene family, ALK and Thada. There is debate regarding the clinical implications of these mutations, and some studies suggest a greater aggressiveness of papillary cancer carrying BRAF mutation for instance. However, the fact that about 50-70% of papillary tumors harbor a BRAF mutation, and most of these cancers are indolent, suggests that there are other important determinants of clinical behavior [3]. In addition, mutations in TERT have been identified in a more aggressive subset of papillary thyroid cancer.

Poorly differentiated and anaplastic thyroid cancer: The loss of the p53 tumor suppressor may allow the further progression to anaplastic carcinoma. The TCGA genomic study on PTC, along with some recent next-generation sequencing studies published in PDTCs and ATC, have provided evidence to support a gradual tumor progression from well-differentiated to poorly differentiated, and ultimately for anaplastic thyroid carcinomas. While RAS and $BRAF^{V600E}$ mutations remain the main drivers in PTCs and PDTCs, TERT promoter mutation, TP53 mutation, as well as alterations PIK3CA-PTEN-AKT-mTOR pathway, complex SWI-SNF, histomethyltransferases, and mismatch repair genes are more frequent in ATC [1].

TCGA studies also allowed the elaboration of the thyroid differentiation score (TDS) and BRAF-RAS score (BRS) [4]. Briefly, BRAF-mutated PTCs are associated with a gene signature BRAF-like and are less differentiated with a low TDS at the RNA level. On the other hand, RAS-mutated PTCs display a RAS-like signature and are highly differentiated with high TDS. This gene expression profile is maintained in PDTCs, i.e. BRAF-mutated PDTCs maintain a BRAF-like signature and are less differentiated than the RAS-mutated PDTCs with high TDS. Anaplastic carcinomas, on the other hand, tends to be BRAF-like and are deeply undifferentiated regardless the RAS-BRAF mutation status. Indeed, the ATC with RAS or other mutations tend to be BRAF-like, as defined by the BRS (a 71 gene panel that distinguishes $BRAF^{V600E}$ from RAS-mutant PTCs, highly correlated to the transcriptional output of the MAPK pathway). The major genomic complexity of ATC may account for the non-perfect correspondence between gene expression and the underlying driver mutation [5].

Novel therapy for PDCT and ATC

Kinase inhibitors: Because of the loss of their typical follicular-cell characteristics, poorly differentiated thyroid and anaplastic thyroid cancer do not respond to conventional treatments with radioiodine.

Hence, the identification of new therapeutic strategies is critical for PDCT and ATC management and the understanding of molecular basis of thyroid cancer, allowed the development of several new-targeted therapeutic options such as kinase inhibitors (Table 1).

Drugs	VEGFR1	VEGRF2	VEGFR3	C-KIT	RET	PDGFR	FGFR1-3	EGFR	Other
Vemurafetrib									$BRAF^{V600E}$, *CRAF*
Everolimus									*mTOR*

Gefitinib								+	
Erlotinib								+	
Dabraferib									*BRAF*^{V600E}
Trametinib									*MEK*
Sorafenib		+	+	+	+	+			*RAF*
Pazoparrib	+		+	+		+		+	
Lenvatinib	+		+	+	+	+	+		
Axitinib	+	+	+	+		+			
Sunitinib	+	+	+	+	+	+			*FLT-3, CSF-1R*
Imatinib				+		+			*ABL, BCR-ABL*

Abbreviation: *VEGFR1,2,3:* vascular endothelial growth factor receptors 1, 2, and 3; KIT, v-kit: Hardy-Zuckerman 4 feline sarcoma viral oncogene; RET: Rearranged during transfection receptor; PDGFR: Platelet-derived growth factor receptor; FGFR: Fibroblast growth factor receptor; EGFR; Epidermal growth factor receptor; BRAF^{V600E}: Valine-to-glutamic acid substitution of BRAF gene; *CRAF*: v-raf murine sarcoma viral oncogene homolog 1; mTOR: Mammalian target of rapamycin; MEK: mitogen-activated protein kinase; ; *RAF*: v-raf murine sarcoma viral oncogene homolog; FLT3: Fms-like tyrosine kinase 3; CSF-1R: Colony stimulating factor 1 receptor; *ABL*: Abelson murine leukemia viral oncogene homolog 1; *BCR-ABL*: Abelson and breakpoint cluster region fusion gene.

