Redifferentiation of iodine-refractory metastatic thyroid cancer: from bench to bedside, through a network analysis

Dottorato di ricerca in Tecnologie Mediche Innovative in Medicina Clinica - XXXIII ciclo

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“Ogni visione è parziale. Non esiste un modo di
vedere la realtà che non dipenda da una prospettiva.
Non c’è punto di vista assoluto, universale.
I punti di vista tuttavia comunicano,
i saperi sono in dialogo tra loro e con la realtà,
nel dialogo si modificano, si arricchiscono, convergono,
la nostra comprensione della realtà si approfondisce.”
(Helgoland. C. Rovelli, 2020)

Outline

ABSTRACT

BACKGROUND: Radioiodine-refractory (RAIR) differentiated thyroid cancers (DTC) lose the ability to efficiently concentrate iodide, rendering RAI (iodine-131; I-131) ineffective. Few studies have shown improved RAI uptake after MEK inhibitors (MEKi) and/or BRAF inhibitors (BRAFi). MicroRNA (miRNA) role in de- and re-differentiation process has been poorly investigated. ***METHODS***: We retrospectively evaluated RAIR DTC patients treated with redifferentiation therapy using kinase inhibitor (KI) therapy with single-agent or combination MEKi and/or BRAFi. Our primary objectives were the percentage of patients with uptake on diagnostic (Dx) and post-treatment (post-Tx) whole body scan (WBS), and the radiologic response to I-131 achieved over that seen with KI. We exploited a bioinformatic approach (SWItchMiner, SWIM) to identify the key genes (switch genes) involved in BRAF-mediated carcinogenesis. MicroRNA Enrichment TURned NETwork (Mienturnet) was used to find the most important miRNAs able to regulate these switch genes. We validated our results by in vitro experiments.

RESULTS: A total of 41 patients with RAIR, advanced DTC (76% BRAF V600E mutant cancer) were included in the study. At post treatment scan, there was uptake, either optimal or suboptimal, in 95% of patients. After I-131, 73% of patients were deemed treatment successes, reaching stable or partial response, that lasted at least one year without treatment on systemic therapy. The median PFS was 29 months from the start of KI and the median TTP was 20 months from RAI administration. The investigation of mutational landscape of BRAF mutated papillaty thiroid cancer (PTC) by SWIM allowed to identify a subset of switch genes (227), 30% of which is regulated by hsa-miR-335-5p. In vitro experiments showed that miRNA-335-5p is lost in primary or continuous BRAF mutated cell lines. Its over-expression in thyroid tumor cell lines harboring BRAF mutation induces the activation of NIS protein and increases intra-cellular iodide uptake.

CONCLUSIONS: Redifferentiation therapy with MEKi and/or BRAFi resensitizes a subset of RAIR DTC patients to RAI and may result in prolonged stable disease off KI. Hsa-miR-335-5p may have a role in redifferentiation of RAIR BRAF-mutated papillary thyroid cancer. MiR-335-5p over-expression may contribute to restoring RAI responsiveness.

BACKGROUND

Radioiodine-refractory (RAIR) differentiated thyroid cancers (DTC) lose the ability to efficiently concentrate iodide, rendering RAI (iodine-131; I-131) ineffective [1]. RAIR thyroid cancer has a dismal prognosis, with an estimated 10-year survival rate of 10% [2]. Management options in this subset of patients include active surveillance, local treatments for metastatic sites, or kinase inhibitor (KI) therapy for rapidly progressing, symptomatic, or life-threatening disease.

A novel strategy, called "redifferentiation", is currently being explored to restore the capacity of RAIR tumors to trap and respond to a single administration of I-131, reducing or deferring the need of long-term KI therapies. Redifferentiation strategy consists of administration of KI (MEK and/or BRAF inhibitors) for a period of time to induce redifferentiation in RAIR DTC, thus re-sensitizes tumours to RAI, followed by therapeutic radioiodine. Patients continue KI until 2-3 days after RAI and then discontinue KI and start follow-up, off systemic treatment.

The first studies with the aim of redifferentiation, using retinoids [3], romidpesin [4], rosiglitazone [5] and sorafenib [6] have been demonstrated to have low clinical value. Grüning *et al*. did not observe any relevant improvement of RAI uptake in patients after isotretinoin treatment, and reported a disappointingly low response rate, in the order of 20% [3]. No major responses were observed in the study with romidepsin and rosiglitazone [4, 5]. An unexpected sudden death was reported during treatment with romidpesin, which led to the temporary suspension of the study. Authors did not recommend further investigation of study compounds as a single agent in RAI-refractory thyroid cancer.

A deeper understanding of the molecular processes who hide behind the lost of sensitivity to RAI (genetic and epigenetic alterations) has allowed to identify better therapeutic targets.

Preclinical data

Mutations in the mitogen-activated protein (MAP) kinase pathway, in particular *BRAF* V600E mutation, have been associated with reduced expression of key genes involved in iodine metabolism (iodide uptake, thyroid hormone synthesis and differentiation, i.e. PAX8, TTF-1, NIS, TPO, TG) [7-9].

Thyroid transcription factor-1 (TTF-1) and human paired box-8 (PAX-8) are involved in thyroid differentiation and control the expression of thyroglobulin (TG), thyroperoxidase (TPO), thyroid-stimulating hormone receptor (TSHr) and sodium/iodide symporter (NIS, also known as solute carrier family 5, member 5, SLC5A5) by binding to the promoters of these genes. PAX8 expression is frequently downregulated in thyroid carcinoma, and this decrease may correlate with the dedifferentiation of thyroid follicular cells, reflected by the loss or downregulated expression of genes involved in the cells' ability to concentrate iodine, thereby contributing to tumor aggressiveness [10]. NIS, located on the basolateral membrane of thyroid follicular cells, is the protein responsible of iodide uptake. Levels and activity of NIS on plasma membrane of thyroid cancer cells are highly variable. Down-regulated expression level of NIS has been observed in RAIR DTC [11]. Different approaches have been used in the attempt to upregulate or re-activate NIS in RAIR tumours. Moreover, human thyroid cancers with *BRAF* mutations show greater reduction of NIS mRNA compared to tumors with other mutations or with no identifiable genetic changes [7]. The restorability of iodide-metabolizing gene expression has been demonstrated using a MEK inhibitor (MEKi) or through the cessation of *BRAF* V600E expression in a thyroid cancer cell line [8]. Chakravarty et al showed that in a thyroid cancer mouse model harbouring a *BRAF* V600E mutation, the pharmacological inhibition of either MEK or BRAF with selective inhibitors, is associated with restoration of gland architecture, tumor regression and recovery of RAI uptake [9].

BRAF is one of the best known driver genes, which encodes for a member of the RAF kinase family. It is a core component of the ERK signalling pathway which is involved in cell growth, differentiation, and survival. The most frequent mutation in the *BRAF* gene is the point mutation c.1799T>A, which is a substitution that results in an aminoacid change from valine (V) to glutamic acid (E) in the activation segment of the BRAF kinase (V600E), promoting its hyperactivation and resulting in constitutive activation of downstream ERK signalling. *BRAF* mutations are present in approximately 8% of human tumours. *BRAF* V600 mutations occur in 37-50% of papillary thyroid cancers. *BRAF* V600 mutations are associated with poor prognosis due to an aggressive disease phenotype, shortened overall survival, and minor response rates [12]. Several BRAFi have been approved since 2011. The first one to be used was vemurafenib, approved first in patients with *BRAF* V600E mutant metastatic melanoma [13].

To date the molecular mechanism behind redifferentiation is not fully understood. MicroRNAs (miRNAs or miRs), small noncoding RNAs (~22 nt) that regulate gene expression at mRNA post-transcriptional level, are involved in physiological and pathological processes, including tumorigenesis, differentiation and progression [14]. They predominantly bind to 3'UTR of target genes resulting in either degradation of target mRNA or inhibition of translation. Several studies reported a general downregulation of miRNAs in tumours, compared with normal tissues. Due to its repression effect, deregulation of specific miRNAs could lead to the repression of tumor suppressor genes and/or increase of oncogene expression. MiRNAs have been shown to be useful predictive biomarkers and targets of therapeutic options in thyroid cancers [15, 16]. Few studies reported the association between RAI-uptake and miRNA expression profiles, and their association with response to RAI-treatment [17].

