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INVESTIGATION OF THE GENETIC BACKGROUND OF PAPILLARY THYROID CANCER

PhD thesis

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List of Abbreviations

Abbreviation	Meaning	Abbreviation	Meaning
AAMKI	anti-angiogenic multi-kinase inhibitor	**MET**	c-Met gene
ABL1	tyrosine-protein kinase ABL1 gene	**miEAA**	miRNA enrichment analysis and annotation
AJCC	American Joint Committee on Cancer	**miRNA / miR**	micro-ribonucleic acid
AKT3	AKT serine/threonine kinase 3 gene	**mRNA**	messenger ribonucleic acid
ALK	anaplastic lymphoma kinase gene	**MTC**	medullary thyroid carcinoma
ATA	American Thyroid Association	**NCBI**	National Center for Biotechnology Information
ATC	anaplastic thyroid carcinoma	**NCOA4**	nuclear receptor coactivator 4
AXL	AXL receptor tyrosine kinase gene	**NGS**	next-generation sequencing
BCR	breakpoint cluster region protein gene	**NIFTP**	neoplasm with papillary-like nuclear features
bp	base pair	**NTRK**	neurotrophic tyrosine receptor kinase gene
BRAF	proto-oncogene B-Raf	**ORA**	over-representation analysis
CA	California	**ORR**	objective response rate
CCDC6	coiled-coil domain-containing protein 6	**PC**	principal component
CDKN1B	cyclin-dependent kinase inhibitor 1B	**PCA**	principal component analysis
CNV	copy number variation	**PCR**	polymerase chain reaction
DICER1	Dcr-1 homolog (Drosophila)	**PDGFRA**	platelet-derived growth factor receptor A gene
DNA	deoxyribonucleic acid	**PD-L1**	programmed death-ligand 1
dsDNA	double-stranded deoxyribonucleic acid	**PFS**	progression-free survival
DTC	differentiated thyroid cancer	**PI3K**	phosphatidylinositol 3-kinase
EBRT	external beam radiation therapy	**PLCγ**	phosphatidylinositol phospholipase C γ
EGFR	epidermal growth factor receptor gene	**PPARG**	peroxisome proliferator-activated receptor γ gene
EML4	echinoderm microtubule-associated protein-like 4	**PTC**	papillary thyroid carcinoma

emPCR	emulsion polymerase chain reaction	**QC**	quality control
ERBB2	erb-b2 receptor tyrosine kinase 2 gene	**qPCR**	quantitative polymerase chain reaction
ERG	ETS-related gene	**R stage**	residual tumor classification
ETV	ETS variant transcription factor gene	**RAF1**	Raf-1 proto-oncogene
FC	fold change	**RAI**	radioiodine therapy
FDR	False Discovery Rate	**RAS**	rat sarcoma virus gene
FFPE	formalin-fixed paraffin-embedded	**RECIST**	response evaluation criteria in solid tumors
FGFR	fibroblast growth factor receptor gene	**RET**	ret proto-oncogene
FISH	fluorescence in situ hybridization	**RNA**	ribonucleic acid
FNAB	fine-needle aspiration biopsy	**ROS1**	ROS proto-oncogene 1
FTC	follicular thyroid carcinoma	**SD**	standard deviation
FuPa	fragmentase universal primer assay	**SE**	standard error
GO	Gene Ontology	**SELECT**	Semaglutide Effects on Cardiovascular Outcomes in People with Overweight or Obesity
H&E	hematoxylin and eosin	**SQSTM1**	Sequestosome-1 gene
H. sapiens	Homo sapiens	**TCGA**	The Cancer Genome Atlas
HIF-1	hypoxia-inducible factor	**Tg**	thyroglobulin
HTLV	human T-lymphotropic virus	**TgAb**	thyroglobulin antibody
I^{131}	iodine-131 isotope	**TGF-β**	transforming growth factor beta
IGFBP5	insulin-like growth factor binding protein 5	**TKI**	tyrosine kinase inhibitor
IHC	immunohistochemistry	**TNM**	TNM Classification of Malignant Tumors
IL	Illinois	**TP53**	tumor protein p53
ISP	Ion Sphere Particle	**TSHR**	thyroid stimulating hormone receptor
KEGG	Kyoto Encyclopedia of Genes and Genomes	**USA**	United States of America
MA	Massachusetts	**V600E**	valine-glutamic acid substitution at amino acid 600
MAPK	mitogen-activated protein kinase	**ZNRF3**	zinc and ring finger 3

1. Introduction

Thyroid cancer is the most common type of endocrine cancer, with more than 800.000 newly registered cases in 2022 globally (1,2). Its incidence has more than doubled in the last 30 years in the USA (3,4). About 90% of the patients diagnosed with thyroid cancer have its differentiated (DTC) subtype, including, basically, the papillary (PTC) and the follicular (FTC) histological variants (5). For better therapeutic outcomes, the widely known conventional histological categorization of thyroid cancer and its subtypes is increasingly required to be further classified into molecular categories as well (6).