Table 1: Molecular targets of KI used in clinical trials/case reports of aggressive thyroid cancer.

KIs are small molecules that directly inhibit the catalytic activity of different protein-kinases by interfering with the ATP binding or other substrates, thereby preventing its activation. This results in the inhibition of signal transduction sustaining mitogenesis and/or angiogenesis.

In particular, ATCs display mutation in both RAS/BRAF and PIK3CA/AKT, thereby causing the constitutive activation of both MAP kinase and PI3 kinase pathways.

Also, the overexpression of the epidermal growth factor-receptor has been frequently observed in ATCs and may represent an attractive target. Moreover, compared to normal thyroid tissue, thyroid carcinomas express VEGF at higher levels. VEGF is a crucial regulator of both physiologic and pathologic angiogenesis via its binding to the cognate receptor VEGFR. The key role of VEGF in angiogenesis, cancer cell survival, tumour growth, and the development of metastases represent also an attractive target.

Hence, several new molecules targeting mutation-driven cancer cell proliferation and angiogenesis have been evaluated in patients with advanced thyroid cancer (Figure 1) and the following paragraphs will describe the most relevant kinase inhibitors used in clinical trials with PDCT and ATC patients (Table 2).

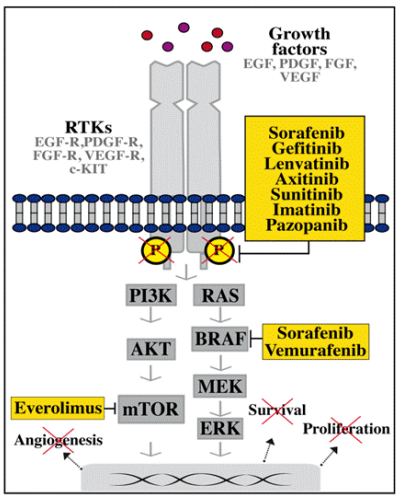

Figure 1: Major signaling pathways involved in proliferation and survival of poorly differentiated and anaplastic thyroid cancer, and clinically relevant inhibitors.

Drugs	Tumour	Phase	Patients (N)	PR (%)	CR (%)	SD>6 months (%)	PD (%)	References/Clinical trials*
Vemurafenib	ATC	II	7	1 (14%)	1(14%)	0	4 (57%)	[6]
		ongoing						NCT02091141 (August 2019)
Dabrafenib	ATC; PDTC	I		NE	0	NE	1 (33%)	Falchoock et al.

Dabrafenib+Trametinib		ongoing						NCT02034110 (March 2020)
Everolimus	ATC	ongoing						NCT00936858 (December 2016)
Gefitinib	ATC	II	5	0	0	1	4	Pemell et al.
Sorafenib	ATC	II	20	2 (10%)	0	5 (25%)	13 (65%)	[13]
	ATC	II	4	0	0	1(25%)	3 (75%)	Kloos
	PDTC	II	2	NE	NE	NE	NE	Kloos
	ATC; PDTC	II	2	0	0	0	2 (100%)	Gupta-Abramson
Pazopanib	ATC	II	15	0	0	0	12	[14]
Pazopanib+paclitaxel	ATC	ongoing						NCT01236547 (October 2018)
Lenvatinib	ATC	II						NCT01728623 (completed)
	PDTC	II	28	NE	NE	NE	NE	Schlumberger et al.
Axitinib	ATC	II	2	1 (50%)	0	0	1 (50%)	[16]
Imatinib	ATC	II	8	2 (25%)	0	4 (50%)	2 (25%)	[19]

*Estimated study completion date; Abbreviation: PR (partial response), CR (complete response), SD (stable disease), PD (progressive disease), PFS (progression-free survival), OS (overall survival), NE (not evaluable)

Table 2: Results of clinical trials with tyrosine kinase inhibitors in thyroid cancer patients.

