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TESI DI DOTTORATO

INSIGHT INTO NOVEL BIOMARKERS AND DIAGNOSTIC APPROACHES FOR THYROID CARCINOMA

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ABSTRACT

Thyroid cancer is the most common endocrine tumor and its prevalence is constantly rising. This rise can be partly attributed to advancements in diagnostic imaging and molecular testing, which have enhanced the early detection of both benign and aggressive forms of the disease. Thyroid cancer is a highly heterogeneous condition, comprising multiple subtypes with distinct genetic profiles and clinical behaviors, as highlighted in the 2022 World Health Organization classification.

Despite advances in diagnostic tools, the clinical management of thyroid cancer patients remains challenging due to the reliance on procedures like ultrasound, fine-needle aspiration (FNA), and liquid biopsies, which can sometimes generate imprecise results. Consequently, there is a growing need to identify non-invasive biomarkers that could help to improve early diagnosis and prognosis.

To address this topic, my research was focused on molecular markers involved in thyroid tumor progression and their potential as biomarkers. In details, my work was focused on exploring the role of selected non-coding RNAs and protein-coding genes in thyroid cancer progression.

Among the potential candidates, microRNAs (miRNAs), small non-coding RNAs that regulate gene expression, have emerged as potential biomarkers in oncology. In detail, my PhD research focused on the characterization of the role of miR-331-5p in thyroid cancer and on assessing its potential as a novel biomarker. Through *in silico* analysis, miR-331-5p was statistically downregulated in thyroid cancer tissues compared to normal samples. Through *in vitro* experiments, I analyzed the effect of miR-331-5p on cellular behavior in both papillary and anaplastic thyroid cancer cell lines. Functional studies demonstrated that the overexpression of miR-331-5p significantly reduced thyroid cancer cell motility, whereas silencing of this miRNA increased cellular migration. These findings highlight the crucial role of miR-331-5p in regulating cellular migration and invasion. Proteomic screening identified eight potential targets down-regulated by miR-331-5p, with BID being validated as a direct target. Data from The Cancer Genome Atlas-Thyroid Cancer (TCGA-THCA) further revealed an inverse correlation between BID levels and miR-331-5p expression in thyroid cancer tissues. These results suggest that the dysregulation of the miR-331-5p/BID axis may enhance tumor aggressiveness, providing novel insight into the biological mechanisms driving thyroid cancer progression.

Finally, my PhD research also explored, through a comprehensive analysis of the literature, the integration of radiomic features and genomic data in thyroid cancer as a novel approach to improve diagnosis of thyroid cancer patients.

Radiomics, which involves extracting quantitative features from medical images, has shown promise in identifying patterns linked to genetic and molecular tumor characteristics. By correlating radiomic features with genomic data, this innovative, non-invasive approach aims to enhance both the diagnosis and prognosis of thyroid cancer, potentially improving clinical decision-making and patient management.

Overall, the findings presented in this thesis shed light on promising novel diagnostic biomarkers for thyroid cancer. Future research should aim to validate these results through *in vivo* studies and to explore their integration with imaging-derived features to enhance personalized approaches to thyroid cancer management.

1. INTRODUCTION

1.1 Thyroid cancer

Over the last few decades, there has been a significant increase in the incidence of thyroid cancer (TC), the most common endocrine malignancy [1]. This rise is partly attributed to advancements in diagnostic techniques such as imaging and molecular testing, which have facilitated earlier detection of both benign and more aggressive forms of the disease [2]. However, the molecular landscape of thyroid cancer underscores its heterogeneity, as it consists of multiple subtypes, each with distinct genetic profiles and clinical behaviors.

According to the 2022 World Health Organization (WHO) classification [3], thyroid tumors derived from follicular cells are primarily categorized into three well-differentiated subtypes:

- papillary thyroid cancer (PTC);
- follicular thyroid cancer (FTC);
- oncocytic carcinoma of the thyroid (OCA, previously known as Hürthle cancer cell).

These subtypes collectively form the group known as differentiated thyroid cancers (DTCs), with PTC being the most prevalent, followed by FTC. Both PTC and FTC are generally associated with favorable prognoses [4], with a 5-year survival rate close to 99% when localized in the thyroid gland [1].

PTC has multiple histological subtypes, including the classic form, the tall-cell variant, and the follicular variant, which can either be encapsulated or infiltrative. The follicular variant of PTC, particularly in its encapsulated form, shares several characteristics with FTC.

Anaplastic or undifferentiated thyroid carcinomas (ATCs), the most aggressive forms of TC, are rare with a poor prognosis and a survival of less than 6 months from diagnosis [5].

Poorly differentiated thyroid cancer (PDTC) and differentiated high-grade thyroid carcinoma (DHGTC), a new category described in the 2022 WHO classification [3], set between well-differentiated thyroid cancers and ATCs, with a 5-year survival rate of around 70% [6].

Clinical features of follicular cell-derived thyroid cancer types are summarized in Table 1.

Table 1. Clinical features of follicular cell-derived TC types.

Type	Prevalence	Differentiation	Histological features	Route of spread; frequency	Prognosis
PTC	80%–85%	Good	Papillae, nuclear features	Local lymph node via lymphatic spread; rare	Good
FTC	5%–10%	Good	Follicles, absence of PTC nuclear features	Distant metastasis via haematogenous spread, usually to lungs and bone; rare	Good
OCA	~5%	Good	Abundance of mitochondria, eosinophilic cytoplasm	Local lymph nodes and distant metastasis; intermediate	Good – intermediate
DHGTC	<5%	Good	Papillary, follicular or solid growth; high mitotic rate and/or necrosis	Local lymph node via lymphatic spread; frequent	Intermediate
PDTC	<5%	Poor	Solid or trabecular or insular growth; absence of PTC features	Invasive growth, lymph node and distant metastasis; frequent	Intermediate
ATC	~2%	Absent	Undifferentiated growth, spindle or squamous cells, macrophage infiltration	Invasive growth, lymph node and distant metastasis; very frequent	Poor

Abbreviations: **ATC**, anaplastic thyroid cancer; **DHGTC**, differentiated high-grade thyroid cancer; **FTC**, follicular thyroid cancer; **OCA**, oncocytic cancer of the thyroid; **PDTC**, poorly differentiated thyroid cancer; **PTC**, papillary thyroid cancer. *Modified from Landa et al., 2024* [7].

Medullary thyroid cancers (MTCs), which arise from the parafollicular or C cells of the thyroid gland, are not part of this thesis.

1.1.1 Overview of diagnosis and management of follicular cell-derived thyroid cancer patients

Thyroid nodules are often detected during routine physical examinations or incidentally during imaging studies. Approximately 7-15% of these nodules are confirmed as malignant based on ultrasound (US) characteristics and cytological evaluation [2, 8]. Current guidelines [4] do not recommend biopsies for nodules smaller than 1 cm unless specific US features, such as macrocalcifications or irregular margins, suggest malignancy. Widely recognized diagnostic international guidelines, including the American Thyroid Association (ATA) guidelines [4], the American College of Radiology Thyroid Imaging and Reporting and Data System (ACRTI-RADS) [9], and the European Thyroid Imaging and Reporting Data System (EU-TIRADS) [10], help to assess malignancy risk of thyroid nodules and to determine the need for fine-needle aspiration (FNA). FNA cytology results are categorized using the Bethesda system, which standardizes cytopathological interpretation. For nodules with indeterminate cytology (Bethesda III and IV) molecular testing is particularly helpful providing additional information to guide management. Conversely, molecular testing is generally not required for clearly benign (Bethesda II) or clearly malignant (Bethesda V or VI) cases [11].

The routine measurement of serum thyroglobulin (Tg) levels is not recommended for evaluating of thyroid nodules, as Tg has limited diagnostic value when the thyroid is intact, being produced by both benign and malignant thyroid cells. [12].

For DTC, cervical US plays an important role in the pretreatment evaluation. It provides detailed information on tumor size, location, lymph node involvement and local invasion. Decisions regarding primary treatment are based on a comprehensive risk assessment that integrates clinical, imaging and cytological findings [13]. For small, unifocal tumors (< 4cm) without extrathyroidal extension or lymph node metastasis, lobectomy is often recommended [4].

After surgery, risk assessment is refined using on pathological findings. These findings help determine whether additional interventions, such as radioiodine (RAI) ablation or thyroid-stimulating hormone (TSH) suppressing therapy, are required. TSH suppression, achieved through thyroid hormone therapy, is a standard component of DTC management, as it reduces recurrence and cancer-related mortality by inhibiting the growth of both normal thyrocytes and most thyroid cancer cells [14].

RAI therapy has traditionally been used after total thyroidectomy to eliminate residual normal thyroid tissue, which could incorporate iodine and produce Tg. This complicates the detection of residual or recurrent cancer using imaging or serum thyroglobulin assays [15]. However, current guidelines advocate a more selective use of RAI, tailoring its application to individual patient risk levels and using the lowest effective dose to minimize adverse effects [4].

Beyond diagnostic, RAI serves as an adjuvant therapy to destroy microscopic neoplastic foci within thyroid remnants or distant sites. While this can improve long-term outcomes, it is not without risk, including short-term morbidity and an increased risk of secondary malignancies [16]. For patients with distant metastatic disease that is RAI-sensitive, this therapy can also serve as a treatment option [17].

ATC, a rare and aggressive subtype of TC, presents unique diagnostic and therapeutic challenges. ATC cells often lose expression of thyroid-specific markers and may exhibit diverse histological patterns, such as squamoid or spindle cell features, making diagnosis difficult. Once diagnosed, the management of ATC prioritizes airway assessment and staging. For patients with resectable ATC (stages IVA and IVB), treatment typically involves aggressive surgical resection followed by chemoradiation. In advanced cases with metastatic disease (stage IVC), surgery is generally limited, and palliative strategies such as radiation or systemic therapy become the focus of care [5].

1.1.2 Molecular profiling of follicular cell-derived thyroid cancer

The Cancer Genome Atlas - THyroid CAncer (TCGA-THCA) project allowed the molecular characterization of TC by analyzing 496 papillary thyroid cancer (PTC) samples. The study highlighted the heterogeneity of PTC by revealing that approximately 90% of cases are driven by events activating the mitogen-activated protein kinase (MAPK) pathway. This activation primarily arises through mutually exclusive mutations in the oncogenes BRAF or RAS, with a smaller subset of thyroid cancers initiated by gene rearrangements involving receptor tyrosine kinase (RTK) genes, most commonly RET [18].

The most frequent genetic alteration in PTCs is the BRAFV600E mutation, present in approximately 40-65% of cases, resulting in a lower response to RAI therapy [19, 20].

In contrast, RAS mutations are identified in approximately 13% of PTCs and are significantly more prevalent in follicular thyroid carcinomas [2]. The RAS genes (HRAS, NRAS and

KRAS) include a family of GTP binding proteins that are involved in the activation of the MAPK pathway and affect cell differentiation, proliferation and survival [21, 22].
Another mutation impacting tumor progression involves the enzyme telomerase reverse transcriptase (TERT). Mutations in the TERT promoter such as C228T and C250T (~9.4%) [18], have been linked to the advancement of TC, with a higher prevalence observed in aggressive form of the disease. These mutations often co-occur with primary driver mutations in BRAF or RAS genes, further contributing to malignancy [7, 23].
PTCs characterized by oncogenic gene fusions often exhibit specific clinicopathologic features. One of the most notable fusions involves the RET receptor tyrosine kinase (5 -10%), commonly referred as RET/PTC. RET is now known to fuse with over 20 distinct genes [24]. Data from TCGA-THCA revealed that RET/PTC fusions consistently preserve and over-express the RET tyrosine kinase domain, which promotes continuous RET activation, resulting in MAPK pathway signaling [18].
Additionally, the TGCA-THCA project has identified other PTC-related fusions mutually exclusive, including those involving NTRK, ALK and PAX8/PPARγ [18].
This molecular heterogeneity in PTC contributes to tumor behavior, response to treatment, and prognosis [25].

In follicular thyroid cancers (FTCs), key molecular alterations primarily consist in point mutations within the RAS gene's GTP-binding or GTPase domains, occurring in nearly 49% of cases and resulting in its loss of function [26]. Approximately 25% of FTCs feature a PAX8/PPARγ rearrangement, where the transcription factor PAX8 regulates the expression of PPARγ, a nuclear receptor. Additionally, smaller groups of FTCs cases exhibit mutation in genes such as EIF1AX (5-20%), PTEN (5-10%) and activating mutations of PIK3CA (~10%) [7, 27].

The oncocytic carcinoma (OCA) subtype is distinct due to specific characteristics, including mutations that impair mitochondrial respiratory complex I genes. This subtype shows ~ 45% frequency of RAS family mutations, with an absence of BRAFV600E or RET/PTC rearrangement. OCA tumors display gene expression patterns enriched in the PI3K/PTEN/Akt signaling and WNT/ β-catenin pathway. Mutations in TP53 (~25%), EIF1AX (~11%) and PTEN (~5%) genes are also observed in this malignancy [22, 28, 29]. Approximately 22% of OCA tumors harbored also TERT promoter mutations [28].

Advanced thyroid cancers, such as differentiated high-grade thyroid cancer (DHGTC), poorly differentiated thyroid cancer (PDTC), and anaplastic thyroid cancer (ATC) are thought to arise directly from DTCs or to share a common cellular origin. Next-generation sequencing (NGS) studies examining mutational landscapes across the spectrum of TC have highlighted that advanced forms frequently retain the main driver mutations found in DTCs [7, 18, 30]. Building upon these foundational mutations, advanced cancers acquire further genetic changes in key oncogenes, with a pattern of mutation accumulation that aligns with tumor aggressiveness: ATC being the most aggressive, PDTC and DHGTC intermediate, and PTC the least.

Whole-exome sequencing on various ATC regions, including areas of well-differentiated thyroid tissue, suggests an early divergence in ATC evolution, marked by shared clonal mutations in genes like BRAF or RAS [31]. This supports a model in which advanced thyroid cancers gradually evolve from well-differentiated cancers through stepwise acquisition of crucial mutations, creating a mutation-driven progression [7].

In PDTC, common mutations mirror those seen in DTC, with BRAFV600E present in ~33% and RAS in ~28% of cases.