The primary choice of treatment for thyroid cancer, if technically feasible, is its surgical removal by partial or total thyroidectomy, depending on the risk of recurrence (7,8). Based on the American Thyroid Association (ATA) guidelines, regular risk assessment is recommended during postoperative management to estimate therapeutic response and prognosis and to prevent relapse. Risk assessment should take factors like histological type, extrathyroidal invasion, (lympho)vascular invasion, presence of distant metastases, and postoperative serum marker (thyroglobulin, anti-thyroglobulin) levels into consideration. Prognostic value is increasingly associated with the molecular profile of the cancer as well (7,9–11). If the cancer falls into the intermediate-risk or high-risk category and contraindication is not present, post-surgical adjuvant I^{131} radioiodine (RAI) is recommended as part of the first-line treatments. RAI can be repeated several times if molecular or structural recurrence happens (7,12).

In the case of therapy-refractory cancer or loss of radioiodine uptake, molecular target therapies can be considered as second-line options depending on the clinical setting (7,12–14). ATA guidelines recommend active surveillance for metastatic DTCs without symptoms with a diameter between 1 and 2 cm and an active intervention if the tumor growth rate increases. Additionally, molecular therapies can be indicated in metastatic DTC cases (12). Molecular therapies taking effect on the mitogen-activated protein kinase (MAPK) signaling pathway can sometimes even reinduce radioiodine sensitivity in tumors previously proved to be refractory to RAI (7,15). Besides those related to MAPK, other significant thyroid cancer signaling pathways include molecules such as phosphatidylinositol 3-kinase (PI3K) and phosphatidylinositol phospholipase C (PLCγ) (16,17). As molecular therapies are usually highly specific, indicating them as second-line approaches can mostly happen after successfully detecting a targetable mutation

within the tumor. Selective molecular therapies are associated with greater efficacy and a more tolerable side effect profile (18). However, in cases where a specifically targetable mutation cannot be found (e.g., *RET*), the usage of anti-angiogenic multi-kinase inhibitors (AAMKIs) might be a viable alternative (15). In general, if first-line approaches fail to achieve the therapeutic goal, and a selectively targetable mutation can be identified while contraindications are not present, application of the appropriate molecular therapy is the recommended choice of treatment; except for the *BRAF* inhibitors against $BRAF^{V600E}$ mutation in DTC. These usually come only after failed or contraindicated AAMKI treatments. It is worth mentioning that the RECIST objective response rate (ORR) and the progression-free survival (PFS) of lenvatinib, an AAMKI, was superior compared to the *BRAF*-selective dabrafenib ± trametinib in the SELECT study (7,15).

Detection of several classes of gene and molecular alterations is possible such as hotspot mutations, copy number variations (CNV), fusion mutations, and microRNA (miRNA) expression differences (17,19). During our original molecular research, we wanted to investigate the relevance and role of frequent gene fusions as well as the miRNA expression patterns within PTC.

Gene fusions basically occur when a single hybrid gene is created by the merger of two distinct genes after an improper chromosomal rearrangement, which causes chromosome segments to break and then reattach incorrectly. Defects of this kind can act as driver mutations and facilitate cancer development. A classic example of such a mutation is the Philadelphia chromosome created by the translocation of the *BCR* and *ABL1* genes between chromosomes 9 and 22, leading to chronic myeloid leukemia (20). However, the oncogenic effect of gene fusions is evident in the case of solid tumors, like PTC (17). The clinically most relevant, frequently occurring, and selectively targetable gene alterations in thyroid cancer are the different types of *BRAF*, *RET*, *NTRK*, and *MET* mutations (19,21–26). An aberrant *RET* gene is quite frequent not only in medullary thyroid cancer but in DTC (~10%) as well (19,27,28). Selective *RET* kinase inhibitors targeting the signaling pathway of *RET* are available to use in such a mutation pattern. They have tolerable side effects and their efficacy, regarding paraneoplastic symptoms as well, is explicit. Members of the neurotrophic tyrosine receptor kinase (*NTRK*) gene family (*NTRK1*, *NTRK2*, *NTRK3*) can also often carry somatic mutations. In these cases, potential oncogenicity is mainly linked to protein fusions of tyrosine receptor kinases on

thyrocytes and their autonomous MAPK, PLCγ, and PI3K signaling activity, consequently. Potent second-line treatments in DTC counteracting the effects of *NTRK* fusions are available as well. These medications showed great therapeutic response rates accompanied by few side effects. In addition, they showed the ability to help tumor tissue regain its previously lost radioiodine uptake ability. The clinical value of inhibiting the molecular pathway related to *MET* in DTC is currently under investigation in clinical trials (13,15,16,24–26,28).