BRAF inhibitors: The RAF kinase inhibitor **vemurafenib** (PLX4032) has been approved for treatment of BRAF-mutated metastatic melanoma. Vemurafenib was also used in patients with metastatic BRAF-mutant colorectal cancer with a limited efficacy.

In a recent phase II "basket" study with vemurafenib in $BRAF^{V600E}$ mutated non-melanoma cancers, two out of the seven patients with ATC achieved a response to therapy (one complete and one partial) and this response persisted for more than one year. Four patients had disease progression and one was not evaluable [6].

Moreover, several case reports documented complete tumour regression and/or partial response to selective BRAF inhibitors in patients with ATC [7,8].

Moreover, the combination of BRAF and MEK inhibition, compared to single-agent BRAF inhibition, demonstrated increased survival in patients with BRAF-mutant metastatic melanoma [9]. Hence, a novel clinical trial is ongoing aimed at evaluating the combination of BRAF inhibitor **dabrafenib** with the MEK inhibitor trametinib in patients with BRAF V600E-mutated rare cancers, including ATC (NCT02034110).

mTOR Inhibitors: The mTOR pathway inhibitor, **Everolimus** (RAD001), has been approved by FDA for treatment of pancreatic, gastrointestinal, and lung origin neuroendocrine tumor, advanced renal cell carcinoma, breast cancer (HR+, HER2-) and non-resectable subependymal giant cell astrocytoma associated with tuberous sclerosis. A phase II trial with everolimus in patients with aggressive RAI refractory thyroid cancer included also five patients with ATC (ClinicalTrials.gov number, NCT00936858). Stable disease for five months and disease progression were observed in one and three patients, respectively. Only one patient achieved a near-complete response that lasted for 18 months, followed by progressive disease [10].

EGFR Inhibitors: Overexpression of the EGFR is frequently detected in poorly differentiated and ATC, although there is no evidence that EGFR is a primary driver of these tumors. A phase II clinical trial showed no response to the EGFR-TK inhibitor gefitinib (ZD1839) in small cohort of five patients with ATC, although one patient had stable disease for 12 months [11].

However, a case report of a 79 year old male patient with metastatic PDTC harbouring EGFR mutation showed a PFS of more than 11 months when treated with the selective EGFR TKI erlotinib (OSI-774) [12].

Multi-tyrosine kinase Inhibitors (mTKIs): Sorafenib (Nexavar), a broad-spectrum kinase inhibitor with activity against VEGFR2, VEGFR3, PDGFR, c-Kit, RAF and RET kinases, was approved in 2013 by the US FDA for the treatment of patient with radioiodine-resistant metastatic differentiated thyroid cancer.

However, a single-arm phase II trial has shown limited activity of sorafenib in patients with anaplastic thyroid carcinoma. Two of the twenty subjects had a partial response with poor progression-free survival (PFS) and overall survival (OS), 1.9 and 3.9 months, respectively [13].

Similarly to sorafenib, **Pazopanib** (GW786034), a mTKI for EGFR, VEGFR, PDGFR and c-kit, demonstrated minimal clinical activity as single agent in patients with ATC [14].

However, a preclinical study reported a synergistic anticancer effect of the combination of pazopanib with the microtubule inhibitor paclitaxel, apparently due to an "off-target" inhibition of Aurora A kinase [15]. A randomized phase II trial (NCT01236547) is recruiting patients with ATC to evaluate the potential benefit of paclitaxel with or without pazopanib in subjects receiving also intensity-modulated radiation therapy (IMRT).

Lenvatinib (E7080) is a novel multi-target kinase inhibitor with a high potency against FGFR1. It has also antiangiogenic properties, as well as direct antitumor effect targeting VEGFR1-3, FGFR1-4, RET, c-kit and PDGFR. Lenvatinib is currently approved for treatment of patients with locally recurrent or metastatic, progressive, radioactive iodine-refractory differentiated thyroid cancer in the United States. Encouraging results were obtained in a phase II trial using lenvatinib in patients with DTC, MTC, and ATC. Three of eleven treated patients with ATC achieved partial response lasting up to 21 months, seven patients had stable disease, and one patient progressed (NCT01728623).