Additional frequent mutations of PDTC include TERT (~40%), EIF1AX (~11%), TP53 (~8%), and histone methyltransferases like KMT2A, KMT2C, and KMT2D. Less common mutations involve mismatch repair genes, such as MSH2, MSH6, and MLH1, along with ARID1B, PIK3CA, and PTEN [32].

ATC shows further genomic instability with features like aneuploidy, alterations in copy number, and higher overall mutation rates, often co-occurring with established driver mutations for DTCs such as mutations in BRAFV600E (19-45%) and in RAS family (9.5-27%) [33]. Compared to PDTC, ATC has higher mutation rates in TP53 (>73%), TERT promoter (~73%), PIK3CA (~18%), PTEN (~15%) and EIF1AX (~9%) [19].

Genes in SWI/SNF chromatin remodeling complexes and histone methyltransferases also frequently harbor mutations in aggressive thyroid cancers, including PDTC (~6%) and ATC (18-36%) [22, 33].

Table 2 summarizes the main genetic alterations found in follicular cell-derived TC.

Table 2. Commonly mutated genes across the follicular cell-derived TC.

	PTC	FTC	OCA	PDTC	ATC
BRAFV600E	40 -65%	-	-	~33%	18 -45%
EIF1AX	~1.5%	5 -20%	~11%	~11%	~9%
PAX8/PPARγ	0.8%	~25%	-	~4%	-
PIK3CA	-	~10%	-	-	~18%
PTEN	~1%	5 -10%	~5%	~4%	~15%
RAS	~13%	~49%	~45%	~28%	9.5 -27%
RET rearrangement	5 -10%	-	-	~14%	-
SWI/SNF	-	-	-	~6%	18 -36%
TERT promoter	9.4%	-	~22%	~40%	~73%
TP53	~6%	5.1 -9.7%	~25%	~8%	>73%

Abbreviations: **ATC**, anaplastic thyroid cancer; **FTC**, follicular thyroid cancer; **OCA**, oncocytic cancer of the thyroid; **PDTC**, poorly differentiated thyroid cancer; **PTC**, papillary thyroid cancer.

1.1.3 Systemic therapies for follicular cell-derived thyroid cancer

Most DTCs patients, have a very positive prognosis, with nearly a 99% 5-year survival rate [1]; however, 5-10% of patients develop distant metastasis [34]. While the prognosis remains good for patients whose metastatic disease still responds to radioactive iodine (RAI) therapy, nearly half of metastatic DTCs eventually become resistant to RAI. This resistance is associated with a significantly worse prognosis, with only about 10% of patients surviving beyond 10 years [4, 35]. Such aggressive, RAI-refractory thyroid cancers present a considerable clinical challenge, creating an urgent need for novel therapies. Systemic treatment with kinase inhibitors becomes an option when disease progresses despite prior treatments, or if there is extensive progression in multiple organs [36].

The growing understanding of the molecular drivers of DTCs, especially in cases where the disease does not respond to RAI, has led to the development of targeted therapies. Mutation profiling has shown value in predicting tumor behavior and RAI resistance [37]. For example, it is well-established that tumors driven by BRAFV600E mutations have increased MAPK pathway activity, which substantially decreases expression of genes related to iodine uptake and metabolism, like the sodium-iodide symporter (NIS) [18, 38].

In 2013, the Food and Drug Administration (FDA) approved Sorafenib [39], followed by Lenvatinib in 2015 [40], as treatments for patients with locally advanced or metastatic RAI-refractory DTC that has progressed. Both are multikinase inhibitors (MKIs) that target angiogenesis through inhibition of the vascular endothelial growth factor receptors (VEGF-R), with additional effects on kinases such as RET, fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) receptors [36].

Later, in 2021, Cabozantinib became the third FDA-approved MKI, offering a second-line option for advanced DTC after prior systemic therapy. Cabozantinib inhibits multiple tyrosine kinases involved in tumor growth and angiogenesis, including VEGF-R2, AXL, c-MET, and RET [41].

While the drugs above do not require target identification, there are additional therapies available that set on identifying specific genetic alterations.

The BRAFV600E mutation, the most common oncogenic driver in papillary thyroid cancers (PTCs), is found in about 60% of cases, making it a prime target for therapies. The BRAF inhibitor Dabrafenib has shown the ability to restore RAI uptake and efficacy, with a partial response rate of ~35% in patients with metastatic or inoperable disease. Additionally, Dabrafenib in combination with Trametinib, a MEK inhibitor, was approved by FDA for BRAFV600E-mutated anaplastic thyroid cancer (ATC) and for BRAFV600E-mutated solid tumors that have progressed after other treatments [42].

RET alterations also contribute to thyroid tumorigenesis, making it a promising therapeutic target. Selpercatinib (LOXO-292) [43] and Pralsetinib (BLU-667) [44], both potent RET kinase inhibitors, were approved by the FDA in 2020 for RET-fusion DTCs and for RET-mutant medullary thyroid carcinomas.

Although rare, it is critical to identify NTRK-fusion thyroid carcinomas, as two FDA-approved tropomyosin receptor kinase (TRK) inhibitors, Larotrectinib [45] and Entrectinib [46], have demonstrated efficacy and safety in metastatic patients harboring this gene fusion.

The development of kinase inhibitors for mutations associated with TC has significantly improved the outcomes of patients with advanced DTC. Table 4 summarizes all FDA-approved kinase inhibitors for advanced DTCs.

Table 4. FDA-approved kinase inhibitors for follicular cell-derived TC.

Drug	Targets	Year of approval
Cabozantinib	VEGFR2, AXL, c-MET, RET	2021
Dabrafenib + trametinib	Dabrafenib: BRAFV600E Trametinib: MEK	2018, 2022
Entrectinib	NTRK fusion	2019
Larotrectinib	NTRK fusion	2018
Lenvatinib	VEGFR, FGFR, PDGFR	2015
Pralsetinib	RET fusion	2020
Selperactinib	RET fusion	2020
Sorafenib	VEGFR, FGFR, PDGFR	2013

Modified from Landa et al., 2024 [7].

Targeted therapies have improved outcomes for patients with advanced DTCs. However, to further optimize patient treatment and enhance the accuracy of TC diagnosis and management, there is increasing evidence supporting the need to develop novel comprehensive diagnostic approaches.

Within this framework, the primary objective of my thesis is to identify novel biomarkers for TC, particularly those correlated with aggressive and treatment-resistant forms of the disease, and to characterize their roles in tumor progression.

Starting on insights from the literature, my research focuses on two promising classes of biomarkers: non-coding RNAs, specifically microRNAs with a focus on miR-331-5p, and protein-coding genes, with a focus on KCTD15, a member of the potassium (K+) channel tetramerization domain (KCTD) protein family.

Furthermore, as part of my research, to gain insight into novel diagnostic approaches for TC, I also conducted a systematic review to evaluate the current evidences on radiogenomics in TC.

1.2 microRNAs as potential biomarkers in thyroid cancer

The prognosis for DTC has improved significantly thanks to advances in early detection, effective surgical methods, and RAI therapy [13]. However, RAI resistance presents a challenge to effective treatment in a subset of DTC patient. To address this challenge, identifying molecular markers that reveal the underlying pathology during tumor progression has become essential.

MicroRNAs (miRNAs) have emerged as promising biomarkers for TC and as regulatory agents influencing cancer-related processes. Studies have demonstrated that miRNAs dysregulation plays a key role in the development of PTC [47]. Moreover, specific miRNA expression patterns are associated with different histopathological subtypes of PTC, as well as distinct recurrence risks, suggesting their potential as markers for prognosis [48].

1.2.1 miRNAs biogenesis

MiRNAs, small non-coding RNAs typically around 22 nucleotides in length, are crucial in modulating gene expression at transcriptional and post-transcriptional levels. By targeting messenger RNAs (mRNAs), they influence various cellular processes, including cell proliferation, differentiation, angiogenesis, and apoptosis [49, 50].

MiRNAs are initially transcribed by RNA polymerases II, resulting in a lengthy hairpin structure known as primary miRNAs (pri-miRNAs) [51]. Around 30% of miRNAs originate from introns of protein-coding genes, while the majority come from specific miRNA gene loci. Each pri-miRNA may yield a single miRNA or clusters of multiple miRNAs that share a common primary transcript. These pri-miRNAs undergo processing by Microprocessor complex, composed of the DROSHA enzyme, a double-stranded (ds) RNase III, and its RNA-binding partner DGCR8 (or Pasha), which converts them into precursor miRNAs (pre-miRNA) of approximately 70 nucleotide long [52]. Pre-miRNAs are then transported from the nucleus to the cytoplasm by Exportin-5, a double-stranded RNA (dsRNA)-binding protein dependent on Ran-GTP [53].

Once in the cytoplasm, pre-miRNAs are further cleaved by the endonuclease DICER1, producing mature miRNA duplex with a ~22-nucleotide length. DICER1 partners with transactivation-responsive RNA-binding protein (TRBP), which helps stabilize dsRNA.

The guide strand of this miRNA duplex associates with Argonaute proteins, forming an RNA-induced silencing complex (RISC) that includes cofactors such as GW182. The RISC complex binds to the 3'untranslated region (UTR) of target mRNA [54]. If there is a perfect match between miRNA and its mRNA target, the mRNA is cleaved. Otherwise, partial binding leads to translational repression, inhibiting protein synthesis and incorporates the target into cytoplasmatic structures called processing bodies (P-bodies) [55].

The partial complementarity of miRNA-mRNA binding allows individual miRNA to influence multiple genes target, with estimates suggesting a single miRNA can regulate over 200 different genes. Furthermore, many genes contain multiple miRNA target sites in their 3'UTR, allowing cooperative regulation by different miRNAs [47].

The process of miRNAs maturation is illustrated in the Figure 1.

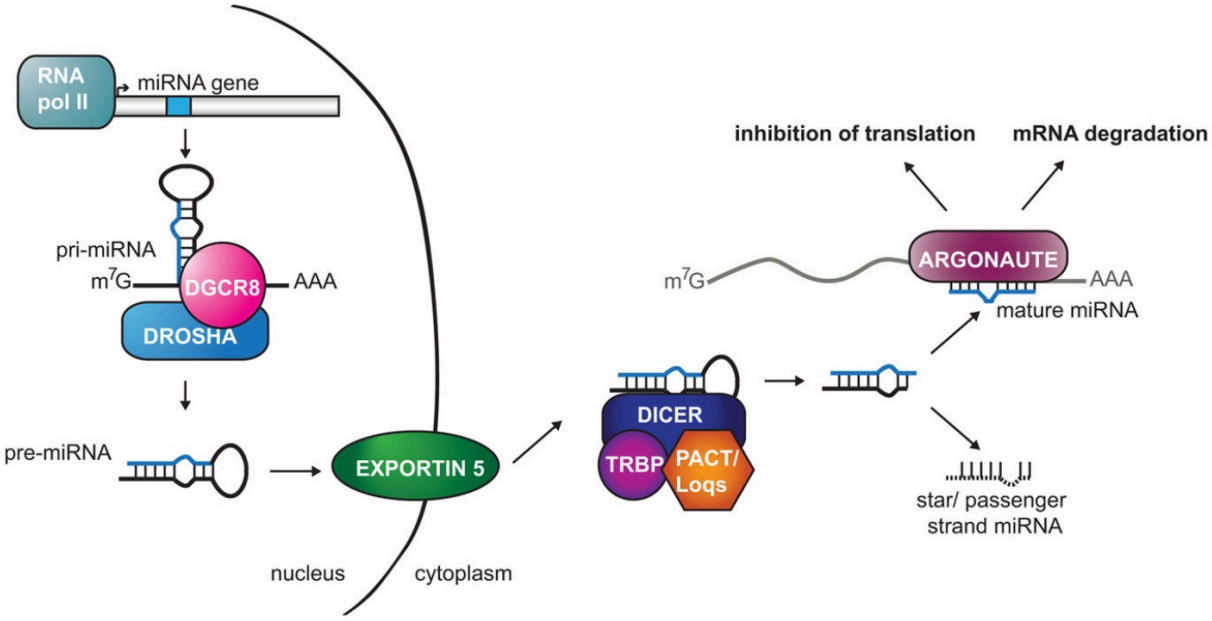

Figure 1. Biogenesis of miRNAs.
MiRNAs are transcribed by RNA polymerase II into primary transcripts (pri-miRNAs), which are then processed by the Microprocessor complex, including DROSHA and DGCR8, to produce precursor miRNAs (pre-miRNAs). These pre-miRNAs are exported to the cytoplasm by Exportin 5, where DICER1 further processes them into a miRNA duplex. One strand is incorporated with Argonaute proteins into the RNA-Induced Silencing Complex (RISC), while the other (called the star or passenger) is degraded. The resulting miRNA-RISC complex can than regulate gene expression by either degrading target mRNAs or inhibiting their translation**.** *Image from Finnegan et al., 2013* [56].

Based on their expression profiles in cancerous versus non-cancerous tissue, and their impact on cell proliferation and programmed cell death, miRNAs can be divided into two groups: oncogenic (oncomiRs) and tumor-suppressor miRNAs [57]. High levels of oncomiRs can downregulate tumor-suppressor genes, while low levels of tumor-suppressor miRNAs can

lead to increased oncogene activity. This dysregulation promotes processes like uncontrolled cell proliferation, resistance to cell death, angiogenesis, and other tumor-promoting mechanisms (Figure 2) [47].