Apart from gene fusions, PTC, characterized by its unique molecular signature, often exhibits alterations in its miRNA expression pattern as well (29–31). miRNAs form a special group of RNAs, being small and non-coding nucleic acids with a fine-tuning regulatory role in gene expression (32). Briefly, miRNAs are around 18–25 nucleotides in length and take effect by binding to their complementary sequences on messenger RNA (mRNA) transcripts, which could result either in target degradation or translational repression and, by that, gene silencing (33). miRNAs are considered to be quite stable molecules, making them accessible from a variety of biological samples such as fresh tissues, fine-needle aspiration biopsy (FNAB) specimens (34,35), blood samples (36,37), or even from formalin-fixed paraffin-embedded (FFPE) tissues (38). miRNAs represent a long-hidden and complex layer of gene regulation that is a crucial component in many physiological and pathological processes including cardiovascular diseases (e.g., cardiac fibrosis and cardiac hypertrophy) (39,40), microbiome homeostasis (41), diabetes mellitus (42,43), calcium and bone metabolism (44,45), as well as in the pharmacodynamics of certain drugs (46). miRNAs influence many cellular processes such as differentiation, proliferation, apoptosis, and metastasis development (29,47–49). In addition, research on the role of miRNAs in anti-cancer drug sensitivity in thyroid cancer has also been published (37).

In oncology, miRNAs are receiving more and more attention due to their duality acting both as oncogenes and tumor suppressors (50). Emerging data suggest that different molecular backgrounds, including aberrant miRNA expressions, are a common feature of various cancers (30,51), including thyroid malignancies (52). PTC is seemingly well known by many clinicians and pathologists, although it is still slowly turning into a more heterogeneous category, which can be further divided not only into clinicopathological subtypes but molecular ones as well. Admittedly, some papers have

already been published investigating the role of miRNAs in PTC pathogenesis (19,53,54); however, establishing the exact depth of the correlation between specific miRNA expressions and the development of the disease lacks original molecular studies with sufficiently large control case numbers even to this day. Also, these studies provide an insufficient coverage of miRNA types. For instance, even The Cancer Genome Atlas (TCGA) project involved PTCs with only 59 matched controls for miRNA analysis and evaluated 1046 different miRNAs while lacking clinicopathological aspects (19). miRNAs' association with the pathogenesis of other conventional thyroid cancer types such as follicular thyroid carcinoma (FTC), medullary thyroid carcinoma (MTC), or anaplastic thyroid carcinoma (ATC) has also been noticed (55). In cases when DTC was diagnosed, tumor development might be traced back to a DICER1 RNase IIIb hotspot mutation generating an unbalanced expression ratio of 5p:3p miRNAs (56).

The link between these miRNA deviations and disease development makes specific miRNAs perfect candidates for being a new generation of diagnostic and prognostic markers of PTC. Indeed, miR-21, miR-127, miR-136, miR-146b, miR-221, and miR-222 are frequently upregulated in PTC and are associated with a more aggressive course of the disease and an overall poorer prognosis (19,29,37,51,55,57–64). Also, *BRAF*-targeting miR-486-5p, miR-9-5p, and miR-708-3p in PTC were previously described as downregulated and – in the case of miR-486-5p – associated with lymph node metastasis development as well as more advanced tumor stage and risk of recurrence (53). Based on previous findings, 5p/3p expression ratio of specific miRNAs might also play a role in the onset of lymph node metastases (54). Upregulation of miR-181a in thyroid cancer has also been identified previously (29). Moreover, expression alterations of miRNAs such as miR-204, miR-146b, miR-221, and miR-222 have been described in association with important cancer features like extrathyroidal invasion and/or metastasis development (29,48,63,64). Alternatively, a decrease in tumor-suppressing miRNA levels, such as let-7, miR-125b, and miR-204-5p (19,56), has also been noted, propagating the malignant conversion of thyroid cells (65). Furthermore, miR-136, miR-21, and miR-127 showed significant correlation with the risk of persistent disease and potential relapse as well as with the risk categories defined by the American Thyroid Association's (ATA) risk stratification system (7,66).