Axitinib (AG-013736), an inhibitor of VEGFR-1, -2 and -3 PDGF-B, and c-Kit, has higher selectivity against VEGFR-2. In a phase II clinical trial exploring the potential benefit of axitinib in thyroid cancer, two patients with ATC were included. This small molecule showed therapeutic efficacy in all histologic subtypes tested and, among the two patients with ATC, one achieved a partial remission and one progressive disease [16].

Sunitinib (SU011248) has inhibitory activity against RET, VEGFR1-3, PDGFR, c-kit, FLT-3 and CSF-1R. A phase II trials with sunitinib in thyroid cancer have been published. Unfortunately, the potential clinical benefit of this compound on ATC treatment remains controversial due to absence of patient recruitment on the basis of tumor pathology [17]. However, a recent case report showed clinical activity of sunitinib as a salvage treatment in an ATC patient who was not suitable to receive systemic chemotherapy treatment. In this patient, although sunitinib treatment has no impact on distant metastases, a complete regression of the neck tumour mass was achieved [18].

Imatinib (STI571), a small molecule that selectively inhibits c-kit, ABL, BCR-ABL, and PDGFR, has been approved by the US FDA and EMA for the treatment of chronic myeloid leukemia (CML) and gastrointestinal stromal tumor (GIST). A phase II study of imatinib was conducted in eleven patients with ATC. None of the eight evaluable patients showed complete response, two patients obtained a partial response and four patients had stable disease, with 27% of six months progression-free survival rate and 46% of six months overall survival rate [19].

Resistance to targeted therapy

Although several small-molecule Ki have been explored for the treatment of advanced, progressive, and radioiodine refractory (RAI-R) thyroid tumours, and some of them have been approved for use in clinical practice (sorafenib and lenvatinib for DTC and PDTC and vandetanib and cabozantinib for MTC), it is still unclear whether ATC patients may benefit from this therapeutic strategy.

Kinase inhibitors, used as single agents, have demonstrated limited clinical benefit for patients with anaplastic thyroid cancer and, thus none of these has gained approval for routine clinical use. In fact, phase II trials with sorafenib, gefitinib, pazopanib, axitinib and imatinib in ATC patients cohorts showed limited or negligible activity.

Here we review the recent insights into the possible mechanisms of resistance to targeted therapies that have been described in thyroid cancer cell lines (Figure 2).

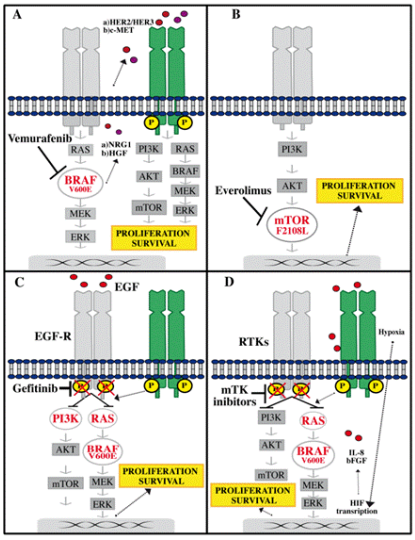

Figure 2: Mechanisms of resistance to BRAF (A), mTOR (B), EGF-R (C) and Tyrosine Kinase Receptors (D) inhibitors in poorly and anaplastic thyroid cancer cells.

Understanding the mechanisms of response and resistance to these drugs *in vitro* may be a prerequisite to a selective patient selection and to design strategies to overcome both initial and acquired resistance to targeted cancer therapies.

Resistance to BRAF inhibitors

Compared with the results obtained in malignant melanoma with BRAF inhibitors, the responses to vemurafenib in thyroid cancer were not satisfactory. This observation suggests that thyroid cancer may have an intrinsic resistance to vemurafenib and, therefore, BRAF mutation by itself may be not a predictor of response to targeted therapy.

Different mechanisms of escape by tumor cells from inhibitory control of these drugs have been described so far *in vitro*.