Clinical evidences

Previous preclinical observations provided the rationale for clinical trials with RAIR DTC patients treated with a MEK inhibitor and/or a BRAF inhibitor [18-21] to restore RAI uptake. These clinical trials

showed an improvement in RAI uptake after MEKi or/and BRAFi in RAS and BRAF mutant cancers, resulting in objective response to I-131 treatment. However, the time to progression and time off KIs is also of importance to physicians and patients. The ability to expose patients to KIs for a short period of time (instead of continuous treatment until it is no longer beneficial), exposes the patient to fewer potential adverse events. Furthermore, resistance to KIs after long-term exposure is inevitable, as demonstrated by the emergence, in thyroid cancer patients exposed to BRAF inhibitors, of mutations that confer resistance to treatment, leading to progression of disease [22].

Bioinformatic analysis

The analysis of the cancer molecular landscape, using bioinformatic tools, is emerging as a powerful approach to identify hidden, tissue-specific pathways [23], to predict effectiveness of existing therapeutic agents (repurposing) or to suggest new ones [24]. Two tools, developed by physics and engineers at our Institution, were exploited for this study purpose: SWitchMiner (SWIM) and Mienturnet. SWIM is a software with a user-friendly graphical interface, developed in MATLAB. SWIM combines topological properties of co-expression networks with transcriptomic data and is able to identify a small pool of regulatory genes, called switch genes, which have been shown to be critically associated with drastic changes in cell phenotypes. This tool has been successfully applied in human cancers to identify genes that could be critically associated with the drastic changes in the physiological state of cells or tissues induced by cancer development, such as breast cancer and glioblastoma [25]. Mienturnet is an interactive web tool for miRNA-target enrichment and network-based analysis [26]. It fetches data of computationally predicted and experimentally validated miRNA-target interactions from two databases (TargetScan and miRTarBase).

AIMS

Here, we describe a population of patients with previously RAIR, advanced DTC, treated with either BRAFi and/or MEKi with the aim to restore RAI avidity (***Section 1- clinical study***). Thus, in this

study we sought to describe the long-term effects of redifferentiation therapy for those patients who were able to discontinue KI after receiving RAI. Assuming the pivotal role of *BRAF* mutation in oncogenesis, as well as in the de-differentiation process resulting in refractoriness to radioiodine therapy in thyroid cancer, we used a bioinformatic approach to identify the key genes involved in *BRAF*-mediated carcinogenesis. Then, we looked for the most important miRNAs able to regulate switch genes that might have a role in the redifferentiation process (***Section 2-bioinformatic study)***. We validated our results by in vitro experiments (***Section 3- in vitro studies***).

SECTION 1 - *clinical study*

MATERIAL AND METHODS

Study design and participants

This was a single center retrospective analysis. Consecutive patients from October 2014 to April 2019 were considered for the study. Eligible patients were required to have RAI refractory DTC, histopathologically confirmed at MD Anderson Cancer Center. One of the following criteria for RAIR disease had to be met: 1) no RAI uptake at known sites of metastases, 2) progressive disease (PD) despite previous RAI treatment within 1 year of RAI therapy. Moreover, to be included in the study, patients needed to be treated with either single-agent or combination BRAFi and/or MEKi, and had to have a diagnostic (Dx) whole-body scan (WBS) while receiving this therapy. Patients who did not complete the redifferentiation strategy, i.e., patients who didn't receive I-131 or who received I-131 but continued the BRAFi and/or MEKi, were not included in the analysis. The study was approved by the institutional review board at The University of Texas MD Anderson Cancer Center, under a waiver of consent. Details about the demographics, tumor characteristics (i.e., histologic subtype and mutational status), volume and sites of disease, prior treatments (surgery, radiotherapy, RAI, kinase inhibitors) before redifferentiation therapy were collected from the charts of patients. Other information obtained from medical records were: name of BRAFi and/or MEKi used to induce

redifferentiation, duration of treatment, uptake on Dx and post treatment (post-Tx) WBS, activity of RAI administered, radiological response to BRAFi and/or MEKi and RAI, thyroglobulin (Tg) trend before, during and after redifferentiation strategy, and survival data.

Treatment schedule

Patients with *RAS* (N-, H-, or K-) and no detectable driver mutations were treated with a MEKi. Patients with BRAF mutated DTC were treated with single agent BRAFi or the combination BRAFi + MEKi. Patients received I-131 after low iodine diet intake of 1 to 2 weeks and after rhTSH injections on two consecutive days. Dx and post-Tx WBS were performed for every patient and a SPECT/CT was available with Dx scan and/or with post-Tx scan (**figure 1**).

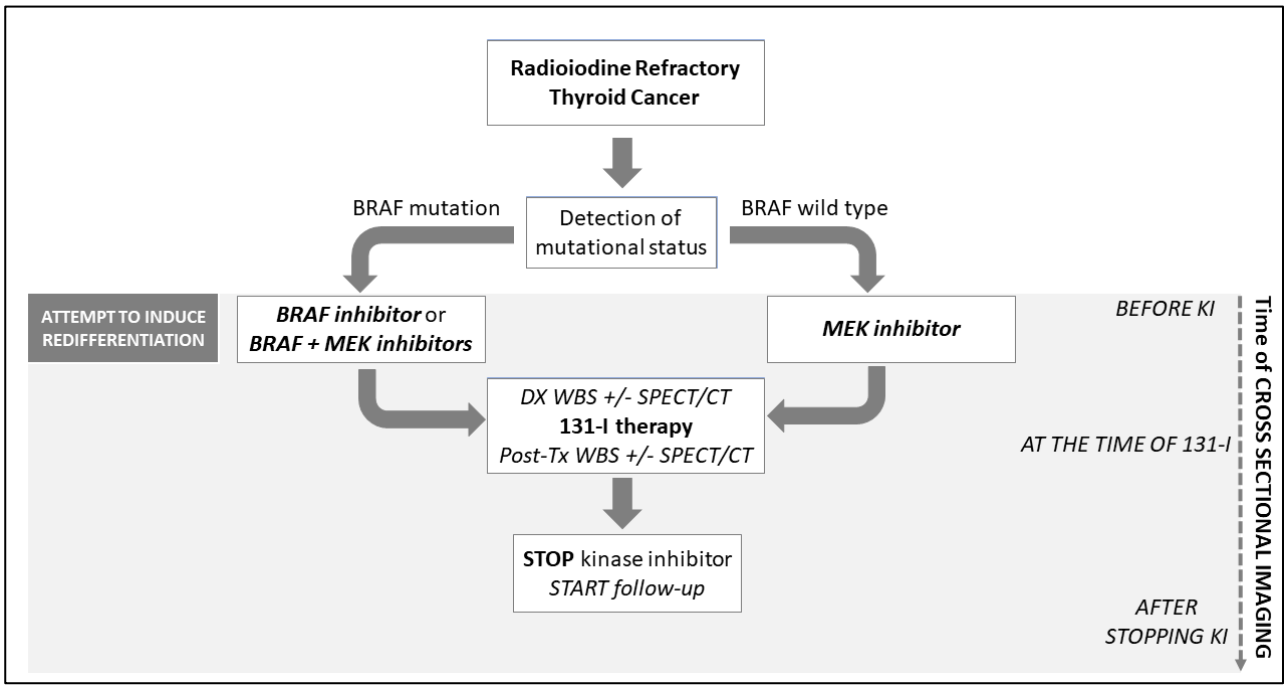

Figure 1. Treatment schedule. Patients with Radioiodine refractory thyroid cancer were treated with MEK inhibitor (MEKi), BRAF inhibitor (BRAFi) or both, according to mutational status, in an attempt to induce redifferentiation (revert to RAI sensitive). Patients received I-131 after a median period of 3.9 months. Diagnostic (Dx) and post-treatment (post-Tx) whole body scan (WBS) were performed for every patient and a SPECT/CT was available with Dx scan and/or with post-Tx scan. The BRAFi and/or MEKi was discontinued 48-72 hours after I-131 administration. Cross-sectional imaging (CT, 18fdg PET/CT) was performed before starting kinase inhibitor (KI) and after I-131 administration. Target lesions were measured before KI, during KI (if available), at the time of RAI administration (using SPECT imaging or other cross-sectional imaging performed close to I-131) and after 131-I therapy (while off KI).