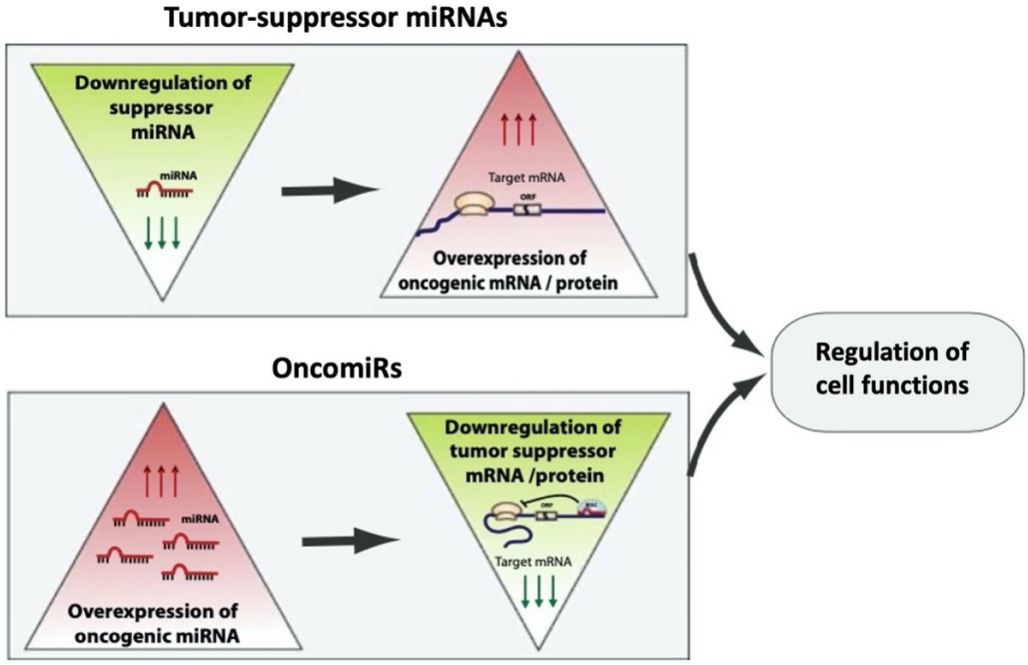

Figure 2. Functions of tumor-suppressor miRNAs and oncomiRs in cancer progression. *Image modified from Nikiforova et al., 2009* [47].

1.2.2 miRNAs dysregulation in thyroid cancer

Studies have highlighted the crucial role of miRNAs in cancer development by modulating target mRNA expression. These small RNA molecules influence various cellular process like tumor growth, invasion, angiogenesis, and immune evasion. In TC, studies have revealed distinct patterns of miRNAs dysregulation, depending on the tumor subtype [47, 58].

In PTC, miR-222 has been identified as an oncomiR, with higher expression levels observed in PTC patients compared to benign thyroid conditions. Interestingly, its expression correlates with risk stratification provided by the American Thyroid Association but not with TNM staging [59]. Together with miR-221, which shares the same seed sequence, miR-222 targets tumor suppressor protein p27, promoting cell cycle progression in PTC and in other cancers [60].

Other miRNAs like miR-146b, miR-21, and miR-181b are over-expressed in PTC tissues compared to normal thyroid tissues [61]. These miRNAs are linked to tumor aggressiveness

and metastasis [62]. For instance, miR-146b is one of the miRNAs most up-regulated in PTC, promoting tumor progression by targeting retinoic acid receptor beta (RARβ) [63, 64].
Similarly, miR-21-5p display over-expression in aggressive variants such as in the tall-cell subtype, suggesting its potential role in molecular classification of PTC [48].
MiR-181b contributes to tumorigenesis by directly targeting the tumor suppressor CYLD, a key negative regulator of NF-kB pathway [65].
Conversely, several miRNAs with tumor-suppressor role exhibit reduced expression levels in PTC. MiR-145, a regulator of PI3K/Akt pathway, is significantly under-expressed, leading to enhanced tumor growth [66]. Likewise, miR-137, miR-451, and miR-613 showed reduced function, correlating with increased proliferation, migration, and invasion [67, 68]. In particular, miR-451a acts as a tumor suppressor by modulating the PI3K/Akt pathway and is inversely associated with aggressive tumor features [69].
The let-7 family of miRNAs, typically expressed in thyroid follicular cells, is also down-regulated in TC [68, 70].

In FTC, miRNAs data are limited, but studies suggest a down-regulation of miRNAs such as miR-542-5p, miR-574-3p, miR-455, and miR-199a, while miR-182, miR-183, miR-221, miR-222, and miR-125a-3p are significantly over-expressed compared to normal thyroid tissues [61]. Among these, miR-199a-5p plays a key role by targeting connective tissue growth factor (CTGF), inhibiting cell cycle progression [71].
Additionally, miR-146b and miR-221 are significantly up-regulated in FTC, indicating their involvement in well-differentiated TC beyond PTC alone [72].

In ATC, only a few miRNAs such as miR-200 family, show dysregulation, highlighting their possible involvement in tumor progression [67]. The miR-200c, a member of this family, is often down-regulated in metastatic cancers, with its loss in ATC associated with TP53 mutations, since miR-200c is transcriptionally regulated by TP53 [73].
A reduction in miR-30 levels has been observed only in ATC. Experimental evidence shows that restoring miR-30 expression reduces levels of vimentin and SMAD2 in ATC cells [74]. Additionally, decreased miR-30a levels correlate with worse outcomes, including aggressive tumor invasion and shorter survival rates [75].
Interestingly, miR-146a-5p and miR-146b-5p, which are over-expressed in PTC, are also highly expressed in ATC. These miRNAs may promote tumor growth in ATC through their interaction with NF-kB signaling pathways [76].

Given the potential role of miRNAs in TC, their expression profiles have become crucial for improving diagnostic accuracy. The thyroid miRNA classifier (ThyraMIR) test is a commercial molecular diagnostic tool designed for indeterminate thyroid cytology. It assesses the expression levels of 10 miRNAs, including miR-31-5p, miR-29b-1-5p, miR-138-1-3p, miR-139-5p, miR-146b-5p, miR-155, miR-204-5p, miR-222-3p, miR-375, and miR-551b-3p to classify thyroid nodules as high or low risk based on their miRNA profile [77].

1.3 The KCTD Protein Family

As part of my research activity, I focused on the study of KCTD15, which belongs to the Potassium (K+) Channel Tetramerization Domain (KCTD) protein family. Given their emerging roles in human diseases, including cancer, KCTD proteins have attracted significant interest as potential biomarkers and therapeutic targets in oncology.

The KCTD protein family is a group of 25 soluble proteins in humans. Although only partially characterized, KCTD proteins are increasingly associated with critical biological processes, including protein degradation and cellular signaling pathways. Their involvement in these functions not only underline their importance in normal physiology but also suggest potential roles in cancer development and prevention, marking some KCTD proteins as promising targets in oncology [78, 79].

KCTD proteins share a conserved N-terminal BTB (Bric-a-brack, Tram-track, Broad complex) domain, also known as the POZ (poxvirus zinc finger) domain of approximately 95 amino acids in length. This domain facilitates protein-protein interactions, enabling the proteins to act as homo- or heterodimers. These interactions are essential for the diverse biological roles attributed to the KCTD family [80, 81]. Through evolutionary divergence from a common ancestral gene, the KCTD proteins have diversified into functionally distinct groups.

Initial analyses clustered the family into seven clades based on sequence similarity [82]. Recent refinements, integrating structural data from the BTB domain and N-terminal regions, have expanded this classification into eight groups, each associated with specific functional and pathological roles [79]. A key feature of some KCTD proteins is their interaction with E3-ubiquitin ligases via their BTB domain, implicating them in protein degradation pathways. In fact, group B, includes KCTD11 (KCASH1), KCTD21 (KCASH2), and KCTD6 (KCASH3), collectively known as the KCASH (KCTD Containing-Cul3 Adaptors, Suppressors of Hedgehog) family. These proteins regulate Hedgehog signaling and are linked to tumor-suppressive activity.

Group C members (KCTD10, TNFAIP1, and KCTD13) are characterized by a proliferating cell nuclear antigen (PCNA)-binding motif, suggesting roles in DNA replication and cell proliferation, processes crucial to both normal cell function and cancer development.

Group D, comprising SHKBP1 and KCTD3, is less characterized. However, SHKBP1 has been implicated in tumorigenesis, while KCTD3 is primarily linked to neurodevelopmental disorders.

Similarly, proteins in Group E (KCTD2, -5, -9, and -17) and Group H (KCTD7 and KCTD14) remain poorly understood, with limited evidences connecting them to specific biological or pathological processes.

Other groups of KCTD proteins (i.e., group A, F and G) reveal others features, including those with functions independent of Cullin3 ligases [78, 83].

For instance, Group A includes KCTD1 and KCTD15, which, despite high sequence homology, diverge in their biological roles. KCTD1 is involved in proteasomal degradation and regulation of aberrant WNT/β-catenin signaling, a key pathway deregulated in many cancers. KCTD15 enhances the inhibitory activity of KCASH2/KCTD21 in medulloblastoma cells, suppressing tumor cell proliferation [84].

Meanwhile, Group F proteins (KCTD12, KCTD8, and KCTD16), serve as auxiliary subunits of the GABA-B receptor, implicating them in neurodegenerative and neuropsychiatric disorders.

Group G proteins (KCTD20 and BTBD10) are linked to AKT signaling, with potential implications in cancer progression.

Several unclassified KCTD proteins, including KCNRG, KCTD19, KCTD4, and KCTD18, remain uncharacterized due to their limited similarity to other family members.

While some members of KCTD families are known to play roles in oncogenic or tumor-suppressive pathways, the precise molecular mechanisms underlying the functions of this family remain to be fully understood.

1.4 Radiogenomics: integrating imaging and genomics data for improving cancer patients' management

In personalized medicine, which focuses on personalizing cancer treatment based on individual molecular profiles, integrating radiomics with genomic data has emerged as a novel promising approach. While genetic testing has become a part of oncology by revealing actionable genetic alterations that guide therapy, incorporating genomics insights into routine clinical practice remains a challenge [85]. The main challenges include the invasive nature of tissue sampling, the high costs, the long processing times, and the complexity of genetic analysis [86]. Moreover, the genomic data obtained from biopsies or surgical samples may not fully describe the tumor's complete genomic profile due to the heterogeneity both between different tumor masses and within the same tumor [87].

Although biopsy and histopathology remain the gold standard for cancer diagnosis, imaging techniques provide significant advantages for non-invasive tumor evaluation. These methods offer a comprehensive view of the tumor, overcoming the limitations of single biopsy samples, and allowing for repeated evaluations of treatment responses and disease progression in clinical settings [85, 86].

Recent advances in medical imaging have introduced algorithms that extract quantitative data from images [88]. Known as radiomics, this process involves selecting regions of interest within high-resolution images and extracting a range of quantitative features, classified into shape-based, first-, second-, and high-order metrics [89].

The emerging field of radiogenomics integrates radiomic data with genomic information to uncover how imaging features correlate with genetic alterations within tumors. By linking specific imaging characteristics to patterns of gene expression, this approach facilitates the prediction of genetic mutations or pathway alterations, providing a deeper understanding of tumor biology [90, 91]. This integrated approach holds the potential to improve diagnostic accuracy [92], to enhance patient outcomes [93], and to provide more accurate prognostic information [94].

2. AIM OF THE THESIS

The aim of this PhD thesis is to identify novel biomarkers, focusing on exploring the role of both non-coding RNAs and protein-coding genes involved in TC progression, and also investigating the emerging role of radiogenomics as a non-invasive diagnostic and prognostic approach for thyroid cancer.

This dissertation is structured around three main objectives:

1. Unveil the involvement of miRNAs in the pathogenesis of thyroid cancer and their potential as biomarkers for this malignancy. In particular, I focused on miR-331-5p, analyzing the effects of its over-expression and silencing on thyroid cancer cells behavior with the aim to uncover its functional role.

2. Characterize the role of KCTD15 in thyroid carcinogenesis, analyzing its expression and function in both thyroid cancer tissues and cell lines.

3. Analyze the potential applications of integrating radiomic signatures with genomic data in thyroid cancer patients.

3. RESULTS

3.1 AIM 1: Identification of miR-331-5p as potential biomarker in thyroid cancer

miR-331-5p has been identified as a potential tumor suppressor in various cancers, including leukemia as well as lung, prostate, gastric, breast, hepatocellular, and colorectal cancers [95]. Previous research conducted in the laboratory where I performed my work, revealed that miR-331-5p is among the miRNAs downregulated in a PTC cell line (TPC-1) ectopically expressing the TWIST1, a key transcription factor involved in the regulation of epithelial-to-mesenchymal transition [96]. However, the specific role of the miR-331-5p in thyroid cancer (TC) pathogenesis remains unclear. Therefore, the primary aim of this study was to investigate the functional role of miR-331-5p in the pathogenesis of TC and its potential as biomarker.

3.1.1 miR-331-5p impairs motility of thyroid cancer cell lines

To evaluate the effects of miR-331-5p on the motility of TC cells, stable transfections were carried out in PTC (TPC-1) and ATC (CAL62 and 8505C) cells. These cell lines were transfected with either a plasmid expressing pre-miR-331-5p (TPC-1 and CAL62) or an Anti miR-331-5p (8505C), and their respective controls vectors (named miR-Null or Anti miR-Null). Following selection with puromycin, the mass population expressing higher transfection efficiency was chosen for further experiments (Figure 3).