A recent report described as a possible mechanism of resistance to BRAF inhibitor vemurafenib, the increase in HER2/HER3 signaling pathway due to autocrine secretion of Neuregulin-1, that was observed in BRAF-mutant undifferentiated thyroid cancer cell lines exposed to vemurafenib. This autocrine loop led to ERK and AKT overactivation that contributed to attenuate the antitumor effect of BRAF inhibitor, which was restored by combination with the selective HER inhibitor lapatinib [20].

Similarly to the previous study, Byeon et al. investigated in thyroid cancer cell lines changes in signalling pathway in response to vemurafenib treatment. They found that $BRAF^{V600E}$ inhibition in 8505C cells led to autocrine c-Met receptor activation, which in turn results in a re-activation of both ERK and PI3K/AKT pathway. Moreover, combined treatment of vemurafenib and c-Met inhibitor PHA665752 resulted in the inhibition of both p-AKT and p-ERK,

thereby implying an enhanced therapeutic efficacy. Similar results were obtained *in vivo* in an orthotopic xenograft mouse model [21].

A recent study evaluated the role of mTOR pathway in the resistance to BRAF inhibitor vemurafenib in thyroid cancer. Hanly et al. found that combination of vemurafenib and mammalian target of rapamycin (mTOR) inhibitors, such as metformin and rapamycin, decreased cell viability and increased apoptosis in thyroid cancer cell lines harbouring $BRAF^{V600E}$ mutation [22].

Also the role of miRNA in resistance to KI was evaluated. Varmeh et al. analysed the effect of long-term exposure of 8505c ATC cell lines to PLX4720, a structurally related progenitor of vemurafenib, on microRNA expression pattern. The Illumina deep sequencing techinique allowed to identify 61 known and 2 novel miRNAs whose expression was significantly altered in the PLX4720-resistant 8505c-R cell line compared with the parental 8505c cell line. The functional annotation and putative target gene analysis revealed that the MAP kinase and PI3K-AKT pathways were among the prominent target of these deregulated miRNAs. Hence, these miRNA may be used as biomarkers of resistance to BRAF inhibitors as well as potential therapeutic targets in combination with BRAF inhibitors to overcome this resistance. Of course, the study is limited to *in vitro* and in silico analysis, so it becomes necessary to validate identified miRNAs in tissues of patients with thyroid cancer treated with BRAF inhibitors [23].

Resistance to mTOR inhibitors

Tumours with the overactivation of PI3-K/AKT/mTOR pathway are very sensitive to mTOR inhibitors such as everolimus. In particular, several activating alterations in the mTOR pathway: such as *MTOR, TSC1* or *TSC2*-have been proposed as biomarkers of response to everolimus in various cancers. Although these biomarkers of sensitivity to everolimus have been identified, mechanisms of clinically acquired resistance remain unknown.

In a recent clinical report, a patient with metastatic ATC treated with everolimus, showed resistance after an extraordinary 18 months of response. Whole-exome sequencing in tumor samples from this patient before and after treatment revealed a novel somatic nonsense mutation in *TSC2* and *mTOR* in resistant tumor. *TSC2* is negative regulator of *mTOR* that confers exceptional response to everolimus. The somatic mutation in MTOR ($MTOR^{F2108L}$) caused resistance to allosteric *mTOR* inhibition by preventing the binding of the drug to the protein. Indeed, human embryonic kidney (HEK) 293T cells stably transfected with $MTOR^{F2108L}$ mutant were significantly more resistant to inhibition with rapamycin than cells expressing wild type *mTOR*. Furthermore, the authors demonstrate that cells expressing $MTOR^{F2108L}$ remain sensitive to treatment with Torin1, a direct TOR kinase inhibitor, suggesting that patients harbouring mTOR mutation would be ideal candidates for clinical trials of selective mTOR kinase inhibitors [10].