Preferably, post-Tx scans should be delayed for 5-14 days post-RAI, however, there was a lot of variability regarding the timing of the post-Tx scans. All scans were reviewed by the same expert nuclear medicine physician. Optimal uptake on WBS was defined as intensive accumulation of radioiodine in all tumor lesions. Suboptimal uptake was defined as either weak uptake or when some, but not all lesions, accumulated radioiodine. The BRAFi and/or MEKi was discontinued 48-72 hours after I-131 administration. Cross-sectional imaging (CT, 18fdg PET/CT) was performed before starting KI and after I-131 administration. For some patients (those who received KI for a longer period of time), cross-sectional imaging were available also during KI therapy. Radiologic response on cross-sectional imaging was measured using Response Evaluation Criteria in Solid Tumors, version 1.1. Target lesions were measured before KI, during KI (if available), at the moment of RAI administration (using SPECT imaging or other cross-sectional imaging performed close to I-131) and after RAI therapy while not receiving KI. Best response were recorded.

Levels of serum non-stimulated thyroglobulin (Tg) were collected before starting KI, before RAI administration, around 3, 6 and 12 months after administration of I-131, and at the moment of progression, when available. We excluded patients with positive anti Tg antibody (Ab) from the Tg analysis.

Data analysis

The primary endpoints of the study were the percentage of patients with uptake on Dx and post-Tx scan, and the best radiologic response to I-131 achieved over that seen with KI, confirmed at least after 6 months from radioiodine. The secondary endpoints of the study were progression free survival (PFS), time to progression (TTP), time on and off kinase inhibitors, percentage of treatment success, serum non-stimulated Tg level during redifferentiation strategy. An additional, exploratory end point was the assessment of differences in PFS and TTP between patients with *BRAF* mutation and patients without *BRAF* mutation (*RAS*-mutated or wild-type for a panel of 400 genes analyzed by Next

Generation Sequencing). PFS was defined as the time from the start of KI to progression according to RECIST 1.1 criteria. TTP is the time from RAI administration to progressive disease. Patients who did not progress, were censored at last follow-up visit. We defined treatment success as the absence of progression within one year from 131-I administration and/or the discontinuation of KI after 131-I administration of at least 12 months. Survival times were calculated and compared using the Log-Rank test. A Wilcoxon signed-rank test for paired samples was used for the comparison of non-stimulated Tg levels at each follow-up assessment. Mann-Withney test was used to compare unpaired groups. P value < 0.05 was considered significant. The statistical analysis was performed using IBM SPSS Statistics 25.

Results

A total of 41 patients were considered for the analysis. Baseline characteristics of patients are listed in **table 1a**. Median age at diagnosis was 56 years. Twenty-four (59%) were women. Papillary thyroid cancer (PTC) was the prevalent histological subtype (80%), 5 (12%) patients had follicular thyroid cancer (FTC) and 3 (8%) patients had poorly differentiated cancer. Thirty-one patients (76%) had *BRAF* V600E mutant cancer, 9 patients had *RAS* mutations (2 *KRAS, 5 NRAS, 2 HRAS*), and one patient was negative for 400 tested genes, including *BRAF* and *RAS*. All but three patients had undergone at least one prior RAI therapy, with a median cumulative activity of 200 mCi. These three patients had no prior RAI treatments: one of them was deemed to be RAI refractory on the basis of a negative diagnostic scan at initial presentation to our institution, in the setting of biopsy-proven and 18FDG-PET avid distant metastases. In the other two cases, the patients had bulky disease and 18FDG-PET avid disease. The latter two patients were started on neoadjuvant systemic therapy prior to their initial thyroidectomy. Sixteen of 41 (39%) had bulky disease, defined as at least one lesion with a diameter ≥ 2 cm. Most of the patients (83%) had disease confined to lungs and nodes (neck and/or mediastinum). Based on the identified somatic mutation, patients were treated with either a

single agent BRAFi (56%) or a MEKi (24%), or the combination of BRAFi and MEKi (COMBO; 20%) (**Table 1b**).

Table 1. a) Study population characteristics (N=41). **b)** Kinase inhibitor (KI) treatment used for redifferentiation therapy.

a)

BASELINE CHARACTERISTICS	**N°**
Age at diagnosis in years, median (range)	56 (34–74)
Sex (%) male	17 (41)
Female	24 (59)
Histology (%) PTC	33 (80)
FTC	5 (12)
PDTC	3 (8)
Driver mutation (%) *BRAF* V600E	31 (76)
RAS (***N***-, ***H***-, or ***K***-)	9 (22)
None detected	1 (2)
Number of prior RAI treatments, median (range)	2 (0-3)
Cumulative administered activity, median (range), mCi	200 (0-517)
Prior treatment for thyroid cancer (%)	
Reoperation	29 (71)
Radiotherapy	17 (41)
Other systemic therapy	8 (20)
Sites of disease (%)	
Lungs, neck and/or mediastinum	34 (83)
Other sites (liver, brain, pleura, bone)	7 (17)
Bulky disease* (%)	16 (39)

b)

Kinase Inhibitor	***N. of patients treated (%)***	***Days of treatment, median (range)***
BRAF inhibitor	23 (56)	147 (24-2344)
MEK inhibitor	10 (24)	58.5 (20-275)
COMBO**	8 (20)	221 (97-559)
Total	*41*	*118 (20-2344)*

PTC, papillary thyroid cancer; FTC, follicular thyroid cancer; PDTC, poorly differentiated thyroid cancer; RAI, radioactive iodine.
*At least one lesion with a diameter ≥ 2 cm
**COMBO=BRAF inhibitor + MEK inhibitor

Of the 31 patients with *BRAF* V600E mutant DTC, 23 were treated with single agent dabrafenib (n=22) or vemurafenib (n=1), and 8 received the COMBO regimen (dabrafenib and trametinib). The other 10 patients (*RAS* mutant and the patient without a detectable mutation) were treated with single

agent MEKi (6 trametinib, 3 cobimetinib, 1 binimetinib). The median duration of KI therapy was 3.9 months **(table 1b**) with a shorter period of treatment for patients treated with MEKi compared to the other 2 groups (p=0.01). This difference can be partly explained by the fact that most patients who received MEKi started the therapy with the intent of redifferentiation.

Uptake on Diagnostic and Post-treatment WBS

Of 41 patients, after redifferentiation therapy, the Dx WBS was positive in 78% (32/41) of patients and it was considered negative in 22% (9/41) of patients. These nine patients with a negative Dx WBX were treated empirically with I-131. The median administered activity of I-131 was 157 mCi (100-255 mCi). The median time between I-131 treatment and post-Tx WBS was 5 days (range, 1-9). On post-Tx WBS, there was uptake in 39/41 (95%) of patients who received therapeutic doses of I-131 (17% of recovered uptake compared to Dx WBS). All *RAS* mutant thyroid cancer patients, who received therapeutic doses of I-131, had uptake, either optimal or suboptimal uptake on post-Tx scan. In *BRAF* mutant DTC, uptake on post-Tx WBS was seen in 29/31 (94%) of patients. In these two cases, the post-Tx WBS was performed within 48 hours from RAI therapy.

Radiological response

The median follow-up period was 13 (1-46) months. Out of 41 patients included in this study, 26 had a long enough follow-up to have at least one CT scan after RAI, thus were evaluable for response. Four out of the 26 patients (all with *BRAF* mutated cancers) had sub-centimeter lesions and were not evaluable with RECIST v1.1 criteria. This left 22 evaluable patients for response by RECIST v1.1 criteria.

The best overall response comparing baseline imaging (prior to KI) to just prior to RAI therapy, was as follows: 32% (7/22) of patients had a partial response, 64% (14/22) had stable disease and 4% of patient (1/22) had progression. After I-131 administration, to evaluate the additional benefit from RAI over that seen on KI, we examined the response rate while off KI comparing the SPECT/CT imaging at the time of RAI administration to the imaging after stopping KI. Most of the study population

(64%; 14/22) had stable disease and 9% (2/22) of patients had a partial response (**figure 2a)**, when compared to imaging taken at the time of RAI administration. Thirty-six percent (8/22) of patients had some degree of decrease in target lesions after RAI. The four patients not evaluable with RECIST criteria had stable disease during and after the redifferentiation strategy and were still off systemic therapy after one year from redifferentiation therapy. Nineteen of 26 patients (73%) were deemed treatment successes. The percentages of treatment successes among patients with optimal (11/13) and suboptimal (7/12) uptake on post-Tx WBS was 85% and 58%, respectively (**figure 2b**). The median (range) time off KI was 15 (2-39) months: 21 (12-39) months among treatment successes and 6 (3-21) months among treatments failures (p=0.02).