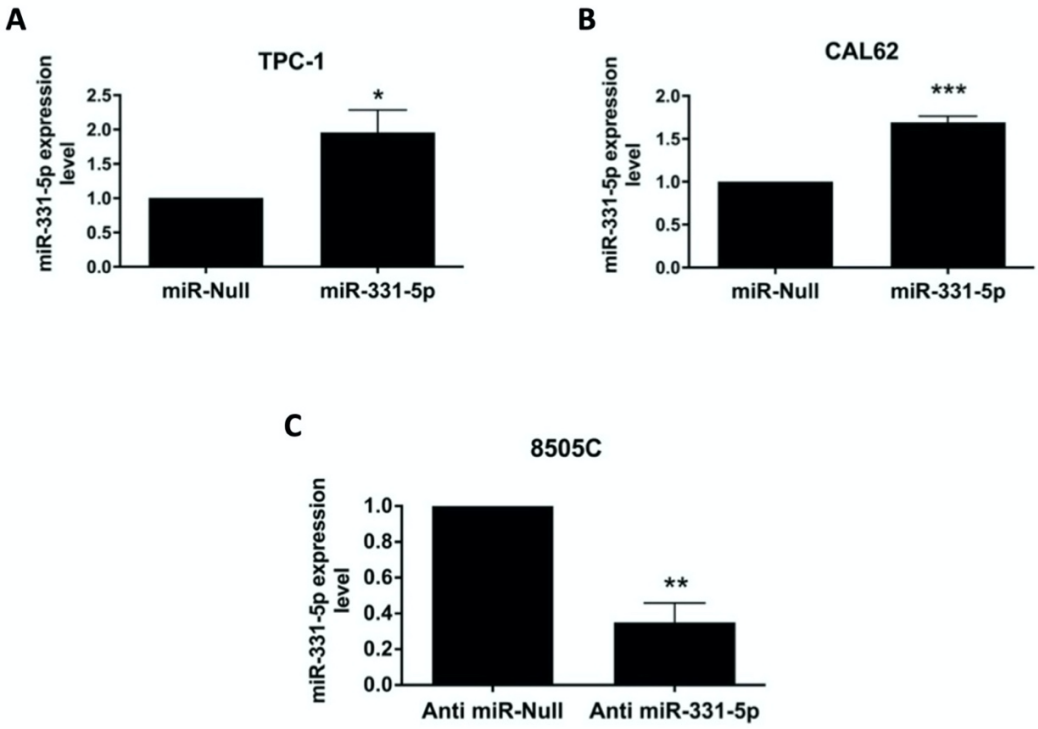

Figure 3. Expression of miR-331-5p after transfection in TC cells. qRT-PCR was performed in the mass population to quantify the expression level of miR-331-5p in TC cells after transfection and selection with puromycin. TPC-1 (**A**) and CAL62 (**B**) cells were stably transfected with a plasmid expressing the precursor of miR-331-5p or a scrambled control plasmid (miR-Null). 8505C cells (**C**) were transfected with plasmid designed to inhibit miR-331-5p (Anti miR-331-5p) or with a scrambled control inhibitor plasmid (Anti miR-Null). The data are presented as mean ± SEM of three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. *Image modified from Orlandella et al, 2024* [97].

To assess the effects of miR-331-5p on the migratory ability of TC cells, a wound closure assay was performed. As shown in Figure 4, in TPC-1 (A) and CAL62 (B) cell lines, over-expression of miR-331-5p significantly reduced cell migration into wound area when compared to cells transfected with the control vector (miR-Null). To further validate these results, a mirror experiment was conducted using the 8505C cells, in which endogenous miR-331-5p was silenced. The inhibition of miR-331-5p significantly enhanced the migratory capacity of 8505C cells relative to the control cells (8505C/Anti miR-Null) (Figure 4C).

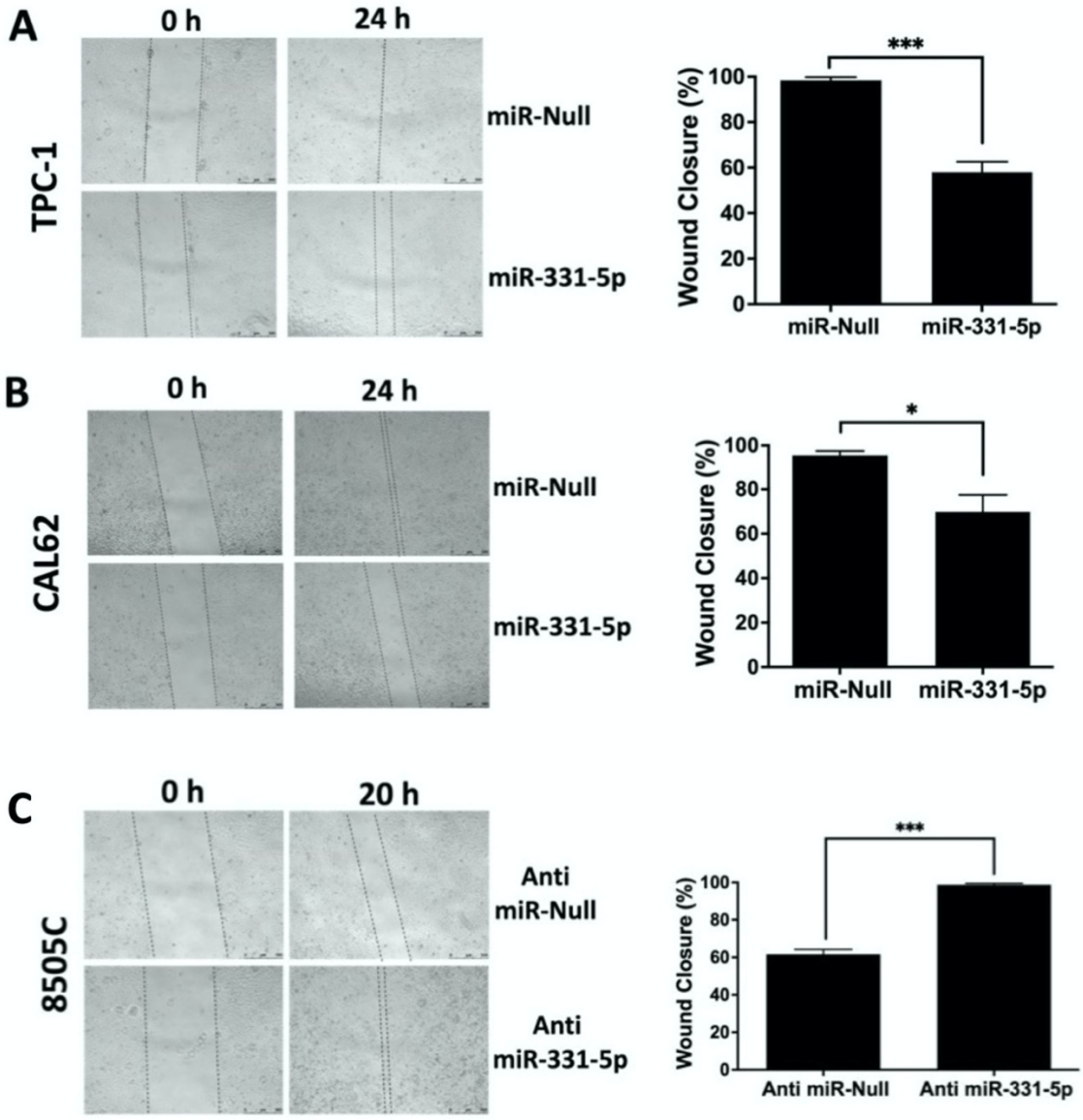

Figure 4. miR-331-5p impairs motility of TC cell lines. A scratch was inflicted into a confluent monolayer of TPC-1 (**A**) and CAL62 (**B**) cells, which were stably transfected with either a plasmid expressing the precursor of miR-331-5p or a control plasmid (miR-Null). Additionally, the migratory ability of ATC cell line 8505C was examined following stable silencing of miR-331-5p expression or transfection with a control plasmid (Anti miR-Null) (**C**). The gap area was monitored until closure, and the percentage of the wound closure was calculated relative to the initial scratch. Scale bar: 500μm. All the experiments were conducted at least three times, and data are presented as mean ± SEM. * $p < 0.05$; *** $p < 0.001$. *Image modified from Orlandella et al, 2024* [97].

Then, I also investigated whether the modulation of miR-331-5p in TC affects cell proliferation rates using the methyl tetrazolium compound assay (MTS) assay. This assay measures cell viability by converting the MTS solution into formazan product that can be quantified spectrophotometrically. However, no significant effects on cell proliferation were observed across all the time points and experimental conditions tested (data not shown).

To further investigate the impact of miR-331-5p on TC cell motility, we evaluated the invasive behavior of PTC (TPC-1) and ATC (CAL62) cells overexpressing miR-331-5p. Using a transwell assay with Matrigel-coated inserts, we observed a marked reduction in the invasion ability of both TPC-1 (Figure 5A) and CAL62 (Figure 5B) cells stably transfected with miR-331-5p compared to their control.

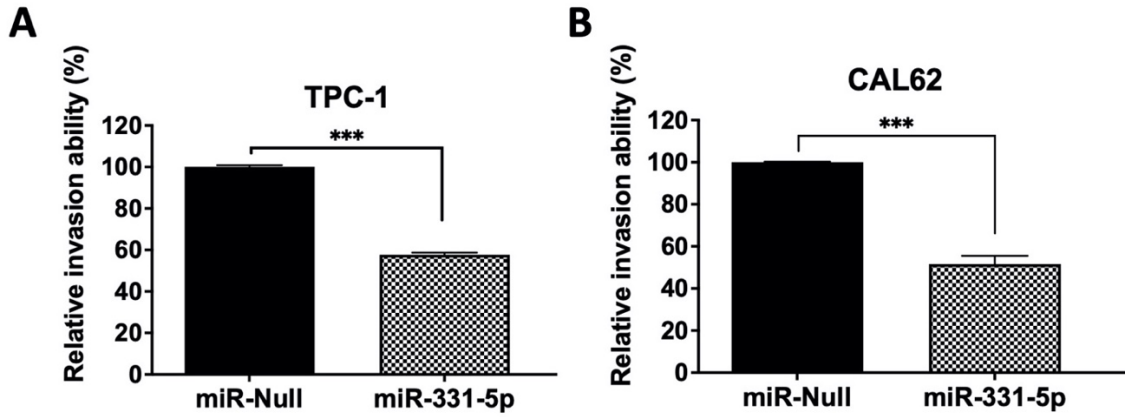

Figure 5. Effects of the miR-331-5p on the invasion of TC cells. The invasive behavior of TC cells was evaluated *in vitro* using Matrigel Matrix-coated permeable inserts with 8.0µm pore. Following incubation, invading cells were stained with crystal violet, and quantification was performed by extracting the dye and measuring absorbance at 550 nm using a spectrophotometer. Results, presented as percentages, reflected the mean ± SEM. *** $p < 0.001$. *Image modified from Orlandella et al, 2024* [97].

In addition, a three-dimensional (3D) invasion assay was performed. Briefly, TPC-1 cells were seeded in 3D Matrigel and monitored over 4, 8, and 12 days. As illustrated in Figure 6, TPC-1 cells over-expressing miR-331-5p formed significantly smaller structure and thus exhibited a reduced invasive ability respect to control cells at all analyzed time points.

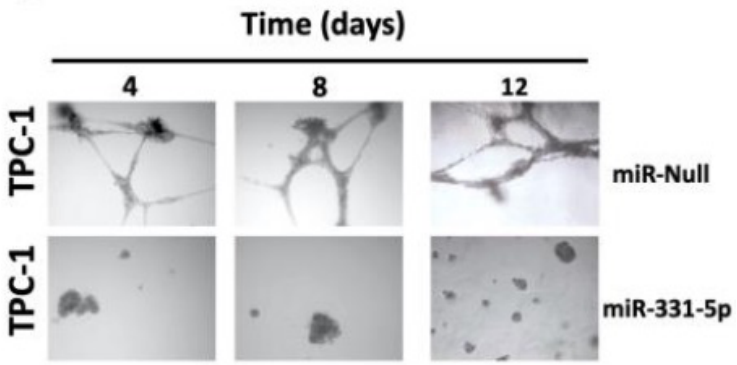

Figure 6. miR-331-5p expression modulates the invasive phenotype of TC cell. Morphological evaluation of TPC-1 cells following miR-331-5p over-expression in a 3D cell culture system. Stably transfected TPC-1 cells were photographed at different times (4, 8, and 12 days). *(Image modified from Orlandella et al, 2024)* [97].

Overall, these data confirm that the modulation of miR-331-5p expression significantly affects the invasive behavior of TC cell lines [97].

3.1.2 Identification of BID as a target of miR-331-5p and their expression in thyroid cancer tissues

To uncover potential targets of miR-331-5p, a label-free proteomic analysis was carried out on CAL62 cells stably transfected with miR-331-5p or with a control vector (miR-Null). This analysis identified 8 proteins, i.e., C3H18, BID, CHMP2B, COMMD10, NLN, NUP54, USP24, and ZC3H18, that were down-regulated in CAL62/miR-331-5p *versus* CAL62/miR-Null cells. Among these proteins, we focused on BID, aa it exhibited the highest fold change (3.97, p= 4.45×10^{-27}) in 510 tumors samples (red box) compared to 58 normal thyroid tissues (turquoise box) in the TCGA-THCA dataset (Figure 7A). Since BID is overexpressed in TC tissues, we also investigated if there is a correlation between the expression of miR-331-5p and BID in this dataset. As shown in Figure 7B, a negative correlation was observed between BID and miR-331-5p expression levels in human TC tissues ($r = -0.11$, $p = 1.1 \times 10^{-2}$), supporting the hypothesis that miR-331-5p regulates BID.

To confirm that BID over-expression corresponds to miR-331-5p down-regulation in TC tissues, the TCGA-THCA dataset was also interrogated. As shown in Figure 7C, miR-331-5p was significantly down-regulated (p-value = $1.1e^{-14}$) in TC samples compared to normal thyroid samples.