Resistance to EGFR inhibitors

EGFR inhibitors as single-agent have not shown significant activity in ATC patients. To date, few studies have attempted to identify mechanisms associated with resistance to EGFR inhibitors in undifferentiated thyroid cancer cell lines. Frasca et al. evaluated the effect of gefitinib in a panel of six thyroid cell lines. They observed that despite the marked inhibition of EGFR phosphorylation, cell viability and apoptosis were not affected by gefitinib in most of the cell lines. The limited cell response to gefitinib was associated with genetic alterations that cause the constitutive activation of the ERK pathway, including $BRAF^{(V600E)}$ and $HRAS^{G12A/Q61R}$ mutations and RET/PTC1 rearrangement. When $BRAF^{(V600E)}$-positive thyroid cancer cells were incubated with the specific BRAF inhibitor PLX4032, sensitivity to gefitinib was restored. Similar results were obtained with tipifarnib and regorafenib, inhibitors of RAS and RET respectively [24].

Moreover, Onoda et al. observed that gefitinib exhibits no activity in OCUT-2, an undifferentiated thyroid cancer cell lines harbouring a mutation in *PI3KCA* gene [25].

However, although EGFR inhibitors were not effective in ATC treatment as single-agent, EGFR remains an attractive target for drugs in combination.

In this respect, Zhang et al performed quantitative high-throughput screening (qHTS) of 3,282-compound library in three ATC cell lines harbouring different genetic mutations, and identified 100 pan-active compounds. Enrichment analysis of qHTS data showed that inhibitors targeting histone deacetylase (HDAC), aurora kinase, mTOR, and EGFR were the most active drug categories. In particular, one of the highly active agents identified was CUDC-101, a dual inhibitor of both EGFR/HER2 and HDACs. Indeed, treatment of thyroid cancer cells lines with CUDC-101 inhibited cellular proliferation and migration, induced cell cycle arrest and caspase-dependent apoptosis *in vitro*. Moreover, CUDC-101 inhibited tumour growth and distant bone metastasis *in vivo*, in a xenograft model resulting in prolonged survival [26].

Taken together, these data suggest that abnormalities in signaling downstream EGFR may cause resistance to EGFR inhibitors and combined treatments with selective drugs may effectively target these disregulated pathways in human ATC.

Resistance to multikinase inhibitors

Although angiogenesis plays an important role in thyroid cancer progression, multikinase inhibitors (MKIs) targeting VEGF pathway and other receptor tyrosine kinases did not display a significant therapeutic effect in patients with ATC.

Currently, few mechanisms underlying poor response to VEGF-targeting mTKi in anaplastic thyroid cancer have been described. Piscazzi et al. found that sunitinib (a multitargeted FLT3, PDGFRs, VEGFRs, and Kit kinase inhibitor) selectively inhibits proliferation in KRAS/BRAF wild-type thyroid cancer cells, harbouring the RET/PTC rearrangement. By contrast, KRAS-or BRAF-mutated thyroid cancer cells are resistant to sunitinib. Pharmacological inhibition of MAPK kinase activity restored sensitivity of KRAS-or BRAF-mutated cells to sunitinib [27].

Tohyama et al. reported significant antitumour activity of lenvatinib against 11 human thyroid xenograft models in nude mice, including five anaplastic thyroid cancers. However, despite the marked inhibition of tumor growth *in vivo*, lenvatinib shown antiproliferative activity *in vitro* in only 2 of 11 thyroid cancer cell lines (RO82-W-1 and TT). The authors discovered that cell response to lenvatinib was associated to direct inhibition of FGFR and RET signaling pathway, which are activated in RO82-W-1 and TT cell lines, respectively [28].

Inhibition of VEGF/VEGFR pathway leads to compensatory upregulation of the other growth factors and cytokines. In solid tumors, such as neuroblastoma [29] and glioblastoma [30], treatment-induced hypoxia mediates resistance to antiangiogenic drugs [31],

including tyrosine kinase inhibitors [32]. Under prolonged exposure to hypoxia, expression of hypoxia-inducible factor 1 alpha (HIF-1α) leads to upregulation of alternative proangiogenic factors, such as basic fibroblast growth factor (bFGF), interleukin 8 (IL-8) or other inflammatory cytokines [33,34]. Also, HIF-1α may influence the recruitment of bone marrow-derived cells (BMDCs) that regulate tumor angiogenesis and invasion [35]. As a result, intratumoral hypoxia followed by HIF-1αoverexpression is associated with tumor invasiveness and metastasis in several cancers [36].