Response by tumor mutation

The single patient with no detectable mutations remained stable after RAI (and off KI) for 15 months. Among *RAS* mutant cancer, three patients (3/4, 75%) met criteria for treatment success, showing stable disease with some regression in target lesions. The other one (25%) failed, progressing after 6 months. In the study population of *BRAF* mutant RAIR DTC, 71% (15/21) were deemed treatment successes: most patients (83%, 10/12) who showed optimal uptake on post-Tx scan, had prolonged (more than one year) stable disease or partial response after RAI; among those patients with suboptimal uptake on post-Tx WBS, treatment success occurred in 50% (4/8).

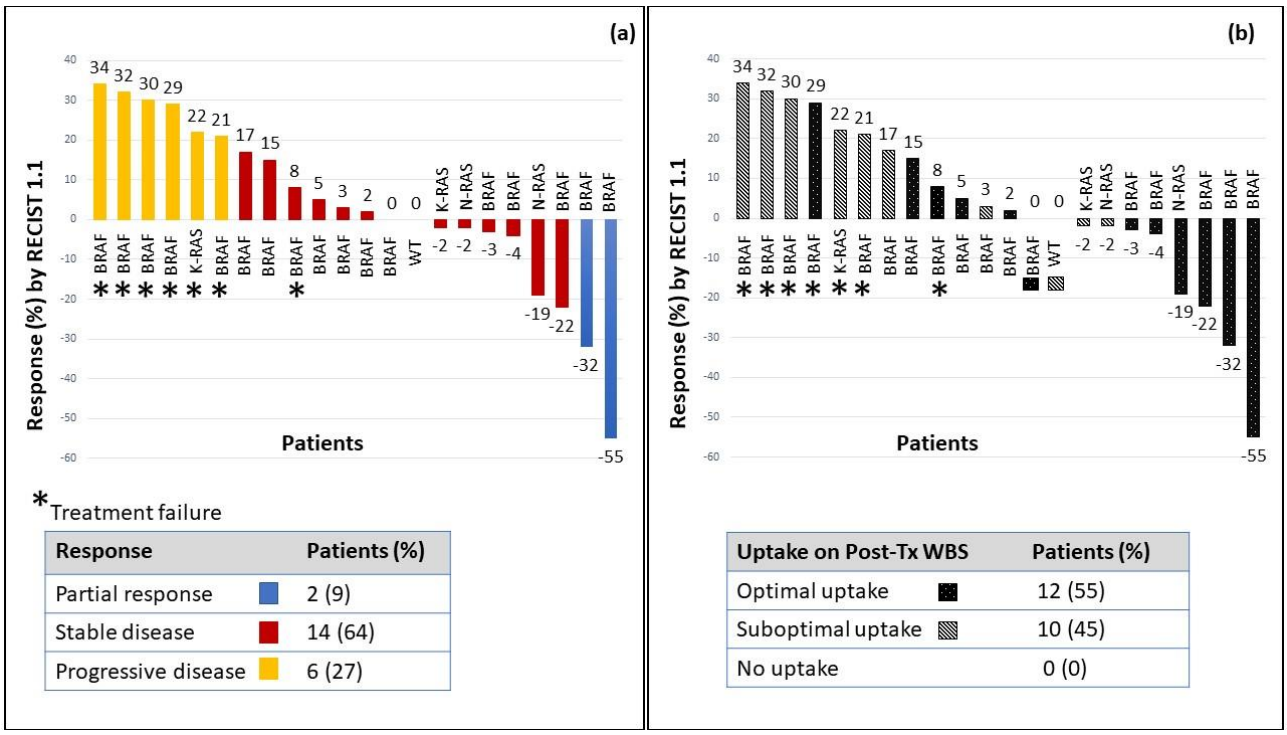

Figure 2. **a) Radiological response to I-131, off KI, according to RECIST 1.1 criteria**. Maximum percentage change in target lesion after RAI administration, using baseline cross sectional images or SPECT-CT at the time of RAI therapy. Blue bars represent patients who had partial response (PR) to RAI, red bars show stable disease (SD) and yellow bars show a response of progressive disease (PD). The figure shows response to RAI according to genotype. The asterisk marks patients who did not meet criteria for treatment success (the absence of progression within one year from 131-I administration and/or the discontinuation of KI after 131-I administration of at least 12 months), thus they are identified as treatment failures. **b) Combination of uptake on post-Treatment whole body scan (post-Tx WBS) and radiological response to 131-I.** Uptake (none, suboptimal or optimal) on post-Tx scan for the cohort of patients (n=22) evaluable for radiological response and according to cancer genotype. Optimal uptake on WBS was defined as intensive accumulation of radioiodine in all tumor lesions (striped bars). When not all lesions accumulated radioiodine or the signal was weak, it was considered as suboptimal uptake (black stippled bars).

Survival outcomes

The median PFS was 29 months (range: 15-43 months) from the start of KI and the median TTP was 20 months (range: 13-27 months) from RAI administration. There was no statistically significant difference in PFS (p=0.2) and TTP (p=0.5) between *BRAF*V600E and non-*BRAF*V600E mutant cancers. Sixty-three percent (26/41) did not restart KI at the time of last follow up visit and/or did not progress. The median PFS and median TTP in patients with optimal versus suboptimal uptake on post-Tx WBS was longer (36 months vs 17 months, 23 months vs 15 months, respectively), however, not statistically significant (p=0.12 and 0.3, respectively).

Biochemical response

A significant increase in non-stimulated Tg level was achieved after redifferentiation therapy, compared to the Tg before starting KI (p=0.01), without evidence of radiological progression. Moreover, a significant reduction in Tg was detected after 3 (p=0.001), 6 (p=0.007) and 12 months (p=0.02) after RAI administration. The Tg level increased again at progression.

SECTION 2 – *Bioinformatic study*

Materials and methods

To identify the switch genes putatively involved in BRAFV600E thyroid carcinogenesis, we analysed RNA-sequencing data, available on The Cancer Genome Atlas (TCGA; *https://cancergenome.nih.gov/*), of thyroid cancers harbouring the *BRAF* V600E mutation. We examined data obtained from 294 thyroid cancers (papillary thyroid cancers). Using SWItchMiner (SWIM), we compared data of *BRAF* V600E mutant thyroid cancers with their corresponding normal samples (59). SWIM software is available online and is downloadable from the supplementary material included in the reference [27]. It works by extracting information contained in complex biological networks. Briefly, SWIM first computes the differentially expressed genes and builds the co-expression network by using the Pearson correlation coefficient between the expression profiles of any pair of genes. In this network, nodes are RNA transcripts and an edge occurs between two nodes if their expression profiles are highly correlated or anti-correlated (i.e. if the absolute value of the Pearson correlation coefficient between the expression profiles of two nodes exceeds a given cut-off). Details about how SWIM works are available online [27]. The topological properties of the co-expression network were investigated by classifying each hub (i.e. nodes with degree at least equal to 5) on the basis of the average Pearson correlation coefficient (APCC) between its expression profile and that of its nearest neighbours. The extent to which hubs are co-expressed with their interaction partners leads to three classifications: date hubs (low positive APCC), party hubs (high positive

APCC), and fight-club hubs (negative APCC). In order to identify disease modules in the network, SWIM makes use of k-means clustering algorithm, employing SSE (sum of squared errors) values to determine the appropriate number of clusters. Then, to assign a role to each node in the network, the heat cartography map is created by evaluating two coordinates related to their intra- and inter-cluster interactions: the clusterphobic coefficient Kp (which measures the links of each node to nodes outside its own cluster) and the within-module degree zg (which measures how "well-connected" each node is within its own cluster). Nodes which have more external than internal links present high Kp values, whereas high zg values denote nodes that are hubs within their community (local hubs). The cartography contains seven regions corresponding to the seven different topological features of the network nodes. In the heat cartography map, each node (i.e. hubs and non-hubs) is coloured according to its APCC value. Switch genes are defined as the subset of fight-club nodes, which are coloured in light blue or blue, present in the R4 region of the map. In particular, switch genes are nodes which have the following features: 1) they mainly interact outside their own cluster (high values of Kp); 2) they are not local hubs (low values of zg), and 3) they are mainly anti-correlated with their interaction partners (negative APCC).