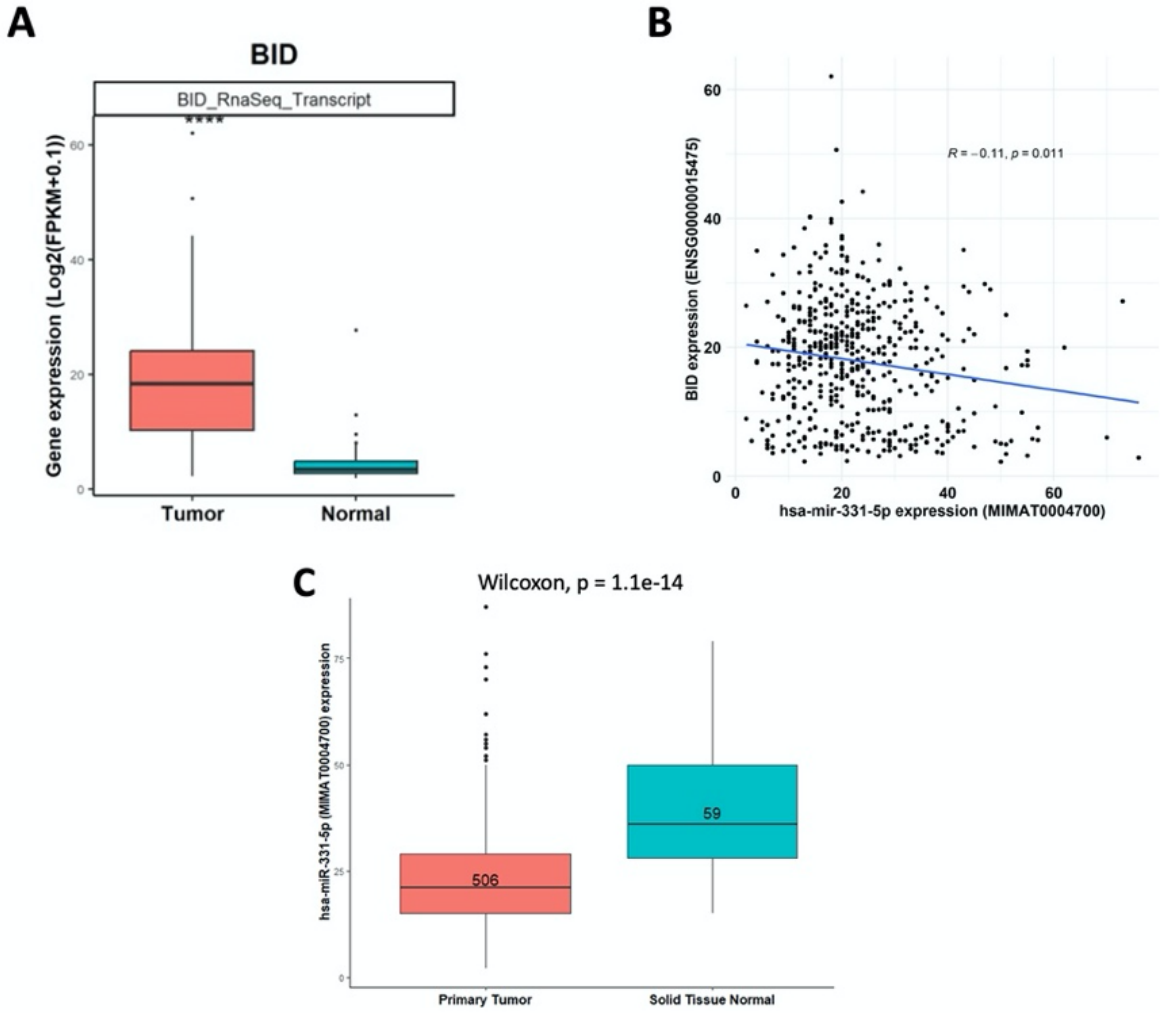

Figure 7. Inverse correlation between BID and miR-331-5p expression in TC. A) The expression of BID was found to be significantly higher in TC tissues (N = 510) compared to normal thyroid tissues (N = 58) based on data from The Cancer Genome Atlas - THyroid CAncer (TCGA-THCA) project. **B**) Co-expression analysis revealed a negative correlation between miR-331-5p and BID mRNA levels in TC tissues. **C**) Downregulation of miR-331-5p in TC tissues compared to normal tissues deposited in TCGA-THCA database. Statistical significance p-values ** $p < 0.01$ and**** $p << 0.001$ according to a Wilcoxon rank sum test with continuity correction. *Image modified from Orlandella et al, 2024 [97].*

Finally, we validated the results from the label-free proteomic analysis through immunoblotting. Figure 8A showed that the BID protein level was reduced in CAL62/miR-331-5p compared to control cells. In addition, the luciferase assay confirmed the direct targeting between miR-331-5p and the mRNA of BID (panel B).

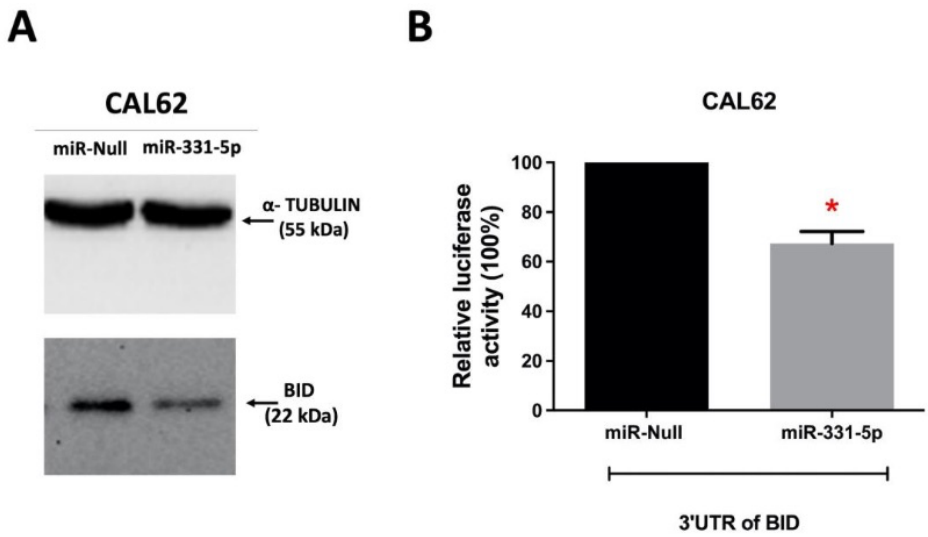

Figure 8. BID is directly targeted by miR-331-5p in ATC cell line model. A) Protein level of BID was analyzed by immunoblotting in CAL62/miR-Null and CAL62/miR-331-5p cells, using α-TUBULIN as an endogenous control. **B**) Luciferase reporter assay was performed in CAL62/miR-Null and CAL62/miR-331-5p cells transfected with a luciferase containing the wild type of the 3′ untranslated region (UTR) of BID. Data are presented as mean ± SEM of two independent experiments, each conducted in triplicate. * $p < 0.05$. *Image from Orlandella et al, 2024* [97].

3.2 AIM 2: [redacted]

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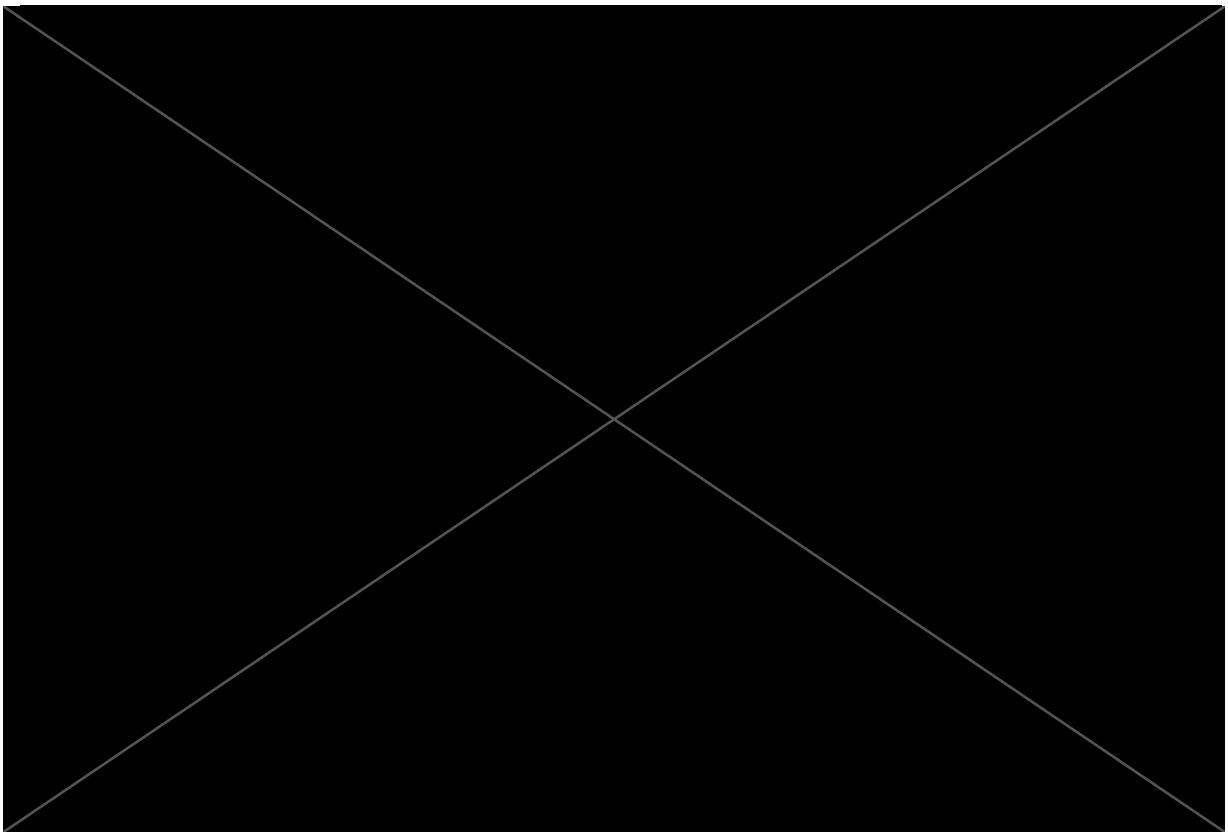

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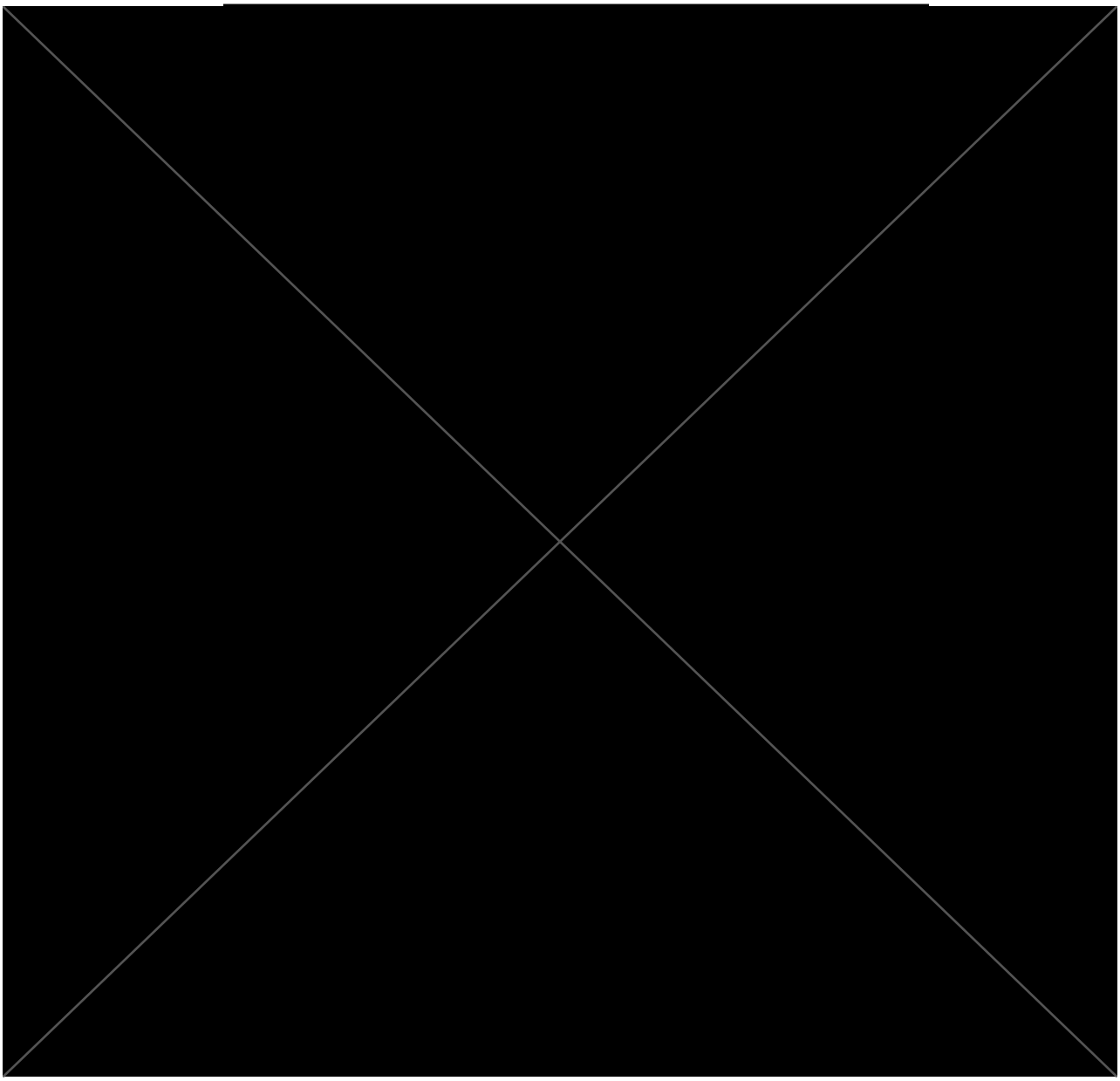