These evidences suggest the crucial role of hypoxia and hypoxia-inducible factors in thyroid cancer progression, aggressiveness, and metastasis [37].

Confirmatory results were obtained by Yang et al that in a panel of different thyroid cancer cell lines, observed epithelial to mesenchymal transition phenotype (EMT), cadherin shift, and increased vimentin expression in a HIF-1α-dependent manner after exposure to hypoxia, which led to increasing cells invasiveness and migration by the regulation of the Twist signal pathway [38].

Inhibition of multiple pathway

Regarding the multiple inhibition of different pathways, several studies have evaluated the synergistic potential of sorafenib combined with other drugs.

Recently, Abdulghani et al. shown that combined treatment with sorafenib and quinacrine, a potent small molecule inhibitor of NFkB signalling, triggers additive/synergistic anti-tumor responses in ATC cell lines.

Besides inhibition of NFKB-p65/RelA, the drug combination targets anti-apoptotic protein Mcl-1, whose expression levels were found to be increased in a subset of clinical ATC specimens compared to normal thyroid tissue. Also, the well-tolerated drug combination improves survival in a mouse orthotopic xenograft model of ATC [39].

Chen et al. demonstrated a synergistic anti-proliferative effect of sorafenib and metformin in anaplastic thyroid cancer cell lines. In particular, metformin addition enabled a 25% dose reduction of sorafenib without loss of its growth inhibitory efficacy. Furthermore, metformin enhanced sorafenib-dependent inhibition of colony and tumour spheres formation, suggesting a specific effect on self-renewal capacity of cancer stem cells [40].

Similarly, Cohen et al. reported a synergistic effect of sorafenib with withaferin A (WA), a natural withanolide, in papillary and anaplastic thyroid cancer cell lines with significant induction of apoptosis. Following 72 hour of treatment with sorafenib alone, IC_{50} value was 7.6 μm for SW1736 anaplastic cell lines. Drug combination with WA decreased IC_{50} to 4 μm, for SW1736, indicating an addictive effect [41].

Taken together, these compounds such as metformin, WA and quinacrine, interacting with multiple prosurvival and proliferative signalling pathways, can be combined with lower doses of kinase inhibitors in order to prevent single-drug resistance and decrease toxicity of mTKi.

Furthermore, in a recent paper Kasaian et al. questioned the role of VEGF pathway as a therapeutic target in anaplastic thyroid cancer.

The authors analysed genomic and transcriptomic profiles of a primary ATC and three anaplastic thyroid cancer cell lines, and compared them to those of 58 papillary thyroid carcinoma and matched normal tissue transcriptomes from The Cancer Genome Atlas (TCGA) study. They found that the main targets of mTK inhibitors, such as FGFRs, VEGFRs, KIT and RET have similar or lower expression levels in anaplastic specimens compared with both papillary thyroid cancers and normal tissues. However, since the study is limited to mRNA expression, further investigations should be performed to validate levels and activation of these proteins in thyroid cancer cells [42].

Conclusions

Targeted therapy with kinase inhibitors is the new challenge in advanced cancer therapy. Although several potential targets have been identified and the efficacy of these compounds has been tested in several clinical trials, results are often controversial. This is particularly true for advanced and poorly differentiated thyroid carcinoma that has been proved refractory to most of these compounds. *In vitro* evidence, suggest that resistance to KI in thyroid carcinomas is often associated to the overactivation of the ERK pathway. Combined treatment with ERK inhibitors restores response to KI in several thyroid cancer cell model, suggesting that combined therapy may be an attractive alternative to cure these malignancies. By the other hand, multikinase inhibitors targeting also tumor angiogenesis, did not display a significant efficacy due to the hypoxia induced resistance mechanisms.

Taken together, these *in vitro* results, suggest that therapy of KI combined with inhibitors targeting crucial steps of intracellular signaling may be efficacious in poorly differentiated and anaplastic thyroid carcinomas.

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