As second step, to identify a putative regulator of switch genes involved in BRAF carcinogenesis of thyroid cancers, we exploited MicroENA Enrichment TURned NETwork (Mienturnet), available at http://userver.bio.uniroma1.it/apps/mienturnet/, to perform miRNA-target enrichment analysis of switch genes identified in the comparison of thyroid cancers with their normal counterpart. This tool, receiving in input a list of genes, infers evidences, both computationally predicted and experimentally validated, of miRNA regulation [26], based on a statistical analysis for over-representation of miRNA-target interactions.

Results

SWIM identified 227 switch genes out of 2167 DEGs in the comparison between thyroid cancers versus normal thyroid tissue [23]. Running SWIM on DEGs, we found that switch genes included protein-coding genes as well as long non-coding RNAs and pseudogenes.

Exploiting Mienturnet to find a regulator of the 227 switch genes involved in BRAF carcinogenesis, miRNA 335-5p was identified as the one with the highest number of interactions. Indeed, about 30% (62 out of 227) switch genes were identified as regulated by miRNA 335-5p (**figure 3, table 2**).

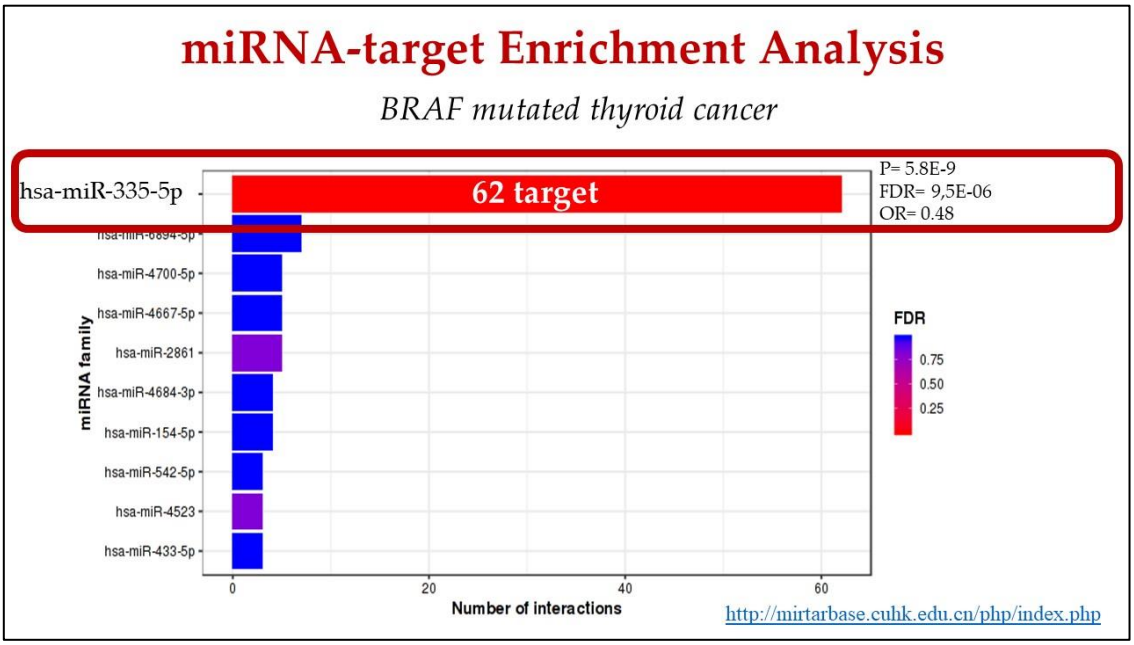

Figure 3: miRNA-target enrichment analysis of switch genes involved in *BRAF*-mediated thyroid cancer carcinogenesis, performed by Mienturnet.

SYT12	GRB7	RNF157	TEKT4	RDH5	PROS1
TNNI1	MFGE8	CDH3	S100A5	VASN	TPD52L1
SERPIN	TACSTD	ABCC3	WDR17	IL36RN	DCSTAMP
SCN1B	DHRS3	PRR15L	HAPLN1	PLEKHG4	PDLIM4
ABTB2	SLC34A	PC	TMC6	SPTBN2	B3GNT8
POU2F	PDE5A	ANO3	PTPRE	SYTL1	MYBPH
KRT19	ACOT6	REN	CATSPERB	B3GNT3	IGF2BP2
SERINC	IYD	CELF4	ST3GAL5	NAB2	UST
DAPK2	ETV5	SLCO4C	NUPR1	MXRA8	SLC2A4
GLS2	NR1D1	CNFN	ASGR1	GDF15	MYOT
				PLCD3	NPTXR

Table 2. List of miR-335-5p targets.

SECTION 3 – *In vitro studies*

Materials and methods

Expression levels of has-miRNA 335-5p were evaluated across several thyroid cell lines, both normal thyroid cell lines and thyroid disease cell lines (benign and tumoral disease).

Cell cultures: With regard to tumor cell lines, we made use of primary and immortalized thyroid cell lines. Primary tumour cell cultures were established, as previously described by our group [28], from the fresh-frozen specimens of patients with papillary thyroid cancer who underwent to surgical resection. Patients provided written informed consent, upon local ethics committee approval (Azienda Universitaria Policlinico Umberto I of Rome; project code Prot. 1184/17). Cell cultures were maintained in culture in specific condition [28-29].

Two commercial immortalized cells lines (8505c and K1; Gibco-BRL Division, Thermo Fisher Scientific, Waltham, Massachusetts, USA) were cultured in DMEM medium; the other ones (BCPAP and SW1736; Gibco-BRL Division, Thermo Fisher Scientific, Waltham, Massachusetts, USA) were cultured in RPMI media. Both of these media containe 10% FBS (Gibco-BRL Division, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and Antibiotic–Antimycotic solution (Gibco-BRL Division, Thermo Fisher Scientific, Waltham, Massachusetts, USA). All cells were incubated at 37°C in an atmosphere of 5% CO2. The gene mutational profile of cell lines used in the present study was reported in Landa et al [30]. All cancer thyroid cell lines harbor *BRAF* mutation.

Characterization of primary tumour cell lines: Patient-derived thyroid cancer cell lines were analysed for the presence of *BRAF* V600E point mutation by Sanger sequencing. DNA from cell lines was isolated using Nucleic Acid and Protein extraction (Macherey-Naghel) and Sanger sequencing analyses of DNA was performed as previously described[31].

Nucleic acid isolation from tissues: DNA and total RNA (containing miRNAs) were simultaneously extracted from DTC tissues using the All Prep DNA/RNA Micro kit (Qiagen) and quantified with Qubit fluorescence-based assays for dsDNA and RNA (Qubit®,Thermo Fisher Scientific).

Cell treatments: Synthetic miR-335-5p (MISSION® microRNA Mimic hsa-miR-335-5p, Sigma Aldrich) or negative control (MISSION® miRNA, Negative Control 1, Sigma Aldrich) were transfected into primary and immortalized thyroid cancer cells lines, at final concentration of 30 nM using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific) for 48h. The control of miR-335-5p overexpression was confirmed using the TaqMan™ MicroRNA Assay in qRT-PCR (Thermo Fisher Scientific).

RNA isolation and quantitative RT-PCR: RNA was isolated from cells using RNeasy Kit (Qiagen, Hilden, Germany) and quantified with Nanodrop 2000 (Thermo Fisher Scientific). The High Capacity cDNA reverse transcription kit (Applied Biosystems Life Technologies, TermoFisher) was used to synthesize cDNA from RNA according to manufacturer's instructions (Applied Biosystems). mRNA expression was analysed on cDNAs using the 7900 Real-Time PCR System, TaqMan Universal Master Mix, TaqMan gene expression Assay-on-Demand according to the manufacturer's instructions (Applied Biosystems). Results were calculated using the $2^{-\Delta\Delta Ct}$ method, normalized to corresponding control samples and expressed as mean±S.D. of three replicates. mRNA expression was evaluated through TaqMan® Gene Expression Assays, using the following assays:

SLC5A5 (Hs00166567_m1), TG (Hs00174974_m1), PAX8 (Hs00247586_m1), TPO (Hs00892519_m1), TSHR (Hs01053846_m1) and GAPDH (Hs02786624_g1) as endogenous control. MiRNA expression was assessed with TaqMan™ MicroRNA Assay for the miR-335-5p (Code: 00546) and U6 (Code: 001973) as endogenous control.