Genetic alterations, depending on their type, can be detected via multiple methods such as polymerase chain reaction (PCR), fluorescence in situ hybridization (FISH), immunohistochemistry (IHC), and next-generation sequencing (NGS). These molecular methods can be applied to fresh tissues and also to FNAB and FFPE specimens (17,20–22,28,67,68).

Compared to miRNAs, the role and importance of fusion mutations in PTC are already quite well understood; however, original molecular studies evaluating their connection to everyday clinical and histological metrics related to PTC are still much needed as well. With our investigations on heterogenous Hungarian PTC cohorts, we would like to provide a comprehensive analysis of fusion mutation occurrences and reveal miRNA expression differences related to the disease by applying NGS. During our research, on one cohort (100 patients), we evaluated the distribution of frequent fusion mutations in PTC determining fusion mutation–clinicopathological metric associations in order to point out the potential of molecular diagnostics in PTC if used more frequently, even in earlier stages or in special clinical scenarios. On a different cohort (118 patients), we compared the miRNA expression patterns of cancerous and non-cancerous thyroid tissue samples.

For fusion mutation analyses, we conducted a retrograde fusion mutation screening in the context of 23 relevant gene fusions in the tumor samples. Then, correlations among molecular and clinicopathological variables of the PTC cases were calculated to find any underlying associations between them.

Based on the results of previous findings, a better understanding of miRNA expression profiles of malignancies – including thyroid cancer – may provide insights into tumorigenesis and disease progression as well as potential diagnostic, prognostic, and therapeutic possibilities in the future (62,64). For this purpose, we conducted a comprehensive molecular study to detect specific miRNAs associated with PTC based on their expression differences between tumor sections and adjacent non-tumor tissues. We evaluated the miRNA profiles of 236 individual thyroid tissue samples (one cancerous and one healthy sample per case) and analyzed the sequencing data of the samples in the context of 2656 types of human miRNAs, doubling the control-matched sample size and more than doubling the examined miRNA cluster size compared to the TCGA study (19).

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2. Objectives

This thesis aims to explore the genetic and molecular underpinnings of DTC, with a specific focus on PTC, the most prevalent histological subtype of the disease. Our research investigates two critical areas: the role of fusion mutations and miRNA dysregulation in the pathogenesis of PTC. We believe that complementary molecular profiling studies in the field can improve PTC diagnostics and treatment, especially if the research comes with a broad clinicopathological dataset simultaneously.

2.1. Descriptive statistics and clinicopathological correlations related to certain gene fusions in PTC

We aimed to be the first to determine the overall distribution of the clinically most relevant (most frequent and/or therapeutically targetable) gene fusions within PTC tumor tissues originating from a sufficiently large Hungarian cohort. We wanted to put our raw molecular data into context by assigning clinicopathological values to it as well.

2.2. The potential role of miRNAs in the development of PTC and their associations with certain clinical scenarios and biological conditions

miRNA research is a rapidly growing field of oncology but it lacks thyroid cancer-related studies. For this reason, we also aimed to perform the most comprehensive analysis of the miRNA expression patterns in a separate Hungarian PTC cohort. The main goal was to highlight the role of certain miRNAs in PTC development by identifying significant miRNA expression differences in cancerous thyroid tissue samples compared to adjacent healthy thyroid tissue. In addition, we planned to further interpret miRNA expression patterns in the context of large-scale international molecular datasets, providing a broader perspective on the role of miRNA dysregulations in PTC. Moreover, similarly to our fusion mutation study, we aimed to find associations between the miRNA-related molecular results and some clinicopathological features as well.

With results originating from extensive datasets, we wished to underscore the importance of integrating molecular profiling into clinical decision-making by highlighting the utility of genetic data in improving cancer diagnostics and prognostics.

3. Methods

3.1. Study population

3.1.1 Study population of the fusion mutation investigation

RNAs were isolated from tissue samples obtained from 107 consecutive patients previously diagnosed with papillary thyroid carcinoma (PTC) who were deemed eligible for the study. However, seven samples were subsequently excluded due to either insufficient RNA quality or incomplete clinical data. This resulted in a final cohort of 100 samples with molecular data suitable for further analysis. The fusion mutation study population exhibited a marked gender disparity, with a significantly higher number of women (n = 71) compared to men (n = 29).