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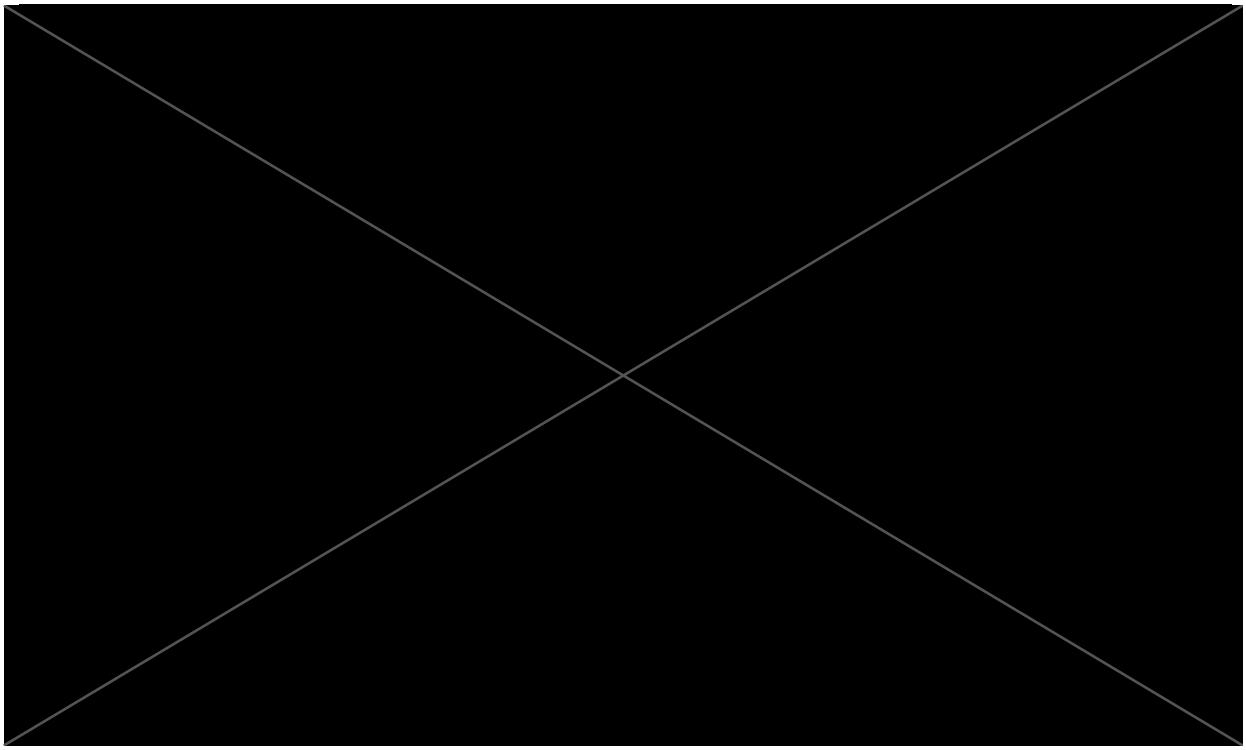
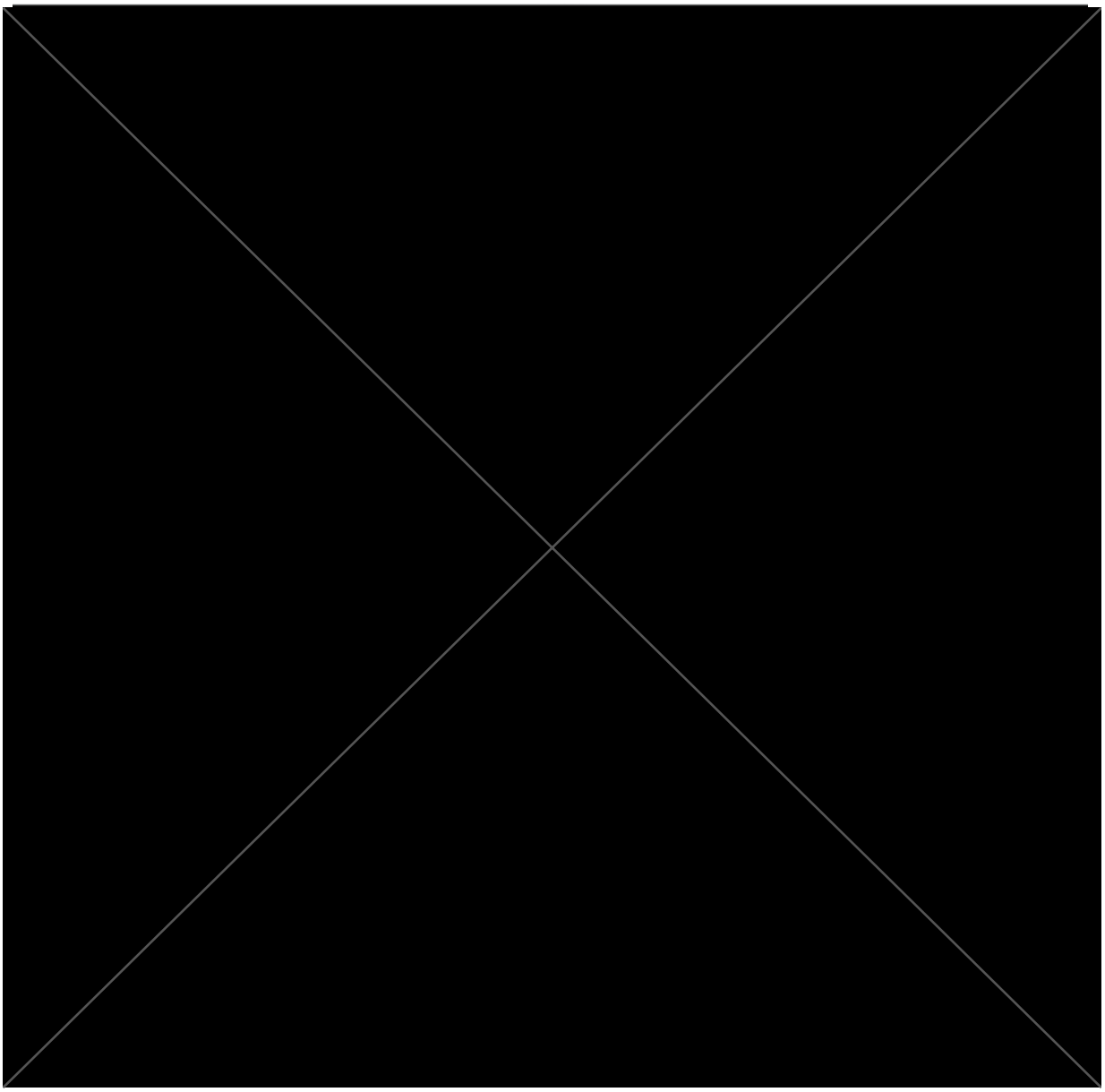




3.3 AIM 3: Identification of novel diagnostic approaches: a systematic review on integration of radiomic and genomic data in thyroid cancer

To uncover novel potential diagnostic strategies, my PhD research also focused on the correlations between imaging and genomic data in TC. As part of this work, I conducted a systematic review of the literature to assess the current state of integrating radiomic features with genomic signatures as a potential diagnostic strategy for TC. By exploring the relationship between data extracted from imaging and molecular profiles, I conducted a systematic review aims to highlight innovative approaches that could improve the accuracy of diagnosis and prognosis for TC patients.

I began by querying three different databases - PubMed, Scopus and Embase - using the following combination of keywords: “radiomics” OR “radiogenomics” OR “genomics” OR “transcriptomics” OR “omics” AND “thyroid cancer” OR “thyroid carcinoma”.

After combining the results from each database, I collected a total of 853 studies, which were reported in an electronic spreadsheet, with duplicates removed. The remaining articles were screened according to several inclusion criteria, such as focusing on TC patients, using imaging techniques to predict genomic signatures, or analyzing gene expression as predictors of imaging biomarkers with focus on human subjects. Studies were excluded if they were review, non-English language, lacked full-text access or only addressed imaging or genomic expression data separately. Non-human studies, such as those involving animals, were also excluded.

After the screening process, 7 articles were included for the final analysis (Figure 12). This systematic review has been registered in the PROSPERO database under registration number CRD42024572292 [102].

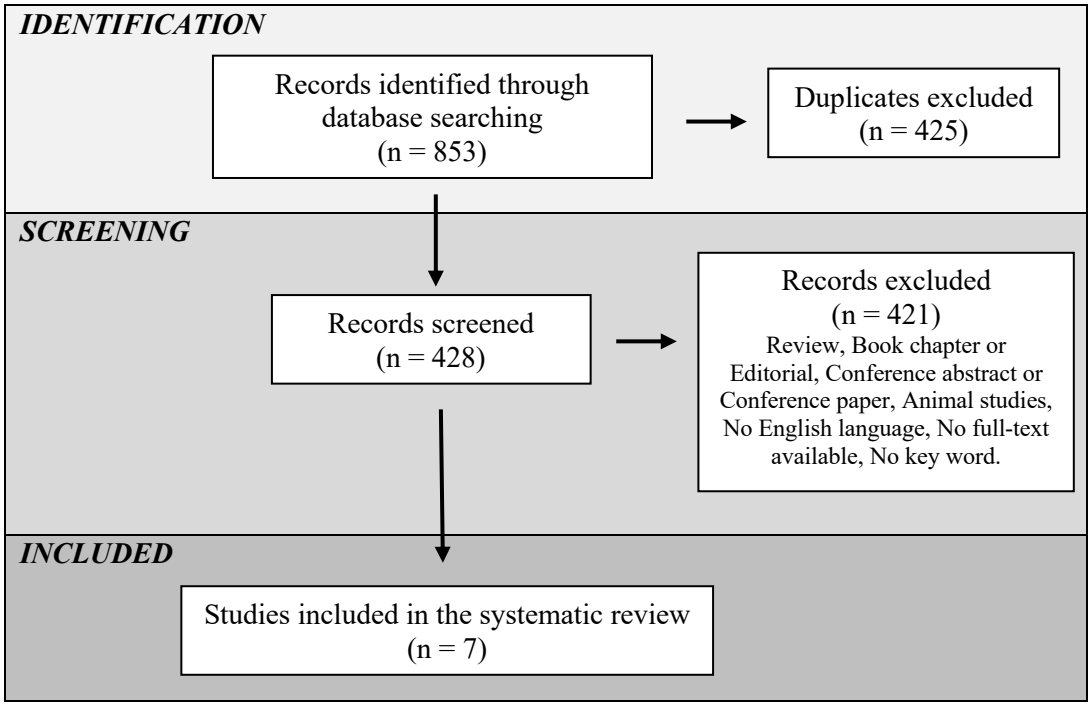


Figure 12. Flowchart illustrating the main research process for the systematic review. *Modified from Luciano et al, 2024* [102].

The quality of the 7 studies included was evaluated using the Newcastle–Ottawa Scale (NOS), a tool designed to assess a methodological quality (rigor) across three domains: selection of participants, comparability of groups and ascertainment of exposure [103]. The scoring system ranges from 0 to 9 points, with higher score correlating with a better study quality.

To determine the risk of bias, three specific factors were considered:

1. Clinical data quality of the enrolled patients.
2. Reliability of radiomic features extracted from imaging.
3. Accuracy in exposure measurement.

Studies scoring lower than 7 were categorized as having a “high risk of bias”; otherwise, it was considered at a “low risk of bias”.

The NOS score of the included studies ranged from 4 to 8, with an average score of 5.86. Specifically, 28.6% of the studies scored 8, indicating high quality with minimal risk of bias. Another 28.6% of the studies scored 6, suggesting moderate quality and low bias. Finally, the remaining 42.8% scored between 4 and 5, reflecting a higher risk of bias.

The included studies in this systematic review mainly focus on integrating radiomic and genomic data to predict metastasis and to predict genetic alterations in TC.

Of seven studies included, three highlighted the potential of radiogenomic strategies to improve the prediction of cervical lymph nodes (CLN) involvement in papillary thyroid

carcinoma (PTC). One study of Dong *et al*. [104] analyzed data from 1298 lateral neck lymph nodes (LNLNs) from PTC patients who underwent preoperative computed tomography (CT) examinations and surgery. By combining radiomics features with clinical risk factors in a nomogram, the study showed strong prognostic ability for predicting LNLN metastasis. Transcriptomic analysis of tumor samples, grouped into high and low-risk categories by the nomogram, revealed gene expression differences between groups, suggesting potential therapeutic targets.

Similarly, Tong *et al*. [105] investigated a radiomics approach using preoperative thyroid ultrasound (US) to predict CLN status in PTC. The study demonstrated higher predictive accuracy of radiomic model compared to conventional US. Gene modules associated with CLN metastasis were identified, and a radiogenomic map showed significant correlation between radiomic features and gene expression profiles, with LAMC1 and THBS genes being linked to the PI3K/Akt signaling pathway.

Lastly, another study explores the use of NGS combined with US-based to improve the predictive accuracy of differentiating between benign and malignant thyroid nodules [106]. NGS identified 592 mutations across 31 genes, with specific mutation in BRAF, TERT and RET found to be more frequent in cancerous tissues and associated with lymph node metastasis. By combining genetic data with radiomic features and clinical information, the study developed a nomogram model that showed superior predictive accuracy for lymph node metastasis compared to single data type models, highlighting the value of integrating multiple data sources for improved diagnostic precision in PTC.

The systematic review I conducted, also highlights the potential of radiomics as a non-invasive tool for predicting key genetic alterations in PTC. Indeed, the remaining four studies included in the systematic review explored these association, specifically focusing on the BRAFV600E mutation and RET rearrangement.

Zheng *et al*. [107], explored the use of Magnetic Resonance Imaging (MRI)-based texture models to identify BRAFV600E mutations in 80 PTC patients. Radiomic features extracted from T2-weighted image and contrast-enhanced T1-weighted image were analyzed, and combined models demonstrated strong accuracy and sensitivity for mutation detection, exceeding individual MRI parameters.

The study of Wang *et al*. [108], investigated US-based radiomic models in 138 PTC patients. Features extracted from grayscale and elasticity US images were combined to construct predictive models, with the combined approach outperforming grayscale alone, highlighting the added value of elasticity radiomics.

Moreover, Yoon *et al.* [109] evaluated US radiomics for predicting BRAFV600E mutations in 527 patients. Radiomic scores derived from selected features were associated with mutation status, particularly in conventional PTC tumors <20 mm. However, validation results were inconsistent, indicating the need for further refinement.

Beyond predicting BRAFV600E mutation, radiomics has been applied to detect RET rearrangements. For instance, Yu *et al*. [110] focused on predicting RET rearrangements using a deep learning radiomics nomogram (DLRN) based on US images of 650 PTC patients. By combining clinical and radiomic features, the DLRN demonstrated superior predictive accuracy in both training and test cohorts, showing the potential of integrating deep learning with radiomic analysis for non-invasive genetic screening.

These findings underscore the potential of radiogenomic models to enhance diagnostic accuracy and support personalized management strategies in PTC.

Table 4 provides an overview of the main characteristics of all the studies included in the systematic review I conducted during my PhD.