Immunofluorescence: Immunofluorescence experiments were performed using Labtek chamber slides as support. Cells were fixed with 4% paraformaldehyde for 20 min at room temperature,

permeabilized with Triton X-100 (0.1%) diluted in phosphate buffered saline (PBS) and incubated in blocking solution (BSA 3% in PBS). Cells were incubated overnight with the primary antibody diluited 1:50 in BSA 3% (rabbit anti-NIS, polyclonal, Genteck) and for 1h with secondary antibodies (dilution 1:200). Secondary antibodies (488-conjugated anti-mouse and anti-rabbit) were purchased from Thermo Fisher Scientific. Nuclei were Hoechst-counterstained. Images were acquired with a Leica microscope using the filter cube GFP ET.

Iodide uptake assay: Iodide uptake assay was performed as described by Walts et al. [32] Cells were seeded in 96-well microplates the day one; the day after, the cells were transfected with MISSION® miRNA and control, as describe above. After 48h from transfection, iodide uptake assay was performed. The culture medium was replaced twice by the uptake buffer (Hanks' Balanced Salt solution, HBSS, and Hepes at concentration 10 mM). After washing cycle, 80 µl of fresh uptake buffer remained in each well of 96-well plate. Immediately 10 µl of NaI solution (at the concentration of 100 µM) was added in each well. The assay plate was left to stand at 20°C for 60 minutes, in the dark. At the end of incubation, buffer was discarded, and cells were washed once with ice-cold HBSS (Thermo Fisher Scientific). Then, cells were lysed with 100 µl of 0,1 M NaOH solution. The whole lysate was used for "Nonradioactive Iodide Assay kit" according to manufacturer's instructions (Bertin pharma). The intracellular iodide concentrations of samples were determined using linear regression of the standard curve. Results were expressed as specific units of iodide accumulation relative to control.

Results

Hsa-miR-335-5p expression level in (both primary and immortalized) thyroid cancer cell lines, harboring *BRAF* V600E mutation, were significantly lower compared to their control (normal thyroid tissue) (**figure 4**).

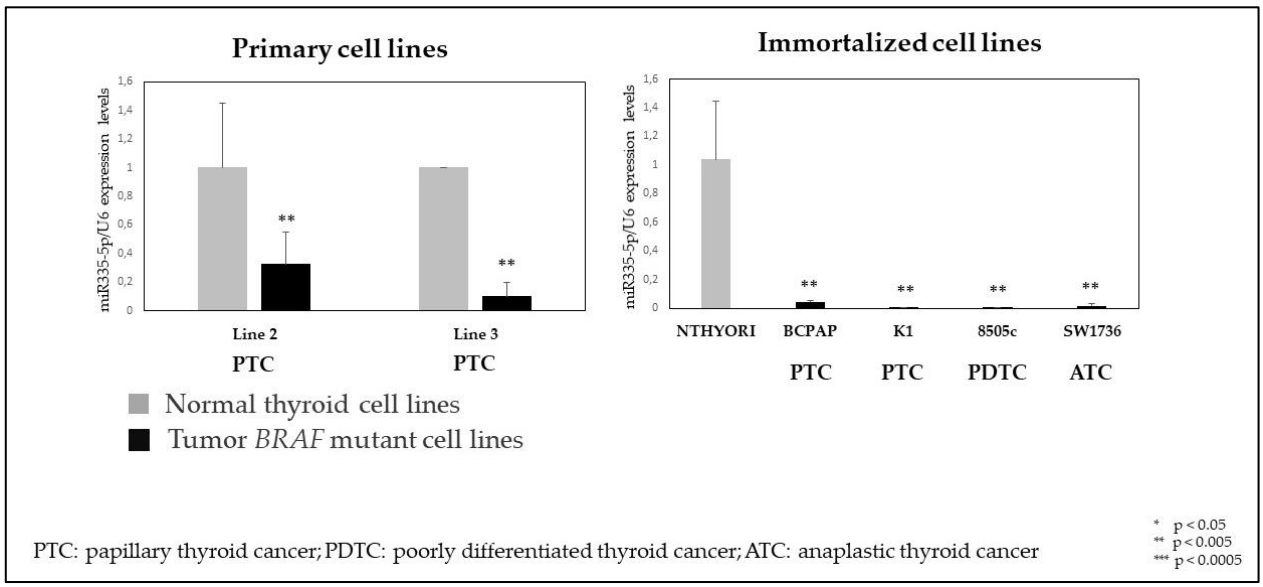

Figure 4. Expression level of hsa-miR-335-5p in thyroid cell lines: normal versus tumor cell lines harboring *BRAF* mutation.

To verify if hsa-miR-335-5p acts on re-differentiation process of *BRAF* mutant thyroid cancer, modifying expression levels and/or function of thyroid specific genes (TG, TPO, NIS, PAX8, TSHR), we performed synthetic miR-335-5p re-expression through transfection.

After transient transfection with miR-335-5p in thyroid cell lines, its over-expression was controlled at transcriptional level in all selected cell lines. After 48 hours from transfection, the analysis of mRNA expression levels of thyroid-specific genes (NIS; thyroglobulin, TG; TSH receptor, TSH-R; TPO; PAX8) revealed that there was no difference in their expression in transfected cells versus controls. This result was similar in primary thyroid cell lines and in the immortalized ones (**figure 5**).