The mean age (± SD) at diagnosis was 45 years (± 15.64 years; women: 46 ± 16.15 years; men: 44 ± 14.52 years). 32 patients had no comorbidities aside from PTC. To investigate the genetic background of PTC, sequencing was performed to identify 23 different types of fusion mutations considered highly relevant to PTC pathogenesis. In the fusion mutation study cohort, the analyzed samples encompassed various PTC subtypes, as detailed in **Table 1**.

Table 1. PTC subtypes in the fusion mutation study cohort.

PTC Subtype	n = 100
conventional	69
follicular variant	17
oncocytic	6
tall cell	3
columnar cell	2
encapsulated conventional	1
trabecular	1
Warthin-like	1

3.1.2 Study population of the miRNA expression investigation

miRNAs were isolated from 258 tissue samples, comprising cancerous and normal tissue specimens from 129 selected PTC patients. Ten tissue sample pairs were excluded due to inapplicable isolate specimens; the remaining ones were then forwarded to sequencing. The sequencing analysis examined expression patterns related to a total of 2656 different miRNAs. During the subsequent bioinformatic assessment, one additional sample pair was excluded due to insufficient sequencing yield. After all necessary exclusions, paired tumor and control samples from a total of 118 PTC patients were included in the final evaluation (**Figure 1**). The tumor samples analyzed in the miRNA study comprised the following histological subtypes of PTC: conventional (n = 96), follicular subtype (n = 16), oncocytic (n = 4), columnar cell (n = 1), and Warthin-like (n = 1) (**Table 2**).

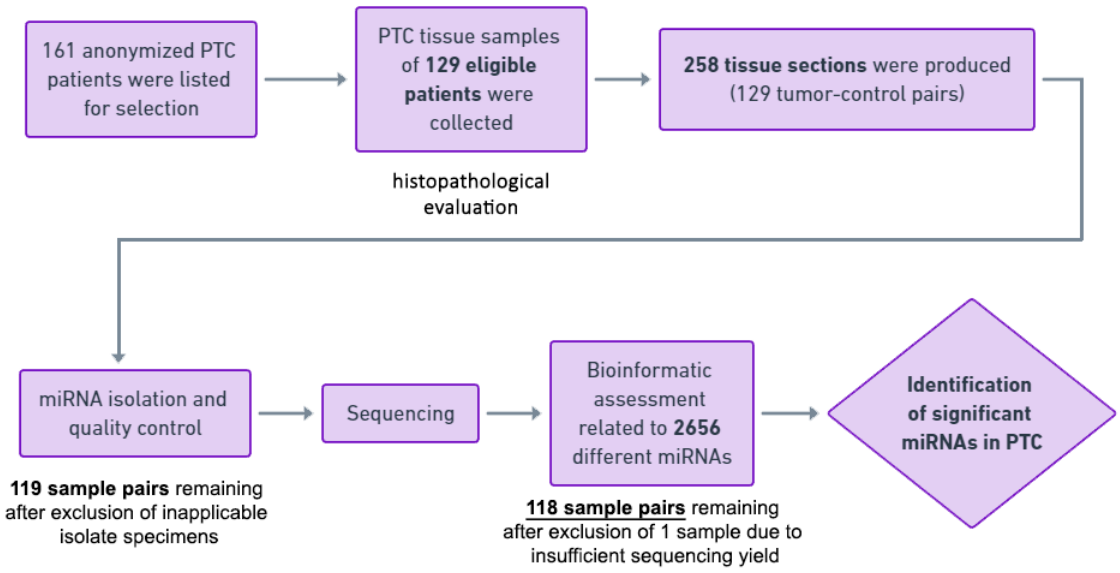

Figure 1. This workflow diagram outlines the steps involved in the miRNA analysis of PTC patients. A total of 161 anonymized PTC cases from the tissue archives were reviewed, of which 129 were deemed eligible based on histopathological evaluation. Paired thyroid tissue samples, including 129 tumor and 129 control specimens, were collected from these cases and subsequently subjected to sectioning for further analysis. Tissue sections underwent miRNA isolation and RNA quality control to assess concentration levels, resulting in the exclusion of samples evaluated as inapplicable

isolate specimens. The remaining samples proceeded to sequencing, where expression patterns were analyzed across 2656 different miRNA types. Following sequencing, a bioinformatic and statistical assessment was performed on the data. During this evaluation, one additional sample pair (tumor and control) was excluded due to insufficient sequencing yield. Finally, we were able to establish those miRNAs that show significantly different expression patterns in PTC and non-PTC tissues related to 118 patients in total.