Table 4: **Main characteristics of the studies included in the systematic review.**

Author, year	Enrolled population, n	Imaging modalities, n	Genomic techniques, n	Outcome
Dong *et al.,* 2023 [104]	471 patients	CT (n = 471)	Transcriptome sequencing (n = 14)	Good predictive value of CT-radiomics features to predict LNLN
Tong *et al.,* 2021 [105]	270 patients	US (n = 270)	LC-MS/MS (n = 49); IHC (n = 40)	Good performance of US-radiomic signature to predict the CLN status
Wang *et al.,* 2022 [108]	138 patients	US (n = 138)	PCR (n = 138)	Good predictive value of gray-scale US to predict BRAFV600E mutation
Yoon *et al.,* 2020 [109]	527 patients	US (n = 527)	DNA sequencing (n = 527)	Limited value of US-radiomic features to predict BRAFV600E mutation
Yu *et al.,* 2022 [110]	650 patients	US (n = 650)	NGS; ARMS RT-PCR; Sanger sequencing (n = 650)	Good predictive value of US-based DLRN to predict RET rearrangement
Zhang *et al.,* 2024 [106]	182 patients	US (n =108)	NGS (n= 182)	Good predictive value of genetic alteration and US-radiomic features to predict the prognosis
Zheng *et al.,* 2023 [107]	80 patients	MRI (n =80)	ARMS RT-PCR; Sanger sequencing (n =80)	Good predictive value of MRI-radiomics features to predict BRAFV600E mutation

Abbreviation: **ARMS RT-PCR**, Amplification Refractory Mutation System Real Time-Polymerase Chain Reaction; **CLN**, Cervical Lymph Node; **CT**, Computed Tomography; **DLRN**, Deep Learning Radiomics Nomogram; **IHC**, Immunohistochemistry; **LC-MS/MS,** Liquid Chromatograph-Mass Spectrometry; **LNLN**, Lateral Neck Lymph Node; **MRI**, Magnetic Resonance Imaging; **n**, Number; **NGS**, Next Generation Sequencing; **US**, Ultrasonography. *Modified from Luciano et al, 2024* [102].

4. DISCUSSION

Thyroid cancer (TC) is the most common malignancy of endocrine system, with incidence increasing worldwide in recent years [1]. While early diagnosis generally has favorable clinical outcome for differentiated types, current diagnostic approaches still based on procedures, such as ultrasound (US), fine-needle aspiration (FNA), liquid biopsy, which can be imprecise in certain cases [58]. Consequently, there is a necessity to identify novel diagnostic biomarkers using advanced techniques that could enhance the early detection of TC.

Tumor biomarkers, whether at RNA or protein level, play a critical role in improving patient management. Over the past decade, advancements in sensitive detection methods have led to the discovery of numerous biomarkers in oncology, providing valuable insights into tumor biology and therapeutic targets [61].

Among these, microRNAs (miRNAs) had significantly increasing attention due to their dual role as biomarkers and as regulators of cancer progression. These ~22 nucleotide transcripts can influence several biological processes, including proliferation, cell cycle, apoptosis, motility by binding the 3' untranslated regions (UTRs) of target messenger RNAs (mRNAs). Depending on their expression patterns in cancerous versus normal tissues, miRNAs are classified as either oncogenic or tumor suppressors [62, 68].

One family of miRNAs implicated in cancer development is miR-331, located on chromosome 12p22. This family results in two mature miRNAs, miR-331-3p and miR-331-5p, with distinct role in tumorigenesis. While miR-331-3p has been reported to act as both tumor suppressor and tumor promoter in different cancers [95], its overexpression in TC specifically inhibits proliferation and motility of PTC cells [111].

Conversely, the role of miR-331-5p in TC remains poorly understood. Previous research from our laboratory, identified miR-331-5p as one of the down-regulated miRNAs in TPC-1 cells over-expressing TWIST1 transcription factor, compared to control cells [96].

Based on this, during my PhD, part of my work was to investigate the functional role of miR-331-5p in TC.

Through a wound healing assay, I demonstrated that PTC (TPC-1) and ATC (CAL62) cell lines over-expressing miR-331-5p exhibit significantly reduced migration rates compared to controls. In contrast, silencing of miR-331-5p in 8505C cells enhanced migration rates, confirming its tumor-suppressive role. Similarly, a Matrigel invasion assay revealed that miR-331-5p expression decreased the invasive capacity of TC transfected cells, further supporting

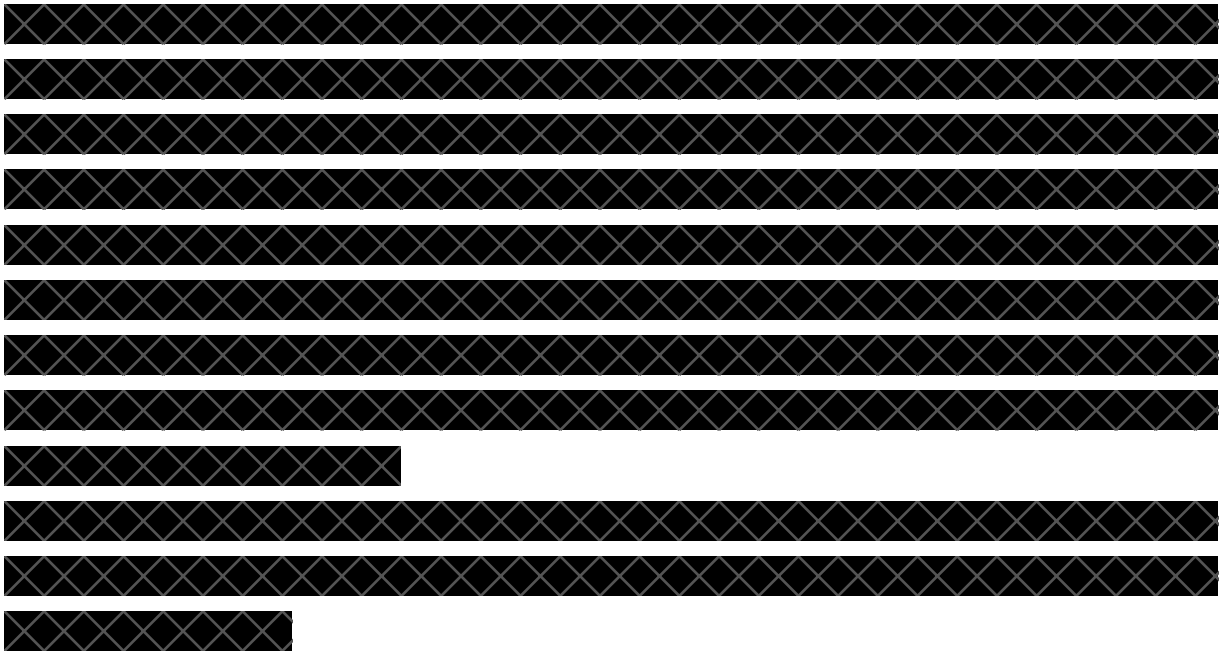
its function as a negative regulator of tumor progression. Interestingly, miR-331-5p modulation did not significantly affect cell proliferation in our experimental conditions, i.e., up to 96 hours' time points. This may be attributed to the involvement of different signaling pathways in proliferation and motility or the need of longer observation time to detect potential effects.

To elucidate the molecular mechanisms underlying miR-331-5p activity, we performed a label-free proteomic analysis and identified BID, a pro-apoptotic protein from BCL-2 family, as a potential target of miR-331-5p. Further, the potential non-canonical binding site between hsa-miR-331-5p and 3'UTR of BID was predicted using several online tools, including miRWalk, RNAhybrid, and STarMir. To validate this prediction, a luciferase reporter assay was conducted, which confirmed the direct interaction between miR-331-5p and 3'UTR of BID.

BID, located on chromosome 22, plays a central role in the intrinsic apoptotic pathway by interacting with BAX protein following DNA damage, as a results by regulating cell death and maintaining genomic stability [112]. Previous studies have also highlighted BID as prognostic marker in PTC, where its up-regulation was associated with poorer outcomes [113].

From our results emerged that BID is significantly over-expressed in TC patients and shows an inverse correlation with miR-331-5p expression levels. Moreover, further *in silico* analysis revealed a significantly downregulation of miR-331-5p in TC tissues.

Collectively, these findings emphasize the tumor-suppressive role of miR-331-5p in TC through the regulation of BID and their potential as promising biomarkers of TC.



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Understanding the genetic mechanisms that drive tumorigenesis is crucial for improving patient management and for personalized medicine.

In oncology, emerging approaches that integrate genetic information with imaging data offers significant opportunities to enhance diagnostic and prognostic accuracy in TC. This integrated approach has the potential to improve patient management by providing clinicians a comprehensive tool for evaluation [91]. Radiogenomics, which combines radiomic features extracted from imaging with genomic data, represents a rapidly advancing field in cancer research, particularly in developing predictive and diagnostic models [115].

Motivated by these assumptions, as further part of my work, I conducted a systematic review to evaluate the current evidences on the association between radiomics and genomics in TC.

The systematic review I conducted includes 7 studies, divided into two primary themes based on clinical outcomes: predicting metastasis and predicting mutation status.

Among the studies addressing metastasis, Dong and colleagues proposed a CT radiomics-based nomogram for predicting lymph node metastasis in PTC [104]. This model demonstrated strengths such as robust lymph node segmentation and performance comparable to those of senior radiologists. However, significant limitations were identified, including the small sample size, manual delineation of regions of interest, and a lack of experimental validation to support the biological relevance of the radiomic features. Similarly, Tong's study explored the potential of combining US radiomics and gene modules to predict cervical lymph nodes (CLN) metastasis in PTC [105]. While the findings highlighted the value of radiomics

compared to conventional imaging, the study was limited by single-center data and non-compliance with imaging standardization protocols.

Advanced genomic tools, such as next generation sequencing, have become essential for identifying genetic alterations, particularly mutations like BRAFV600E, which are associated with aggressive cancer phenotypes. Another study emerged from my analysis was performed by Zhang *et al.* that demonstrated that integrating genetic mutations with US-based radiomic features improved the prediction of lymph node metastasis [106]. However, this study was also limited by a small sample size and the need for long-term validation to confirm its reliability in broader clinical settings.

In addition to metastasis prediction, the systematic review I conducted examined the role of integration of radiomics an genomics data in predicting mutation status. Currently, FNA and gene sequencing are the standard for determining mutation status, but these methods have limitations, including false negatives. Non-invasive approaches using radiogenomics could address this weakness. For instance, Zheng's study utilized MRI radiomics to predict the BRAFV600E mutation in PTC, showing promising results despite limitations such as the exclusion of smaller lesions and potential overfitting due to limited sample size [107].

Similarly, Wang and colleagues investigated US radiomics to assess BRAFV600E mutations, combining grayscale and elasticity features [108]. While the study highlighted the potential of this approach, selection bias, limited genetic analysis, and a lack of external validation have been reported.

Interestingly, Yoon's study showed that US radiomic features were not reliable for predicting BRAFV600E mutation status, except in tumors smaller than 20mm [109]. This variability underscores the influence of imaging modality and methodology on the reliability of radiogenomic models.

A study incorporating deep learning demonstrated the potential of a radiomics-based nomogram for detecting RET/PTC rearrangements preoperatively, further emphasizing the role of advanced computational tools in improving predictive capabilities [110]. However, these findings require larger-scale validation to assess their broader applicability.

Overall, these studies analyzed in the systematic review underscore the potential of integration of radiomic features with genomic signatures as a non-invasive tool to enhance TC diagnosis and prognosis. However, several limitations emerged from the systematic review, including the limited number of studies available on TC radiogenomics, which prevents a consistent evaluation and comparison of results. Tool like The Cancer Imaging Archive

(TCIA), which combine imaging data with genomic information from The Cancer Genome Atlas (TCGA), could serve as valuable resources for advancing radiogenomic research in TC.

During the analysis of the 7 articles included in this systematic review, I observed a significant methodological heterogeneity in the field of radiomics. This variability, extending across imaging techniques such as US, CT, and MRI, reflects the complexity of integrating imaging and genomic data. Each modality offers unique advantages but also presents limitations. For instance, US remains the gold standard for diagnosing TC when matched with FNA. However, its high dependency on the operator makes it less reliable compared to CT or MRI. Despite this, its accessibility highlights the need to enhance its reproducibility and standardization to fully exploit its potential in clinical practice.

I also observed a lack of consistency in the genomic methods employed, such as NGS, Sanger sequencing, and PCR, which adds an additional layer of variability. This heterogeneity makes it difficult to draw robust and comparable conclusions across studies. Moreover, the use of diverse software and methods for radiomic feature extraction complicates the translation of these insights into clinical practice, where standardization and reliability are essential.

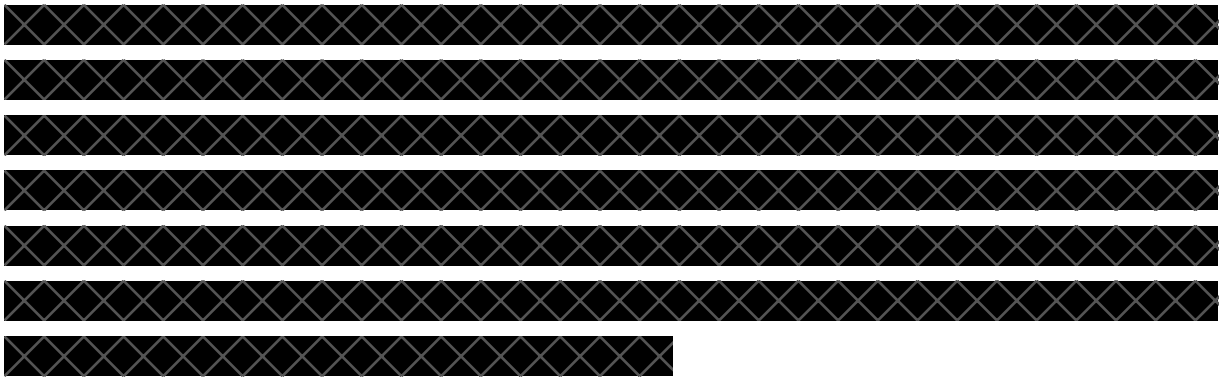
Another recurring issue is the small sample sizes used in all the studies, which inevitably restricts the generalizability of their results. Finally, PTC, while being the most common subtype of TC, is inherently heterogeneous [25], further complicating the ability to extrapolate findings to other subtypes.