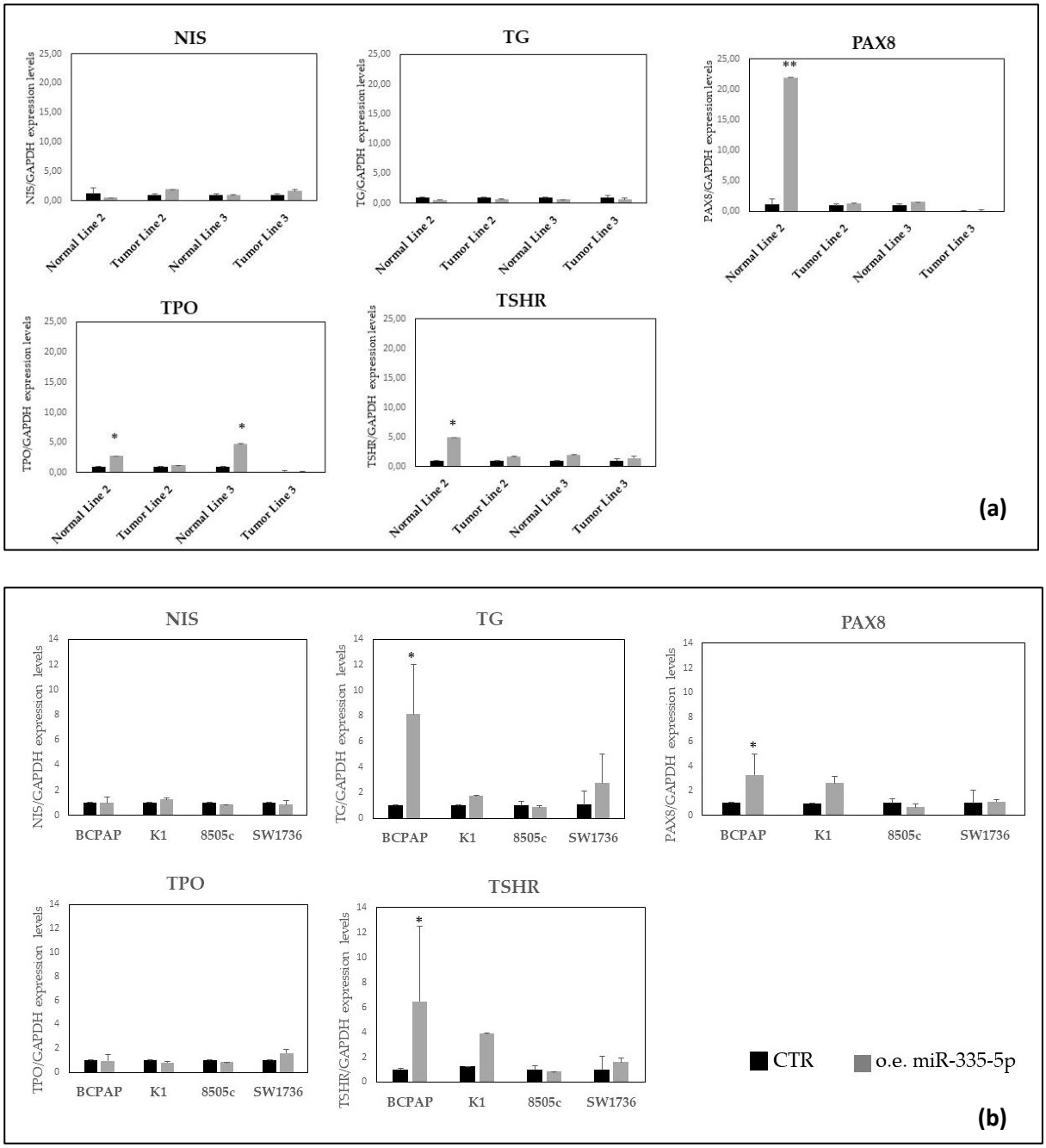

Figure 5: Expression levels of thyroid specific genes in primary thyroid cell lines (a) and immortalized cell lines (b). *CTR: control, o.e.:over-expression*

To further understand the role miR-335-5p on functional differentiation of thyroid cells, we performed immunofluorescence experiments and iodide uptake assay.

Immunofluorescence revealed anti-NIS staining in normal thyroid cell, as expected, and regardless of miR-335-5p overexpression (**figure 6a**). NIS appears at the plasma membrane level and in the

cytoplasm, that suggests the active and inactive form of NIS protein, respectively. NIS staining is absent in primary cell line 3 but it shows up after 48 hours from miR-335-5p transfection (**figure 6b**). A similar result was observed in K1 immortalized cell line (**figure 7a**). On the contrary, in less differentiated thyroid cell lines with a bulkier, thus more aggressive, mutational profile (8505c and SW1736), no substantial differences were observed between control cell lines and the ones over-expressing miR-335-5p (**figures 7b, 8a**).

Looking at quantity of intracellular iodide, evaluated by the uptake assay, in primary cell line 3 and K1, in line with the experiments of immunofluorescence, a statistically significant increase in intracellular iodide was observed after 48 hours from miR335-5p overexpression, compared to control (**figures 6c, 7c**). Iodide uptake was not significantly increased in poorly differentiated tumor lines (8505c and SW1736) (**figures 7d, 8b**).

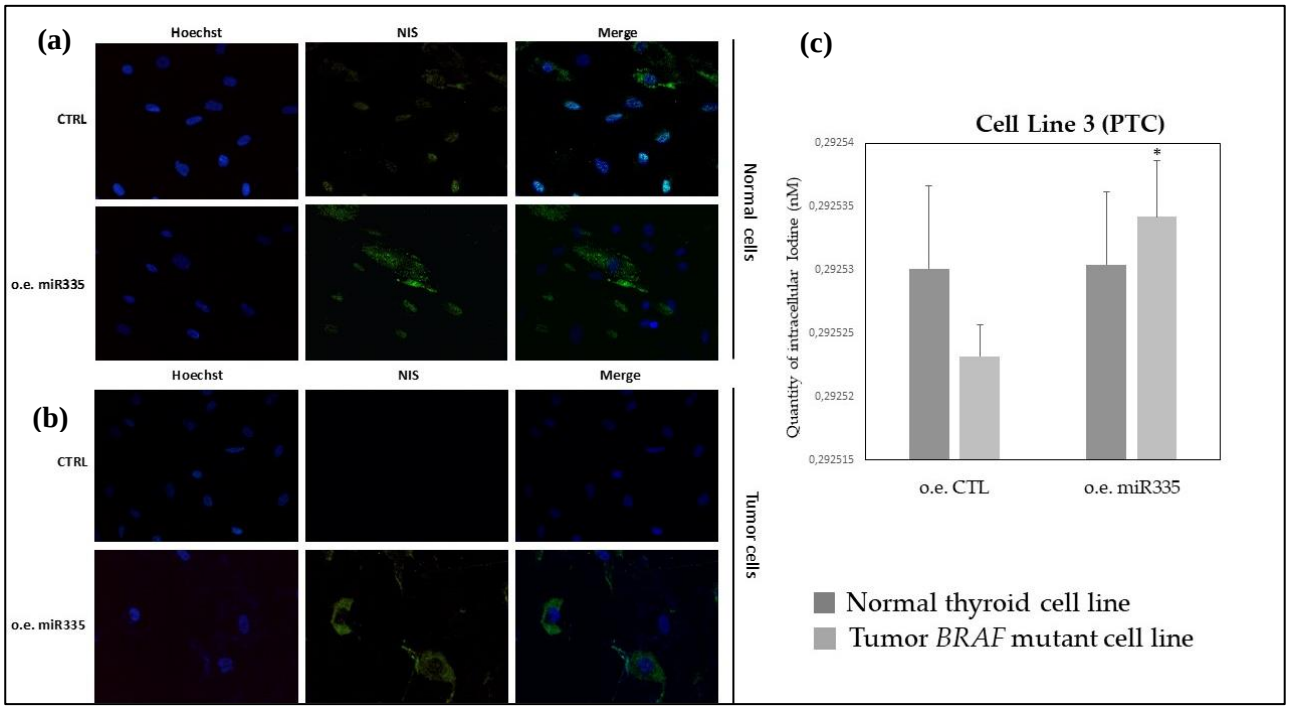

Figure 6. (a) Immunofluorescence revealed anti-NIS staining in normal thyroid cell and regardless of miR-335-5p overexpression; **(b)** NIS staining is absent in primary cell line 3 (ctrl: control) but it shows up after 48 hours from miR-335-5p transfection; **(c)** intracellular iodide, evaluated by the uptake assay, in primary tumor cell line 3 versus normal thyroid tissue, without (control) and after miR335-5p overexpression.

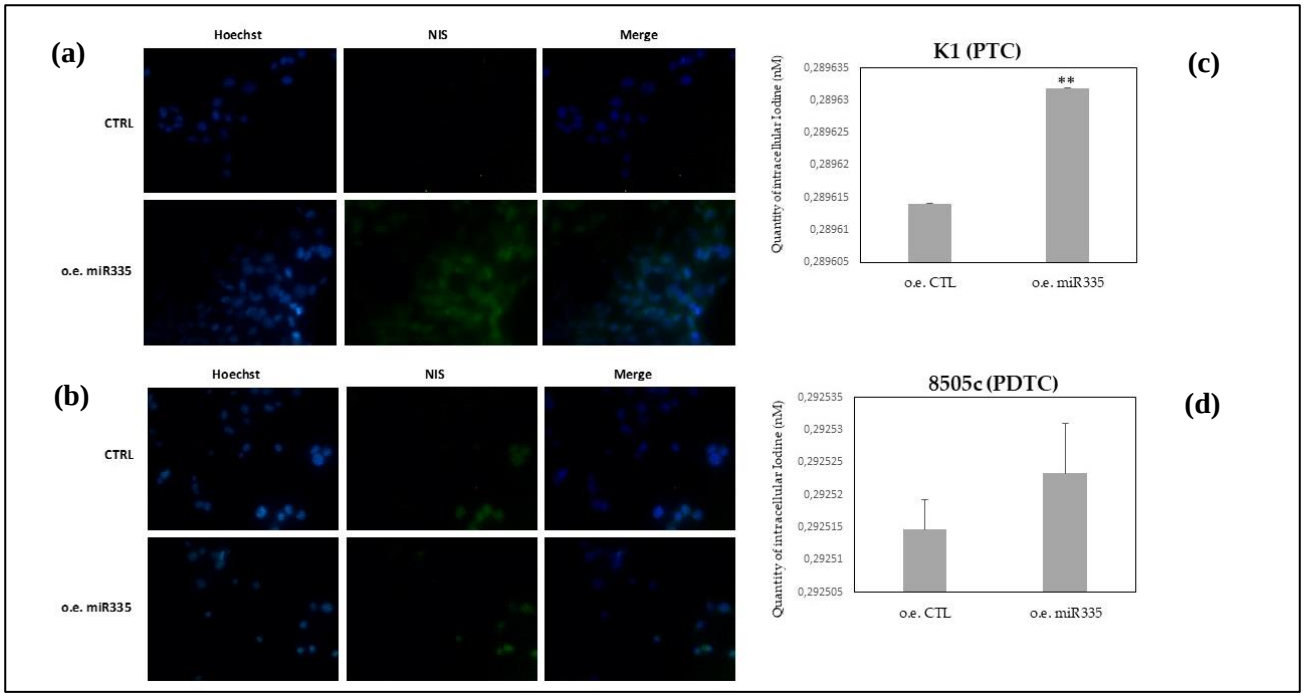

Figure 7. (**a-b)** NIS staining by immunofluorescence in K1 (PTC: papillary thyroid cell) cell line and 8505c (PDTC: poorly differentiated thyroid cancer); **(c-d)** intracellular iodide, evaluated by the uptake assay, in these cell lines, after 48 hours from miR335-5p overexpression, compared to control.