Table 2. PTC subtypes in the miRNA study cohort. The distribution of PTC subtypes in the studied cohort revealed that, among the 118 patients, the conventional subtype was the most prevalent, representing 81.36% of cases, followed by the follicular subtype, which accounted for 13.56% of cases. These findings underscore the dominance of the conventional subtype in PTC incidence while reflecting the variability in PTC presentations.

PTC Subtype	n =	%
Conventional	96	81.35
Follicular subtype	16	13.56
Oncocytic	4	3.39
Columnar cell	1	0.85
Warthin-like	1	0.85
All PTC subtypes	118	100

3.2. Sample collection, and histopathological processing for both fusion mutation and miRNA expression analysis

After preliminary filtering, a total of 100 – for the fusion mutation investigation – and 118 – for the miRNA expression analysis – PTC tissue blocks derived from thyroid surgical materials, were included in our research. These samples of consecutive cases were collected from the histopathological archives of the Department of Internal Medicine and Oncology, Semmelweis University, Hungary, and the National Institute of Oncology, Hungary. We queried the related medical records and pathological data from clinical databases (e.g., eMedSolution®, MedRec®) in an anonymized manner.

In both studies, we included the following clinicopathological data as variables: age at diagnosis, sex, ATA risk score, TNM stage, and cancer stage based on the 8th edition of the American Joint Committee on Cancer (AJCC). Exclusively in the fusion mutation study, additional variables were included; namely histological subclassess of PTC, aggressiveness of the PTC variant, R stage, tumor size, (lympho)vascular invasion, perineural spread, capsule invasion, extrathyroidal spread, focality, microcarcinoma character, cancer localization and sidedness within the thyroid lobes, preoperative imaging features (tumor size, sidedness, infiltrative character, lymph node involvement, presence of distant metastasis), type of surgical procedure performed (including lymph node dissection), features of indicated treatments (radioiodine, external beam radiation therapy (EBRT), molecular therapy, relapse after treatment (molecular and/or structural), complication after treatment), data from medical history (family history of thyroid disease, prior chemo-therapy, Hashimoto's disease, prior hyperthyroidism or hypothyroidism, prior goiter, association with thyroid or parathyroid adenoma, and association with comorbidity clusters such as other malignancy, breast cancer, benign tumor, uterine myoma, diabetes, cardiovascular morbidity, respiratory disease, gastric acid related disorder, appendicitis, autoimmune disease, gallstones, musculoskeletal disorder, kidney stones, endometriosis).

The collected tissue samples were FFPE blocks. Hematoxylin and eosin (H&E)-stained trial sections were prepared from these blocks to confirm the presence of tumor tissue, verify the histological subtype, and determine the percentage of tumor volume within the samples by an expert pathologist. Only PTC samples with a sufficient tumor burden at the site of interest within the FFPE block, relative to the overall tissue volume,

were included for further processing. Samples containing less than 80% tumor volume were excluded from the subsequent analyses.

In the case of the miRNA expression study, the involvement of non-tumor control samples was also required. For this purpose, we used thyroid tissues adjacent to cancer within the same thyroid specimens. The control samples were collected by targeting tissue areas as distant as technically feasible from the PTC sites, typically from the contralateral lobe of the thyroid, to ensure minimal contamination and reliable comparison. In miRNA analysis, both the tumor-containing and the normal tissue samples were dissected from the same surgical material, thus resulting in a double amount of tissue specimens per patient relative to the fusion mutation study.

Macrodissections of the pre-selected and approved tissue blocks were performed, yielding 10 μm thick tissue curls, with four curls obtained per sample. These tissue curls were subsequently processed for molecular analysis.

3.3. Fusion mutations in PTC: relative frequency and their correlation with clinicopathological characteristics and patient outcomes

3.3.1 Molecular processing for fusion mutation detection (RNA isolation, quality control (QC), RNA quantification and sequencing)

The RecoverAll™ Total Nucleic Acid Isolation Kit (Life Technologies, Carlsbad, CA, USA) was used for the isolation of DNA-free RNA. In short, the paraffin was removed with xylene and ethanol treatment. The pellets were digested with proteinase K solution in heat blocks for 15 minutes at 50 °C, then 15 minutes at 80 °C. Samples were combined with Isolation Additive, ethanol, and then RNAs were captured by the column. After several washes and incubation with the presence of DNAse, purified RNA was eluted into a 60 μL elution buffer. The concentration of the isolated RNA was measured with the Qubit RNA HS Assay Kit (Life Technologies, Carlsbad, CA, USA).