Six out of seven studies reported favorable outcomes for the integration of radiomic and genomic features, while only one study highlighted limited predictive value for US-based radiomics. This bias could be due to the low number of studies available on TC radiogenomics.

In the future, addressing these limitations and advancing the integration of radiomics and genomics data could enable radiogenomics to play a growing role in the evaluation of TC cancer. This approach holds the potential to offer a non-invasive and personalized approach in the field of personalized medicine [116]. However, further efforts are required to fully integrate this promising approach in the decision-making process for TC management.

5. CONCLUSION

During my PhD, I explored various aspects of thyroid cancer (TC) biology, focusing on molecular mechanisms underlying TC pathogenesis with the aim of identifying novel biomarkers. The findings emerged from my thesis, underscore the importance of miRNAs as key regulators and as potential biomarkers in TC. Specifically, miR-331-5p was shown to act as a tumor suppressor by regulating the pro-apoptotic protein BID, inhibiting migration and invasion of TC cells, and demonstrating inverse correlation with BID expression in PTC tissue samples. These results highlight the potential of miR-331-5p and BID as a promising novel biomarkers in TC. Future *in vivo* studies could help to validate their role in modulating tumor growth and metastasis.



In parallel, a systematic review I conducted on the associations between radiomic and genomic data in TC highlight the emerging potential of this integration for non-invasive diagnosis and prognosis of TC. While promising results were reported in predicting metastasis and mutation status (such as BRAFV600E mutation), significant methodological limitations, including small sample sizes, imaging heterogeneity, and lack of standardization, were evident. Addressing these challenges and using databases such as TCIA and TCGA could enable broader use of radiogenomics in TC, improving diagnostic accuracy and personalized treatment strategies.

Overall, the findings presented in this PhD thesis provide insights into the molecular mechanisms and into biomarkers involved TC progression, and highlight the potential of emerging diagnostic approach, like radiogenomics, to improve disease management.

Future studies aimed at combining molecular and imaging-based approaches will be essential to develop more effective, non-invasive, and personalized strategies for TC diagnosis and treatment.

6. MATERIALS AND METHODS

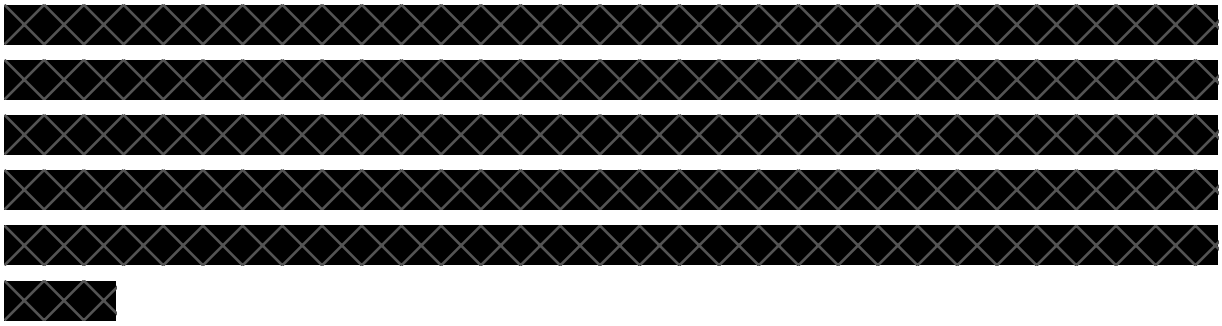
Stable cell transfection - To modulate miR-331-5p expression, plasmid designed to over-express the precursor of miR-331-5p (HmiR0192-MR04) or to inhibit its activity (HmiR-AN0419-AM01) were purchased from Gene-Copoeia (Nivelles, Belgium). Corresponding scrambled controls for over-expression (CMIR0001-MR04, miR-Null) and inhibition (CMIR-AN0001-AM01, Anti-miR-Null) were used as negative controls (Gene-Copoeia).

The cells employed in this study, were the human papillary (TPC-1) and two anaplastic (CAL62 and 8505C) thyroid cancer cell lines. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine, and penicillin/streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

Stable transfections were carried out using Lipofectamine 2000 reagent following standard protocol and after a selection with puromycin, a mass population was chosen on the basis of transfection efficiency.

All the reagents were purchased from Thermo Fisher Scientific, Waltham, MA, USA.

Transient cell transfection - Human papillary thyroid cancer (K1) cell lines were cultured in DMEM (Thermo Fisher Scientific) supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin (Thermo Fisher Scientific) at 37 °C in a humidified atmosphere of 5% CO_2.



qRT-PCR - To assess transfection efficiency in TC cells, total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific). The RNA concentration was assessed with NanoDrop spectrophotometer (Thermo Fisher Scientific).

For miRNA detection, 10 ng of RNA was reverse-transcribed using the TaqMan MicroRNA RT Kit (Thermo Fisher Scientific), following the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed using a TaqMan probe specific for miR-331-5p, with U6 small nuclear RNA serving as the endogenous control. The reaction utilized the TaqMan

Universal Master Mix II. Relative miR-331-5p expression changes between experimental samples and control (identified as cell lines transfected with the control plasmid miR-Null or Anti miR-Null and placed equal to 1) were calculated via $2^{-(\text{sample 1 } \Delta Ct - \text{control } \Delta Ct)}$.

[redacted]

Cell viability and proliferation - To evaluate cell viability, The CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS, Promega, WI, USA) was employed. This colorimetric technique quantifies metabolically active cells by measuring the conversion of MTS into a formazan product. Specifically, $1x10^3$ cells were seeded into each well of a 96-well plate. At defined time points (24, 48, 72 and 96 hours), 20µL of the MTS reagent was added to each well. Following a 30-minute incubation at 37°C, the absorbance at 490nm was measured using an automated microplate reader (Microplate Manager, Bio-Rad).

[redacted]

Cell migration and invasion assay - To assess cell migration ability, a wound healing assay was performed. Cells stably transfected were seeded in 6-well plates and cultured at 37°C for 24 hours. A scratch was carefully made across the monolayer using a sterile pipette tip. The wound area was observed under a Leica DM4000 phase-contrast microscope (5x magnification) and photographed from the moment of the scratch until closure.

ImageJ software (version 1.53t) was used to calculate the percentage of the closure, comparing transfected cells to relative controls.

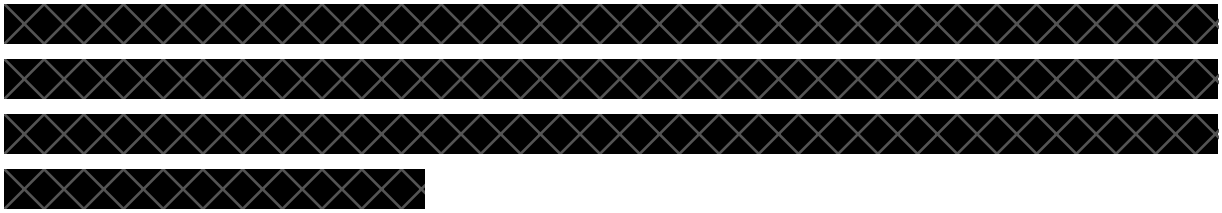
Briefly, as described in Orlandella *et al.* [97], For evaluating invasion capacity, both Matrigel Matrix and 3D Matrigel assay were employed. In the Matrigel Matrix assay, $1x10^5$ cells were suspended in DMEM supplemented with 5% FBS and seeded into the upper chamber of a polycarbonate membrane with 8µm pores coated with 35µg of Matrigel extracellular matrix (BD Biosciences, San Jose, CA, USA). Cells were incubated for 24 hours (TPC-1) or 48 hours (CAL62). Invading cells were fixed using an 11% glutaraldehyde solution, stained with crystal violet, and subsequently eluted for quantification at 550nm using a spectrophotometer.

For TPC-1 cells, the invasion was further evaluated using a 3D Matrigel assay. A 45µL layer of Matrigel Basement Membrane Matrix (BD Biosciences) was applied to Nunc Lab-Teck chambered coverglasses (Thermo Fischer Scientific) and polymerized at 37°C for 30 minutes. TPC-1 transfected cells (5×10^5) were suspended in 500µL of DMEM containing 10% FBS and 2% Matrigel and then plated on the chambered slides. Cells were incubated for up to two weeks, with images captured at defined intervals (4, 8, and 12 days) to evaluate their invasive capability.

Proteomic analysis - Proteomic profiling of CAL62 cells transfected with miR-331-5p or control (miR-Null) constructs was conducted using a label-free quantitative approach. Proteins were extracted, separated by SDS-PAGE, and analyzed via mass spectrometry.

Data processing and quantification were performed using a label-free approach, with differentially expressed proteins identified based on a fold change threshold (≥ 1.20 or ≤ -1.20). Results were validated and submitted to the ProteomeXchange repository (ID: PXD039884) [97].

Computational analysis - For the analysis of gene expression differences, ingenuity pathway analysis (IPA) was employed (QIAGEN, version 107193442, accessed on December 18, 2023), using RNA-seq data from the The Cancer Genome Atlas - Thyroid Cancer (TCGA-THCA) cohort. The data were processed using R software (version 4.1.3) and quantification was based on Human Genome build 38 with GenCode Release 33 [97].



[redacted]

Western blotting analysis - For protein analysis, cell lysates were prepared using JS Buffer, and protein concentrations were determined using the Bradford assay (Bio-Rad). Protein extracts were resolved via SDS-PAGE using 14% (for BID protein) [redacted] polyacrylamide gel and then transferred to nitrocellulose membranes.

After blocking, membranes were incubated overnight at 4°C with the following primary antibodies:

- anti-BID (B-3) monoclonal antibody (sc-373939, Santa Cruz Biotechnology);
- anti-α-TUBULIN (T9026) monoclonal antibody (Merck KGaA);
- [redacted]

Following washing, species-specific secondary antibodies conjugated with horseradish peroxidase were applied. The antigen-antibody complexes were detected using a chemiluminescence substrate (Thermo Fisher Scientific) and analyzed with a Bio-Rad Chemidoc imaging system.

Luciferase reporter assay - The potential binding sites of miR-331-5p to BID was identify by miRWalk (http://mirw alk.umm.uni-heidelberg.de/), RNAhybrid version 2.2 (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid) and STarMir (https://sfold.wadsworth.org/cgi-bin/starmirWeb.pl) online tools.

The interaction between BID and miR-331-5p was verified by the luciferase reported assay. This involved the use of a plasmid (LightSwitchTM, Product #S809507) where luciferase wase fused with the 3'UTR of BID under the control of a constitutive promoter. An empty vector (EMPTY_3UTR, Product #S890005), which only contains luciferase, served as positive control. Additionally, the 3'UTR of β-ACTIN gene (ACTB_3UTR Product #S804753), included for transfection normalization, was co-transfected.

Briefly, as described in Orlandella *et al.* [97], $1x10^3$ CAL62/miR-331-5p and CAL62/miR-Null were plated in a 96-well plate in triplicate. After 24 hours, cells were transfected with 50ng of plasmid DNA per well, using FuGENE HD reagent (3:1 Ratio of DNA to reagent). After 24 hours, luciferase activity was measured by adding 100μL of LighSwitch Assay Solution (Cat. Number, 32031) for 30 minutes. The luminescence was then read with a luminometer (EnSpire® Multimode Plate Reader, PerkinElmer, Waltham, MA, USA).

All reagents were purchased from SwitchGear Genomics (La Hulpe, Belgium).

Statistical analysis - All statistical analyses were conducted using GraphPad Prism 9 software. Results are presented as mean values with standard error of the mean (±SEMs).
Statistical significance was assessed using Student's t-test for pairwise comparisons, and the Wilcoxon test for non-parametric comparisons.
For comparison of more than two groups, the Kruskal-Wallis test was used. A p-value of <0.05 was considered statistically significant.
Additionally, Spearman's correlation was performed on the expression levels of genes and miRNAs, using RNA-seq and miRNA-seq data from the TCGA-THCA dataset, specifically for primary tumor samples.

7. ABBREVATIONS

ACRTI-RADS: American College of Radiology Thyroid Imaging and Reporting and Data System
ATA: American Thyroid Association
ATC: Anaplastic Thyroid Cancer
CLN: Cervical Lymph Nodes
CT: Computed Tomography
DHGTC: Differentiated High-Grade Thyroid Carcinoma
DLRN: Deep Learning Radiomics Nomogram
dsRNA: double-stranded RNA
DTCs: Differentiated Thyroid Cancers
EU-TIRADS: European Thyroid Imaging and Reporting Data System
FDA: Food and Drug Administration
FGF: Fibroblast Growth Factor
FNA: Fine-Needle Aspiration
FTC: Follicular Thyroid Cancer
GEPIA2: Gene Expression Profiling Interactive Analysis 2
GTEx: Genotype Tissue Expression
KCTD: Potassium (K+) Channel Tetramerization Domain
LNLNs: Lateral Neck Lymph Nodes
MAPK: Mitogen-Activated Protein Kinase
miRNAs: MicroRNAs
MKIs: Multikinase Inhibitors
MRI: Magnetic Resonance Imaging
mRNAs: messenger RNAs
MTCs: Medullary thyroid cancers
NGS: Next Generation Sequencing
OCA: Oncocytic Carcinoma of the Thyroid
PDGF: Platelet-Derived Growth Factor
PDTC: Poorly Differentiated Thyroid Cancer
PTC: Papillary Thyroid Cancer
qRT-PCR: Quantitative Reverse Transcription Polymerase Chain Reaction
RAI: Radioiodine
RISC: RNA-Induced Silencing Complex
RTK: Receptor Tyrosine Kinase
TC: Thyroid Cancer
TCIA: The Cancer Imaging Archive
TERT: Telomerase Reverse Transcriptase
Tg: Thyroglobulin
TGCA-THCA: The Cancer Genome Atlas-THyroid CAncer
TGCA: The Cancer Genome Atlas
ThyraMIR: Thyroid miRNA classifier
TRBP: Transactivation-Responsive RNA-Binding Protein
TRK: Tropomyosin Receptor Kinase
TSH: Thyroid-Stimulating Hormone
US: Ultrasound
UTRs: Untranslated Regions
VEGF-R: Vascular Endothelial Growth Factor Receptors
WHO: World Health Organization

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