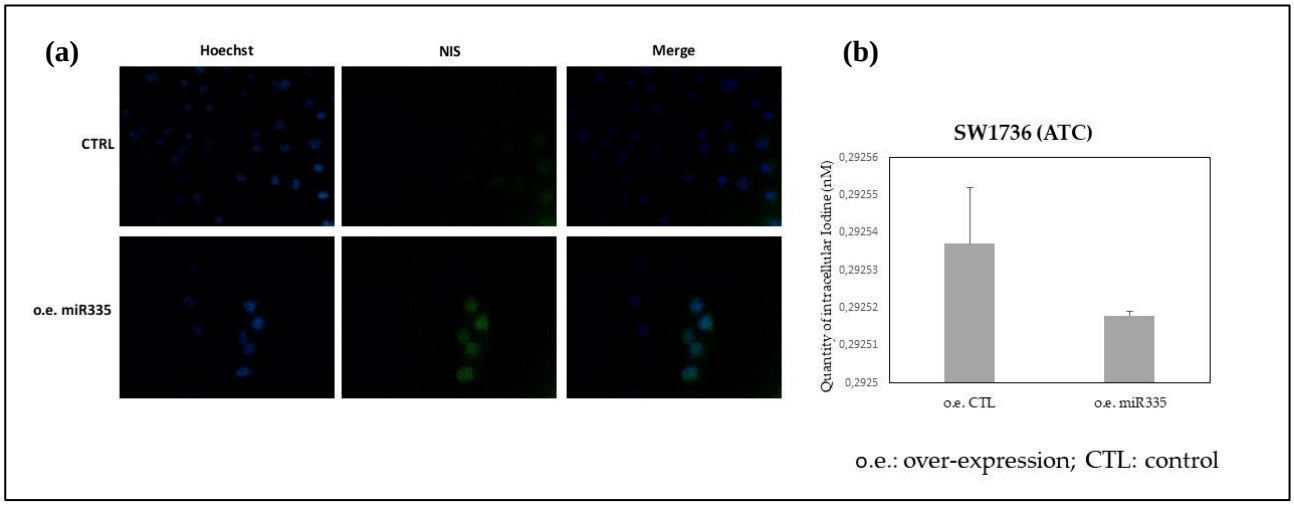

Figure 8. **(a)** NIS staining by immunofluorescence in SW1736 cell line (ATC: anaplastic thyroid cancer) in control (CTRL) and after 48 hours from miR-335-5p transfection; **(b)** intracellular iodide, evaluated by the uptake assay, in SW1736 cell line without (control) and after miR335-5p overexpression.

DISCUSSION

Redifferentiation strategy with BRAFi and MEKi in metastatic RAIR DTC has been demonstrated to restore RAI uptake and result in objective response, both in pilot studies and retrospective experiences [18, 19, 21, 33]. These studies showed an objective response to resensitization treatment (KI plus RAI) in a percentage ranging between 25 and 62% of patients who successfully completed the protocol schedule. These studies had small sample size and they did not report if the efficacy in redifferentiation therapy was associated with prolongation of TTP, PFS, time to start other KIs and overall survival. Our larger and retrospective experience with redifferentiation therapy followed by RAI administration confirms some previous finding and adds some data to the topic. We saw uptake, optimal or suboptimal, on post-Tx scan in 95% of patients included in the analysis. Ninety-four percent of *BRAF* mutated DTC patients had RAI uptake after redifferentiation therapy. For patients evaluable for radiological response after I-131, 73% of patients benefited from redifferentiation strategy, reaching stable or partial response for more than 12 months. TTP and PFS after redifferentiation therapy plus RAI were 20 and 29 months, respectively, with a median time off KI of 15 months. The results of this study should be interpreted with caution due to several limitations, including the retrospective nature of this study, the lack of control arm, the variability of the redifferentiation protocol, the different intent of starting KI therapy (not only for the purpose redifferentiation), and the timing of radiological evaluation. Development in the area of redifferentiation therapy is hampered by an absence of consensus regarding the definition of radioactive iodine-refractory differentiated thyroid cancer.

The investigation of mutational landscape of *BRAF* mutated DTC, allowed to identify a subset of switch genes, 30% of which is regulated by hsa-miR-335-5p.

A growing number of studies have been conducted to show that miR-335 is a significant cancer-associated miRNA. It has been demonstrated to be widely dysregulated in various human cancers

(breast cancer, hepatocellular cancer, colorectal cancer, meningioma and prostate cancer), playing critical roles in tumorigenesis and cancer progression [34]. The expression level of miR-335 is upregulated or down-regulated depending on the type of cancer. MiR-335-5p role has been studied in various biological process: tumorigenesis, proliferation, apoptosis, migration, metastasis, invasion[34]. It has also been studied as prognostic and diagnostic marker. Indeed, miR-335 expression levels have been correlated to clinical and pathological (TNM) stage, local recurrence, histologic grade, lymph node and distant metastases.

With regard to the issue of differentiation, studies on neuroblastoma showed that downregulation of miR-335 in non-neuronal cells (glial/smooth muscle precursor cells) modulates expression levels of HAND1 and JAG1, known modulators of neuronal differentiation[35]. Evidence suggests miR-335 maintains the non-neuronal features possibly by blocking neuronal differentiation. Other findings suggest that miR-335 is potently required for differentiation of malignant glioma cells induced by cAMP/PKA pathway activation, and it may act as an important fate determinant to control the differentiation status of malignant gliomas[36].

A few studies have explored miR-335-5p role in thyroid cancer. Previous experiences showed that the expression level of miR-335-5p in TPC-1 (human thyroid cancer cell line) was down-regulated, and miR-335-5p could inhibit the proliferation of thyroid cancer cells [37]. MiR-335-5p has been demonstrated to be the most significant down-regulated miR comparing papillary thyroid cancer to adjacent non-tumor tissues[38]. A network-based integrative analysis of follicular thyroid carcinoma and benign follicular thyroid adenoma identified miR-335-5p as a molecular mechanism that differs between the two subtypes [39].

So far, to the best of our knowledge, mir-335-5p role in dedifferentiation and redifferentiation process of thyroid cancer has not been investigated. We show for the first time that miRNA-335-5p is lost from normal thyroid tissue to primary or continuous *BRAF* mutated cell lines. Its over-expression in

thyroid tumor cell lines harboring *BRAF* mutation induces the activation of NIS protein and increases intra-cellular iodide uptake, supporting our hypothesis of miR-335 role in redifferentiation of *BRAF* mutated papillary thyroid cancer. This role seems to be more prominent in papillary thyroid cancer than in undifferentiated forms of thyroid cancer. The last result was predictable because the identification of miR-335-5p came from an analysis carried out in PTC from TCGA, where poorly differentiated and undifferentiated forms of cancer were not included. Further studies based on the evaluation of miR-335-5p levels in RAI-refractory thyroid cancer patients are needed to confirm that over-expression of this miRNA may have a role in restoring RAI responsiveness. At that point, a combinational approach, based on the inhibition of BRAF pathway and over-expression of miR-335-5p may be proposed to fight RAI refractoriness in thyroid cancer.

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