The Oncomine Focus amplicon library was prepared using the Ion AmpliSeq Library Kit 2.0 (Life Technologies, CA, USA); briefly, multiplex primer pools were added to 10 ng of genomic DNA, and after reverse transcription, to 10 ng of total RNA, then amplified with the following PCR cycles: at 99 °C for 2 minutes, at 99 °C for 15 seconds, and at 60 °C for 4 minutes (23 cycles), and holding on at 10 °C. Primers were partially digested using a FuPa reagent, then sequencing adapters were ligated to the amplicons. Agencourt

AMPure XP Reagent (Beckmann Coulter, CA, USA) was selected for library purification. The concentration of the final library was determined by the qPCR method run by the QuantStudio instrument (Life Technologies, CA, USA).

Template preparation was performed with the Ion OneTouch kit (Life Technologies, CA, USA) on a semiautomated Ion OneTouch instrument using an emPCR method. After breaking the emulsion, the non-templated beads were removed from the solution during the semiautomated enrichment process on an Ion OneTouch ES (Life Technologies, CA, USA) machine. After adding the sequencing primer and enzyme, the Ion Sphere Particle (ISP) beads were loaded into an Ion 520 sequencing chip, and the sequencing runs were performed using the Ion S5 Sequencing kit (Life Technologies, CA, USA) with 500 flows.

3.3.2 Data processing for gene fusion assessment and correlation analysis

In the PTC tumor samples, we aimed to detect the presence or absence of the following 23 different oncogenic driver gene fusion mutations: *ABL1*, *AKT3*, *ALK*, *AXL*, *BRAF*, *EGFR*, *ERBB2*, *ERG*, *ETV1*, *ETV4*, *ETV5*, *FGFR1*, *FGFR2*, *FGFR3*, *MET*, *NTRK1*, *NTRK2*, *NTRK3*, *PDGFRA*, *PPARG*, *RAF1*, *RET*, *ROS1*.

To explore the distribution of the listed fusion gene partners compared to other gene partners, other fusion mutations, and fusion-negative cases, variant annotation and cloud-based data analysis were performed using Ion Reporter 5.18 platform (Life Technologies, CA, USA) with pre-defined parameters followed by descriptive statistics.

After successfully collecting molecular data, we complemented the clinicopathological dataset with sequencing information regarding the fusion mutation status for each case. Due to the large number of variables and the heterogeneity of the variable types within the clinicopathological dataset, which contains nominal, ordinal, and ratio-scale types, we applied multiple statistical tests to improve the quality of our multivariate analysis.

Descriptive statistics of basic clinical data were performed using IBM SPSS 27.0 (SPSS Inc., Chicago, IL, USA).

Association mapping within the entire dataset (including the listed fusion mutations and all the clinicopathological data) could not have been achieved by conventional multivariate techniques. The reason is that the variables are heterogeneous in terms of

measurement scale. Fusion mutation-related variables are binary nominal as well as many other clinicopathological variables (e.g., aggressiveness, multifocality, relapse). However, other variables belonged to ordinal (e.g., clinical stage, TNM stage, ATA risk score) and ratio (e.g., age, tumor diameter) types. In the case of nominal variables (or binary/nominal variables), only the equality or non-equality of character states can be established. Arithmetic variances only make sense in the case of ratio-type variables, while for ordinal variables, only the sequence of states can be interpreted. Therefore, special methods need to be used to uncover variable associations in the most effective way possible. A new method, the *d*-correlation formula, recently published by Podani et al., was applied to determine correlations across mixed-type variables by reducing the number of dimensions of the dataset. Then, the comprehensive correlation matrix, which was generated by this formula, could be visualized with principal component analysis (PCA) with which we were able to reduce data dimensionality by identifying the most important patterns that describe the data variability. PCA also reorients the data into principal components, which are new, uncorrelated variables ordered by their importance.

For specific subsets of data, we also performed more conventional statistical analyses to extend the scope of the investigation to potential correlations that were not apparent from PCA. For this, we mainly focused on associations of *ETV6* and *NTRK3* partner genes as they were seemingly not clustering well with other variables via PCA. Moreover, we assessed the relation of fusion mutation status (including general fusion mutation positivity and those specific fusions occurring at least six times in the dataset) to the patients' age at diagnosis, and the association pattern exclusively between not fusion-related, binary-type clinicopathological variables. For the reasons detailed above, we were limited in terms of effectively comparing multiple variable types with each other in such a way. This statistical analysis was conducted using the Python v3.8 programming environment. The significance of associations between fusion mutations (binary/nominal) and other nominal-, and ordinal-type clinicopathological variables was calculated by the Chi-square test ($p < 0.05$) using the SciPy v1.7.3 package. Associations found to be significant from these subsets of data were then further evaluated with logistic regression models to determine the strength and direction of the correlations, for binary variables exploiting the scikit-learn v1.2.2 package. We used multinomial logistic regression for not-binary nominal variables, as it handled multiple different categories of the same

variable better. For ordinal variables, we applied the Ordered Logit model using the Statsmodels package v0.13.2.

For ratio-type variables, we first checked the normal distribution of the values. Only age at diagnosis showed a normal distribution, while the diameter of the tumor, the cumulative dose of RAI therapy, and the cumulative dose of EBRT were not-normally distributed. Given the size of the dataset, the evaluation of normal distribution was performed by the Shapiro–Wilk test. The normally distributed variable (age at diagnosis) was further tested for the homogeneity of variances. Next, the mean age at diagnosis was investigated in the context of fusion mutation status by applying an independent two-sample t-test or, in the case of *ETV6* fusion, a Welch's t-test due to potential violation of the homogeneity of variances assumption. With the t-tests, we were able to determine any significant differences between the mean age of the fusion-positive and fusion-negative groups of patients. In cases of not-normally distributed variables, the Kruskal–Wallis test was used to determine any significant associations to fusion mutations. As no significant association pairs could be identified, we did not continue with a deeper analysis in this direction. For statistical evaluations of ratio-type variables, we applied the SciPy package as well.

3.4. miRNA expression profile of PTC compared to normal thyroid tissue and its association with clinicopathological features, diagnostics and prognostics

3.4.1 Molecular processing for miRNA detection (miRNA isolation, quality control (QC), miRNA quantification, and sequencing)

Zymo Quick RNA FFPE kit (Zymo Research, Irvine, CA, USA) was employed for the isolation of miRNAs from the prepared FFPE tissue sections. The process started with the removal of paraffin using a proprietary deparaffinization solution, which was followed by rehydration of the tissue. The tissue was then subjected to proteinase K digestion at 55 °C for 2 hours and subsequently at 65 °C for an additional 15 minutes to ensure thorough lysis. After digestion, the samples were treated with RNA lysis buffer, which facilitated the selective binding of miRNAs to the kit's Zymo-Spin IICR column. The column was then washed multiple times to remove contaminants. To ensure the elimination of genomic DNA, the samples were treated with DNase. Finally, the miRNAs were eluted in 50 μL of elution buffer. During molecular analysis, we performed RNA

quality assessment. Initially, we determined RNA concentrations using the Qubit™ HS RNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) on a Qubit™ 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). When necessary, the concentration of miRNAs was measured again using the Qubit™ microRNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) also on a Qubit™ 3.0 fluorometer.

Then, we prepared miRNA libraries in the following multi-step process using NEXTFLEX® Small RNA-Seq Kit v4 (PerkinElmer Inc. Waltham, MA, USA). We started with an input of 50 ng of RNA, followed by the ligation of the NEXTFLEX® 3′ adenylated adapters. After that, we removed the excess 3′ adapters, then we ligated the NEXTFLEX® 5′ adapters. This was succeeded by the reverse transcription, first-strand synthesis; post-synthesis, we conducted bead cleanup, and then PCR amplification was performed using barcoded primers (19 cycles). Lastly, we finished the miRNA library preparation with size selection and cleanup.

The next step was the quality control of the miRNA libraries involving DNA concentration measurement using the Quant-iT™ 1X HS dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) on either a FLUOstar Omega fluorometer (BMG Labtech, Ortenberg, Germany) or a Qubit™ 3.0 fluorometer, along with assessment of the fragment sizes using the LabChip® GX Touch™ nucleic acid analyzer (PerkinElmer Inc. Waltham, MA, USA) with an HT DNA X-Mark Chip (CLS144006) (PerkinElmer Inc. Waltham, MA, USA) with the HT DNA NGS 3K Reagent Kit (PerkinElmer Inc. Waltham, MA, USA).

For pooling the libraries, we calculated the molar concentrations based on the overall concentrations and the fragment sizes. Then, equal molar quantities were pooled from the libraries. The concentration of this pool was measured using the Quant-iT™ 1X HS dsDNA Assay Kit again and diluted to the final concentration of 2 nM.

Finally, the sequencing was carried out on a NextSeq 2000 system (Illumina Inc., San Diego, CA, USA) using a P3 (1 × 50 cycles) kit. This setup allowed us to generate 2 × 40 base pair (bp) paired-end reads. miRNAs showing expression levels below the set threshold of the applied NGS platform both in the PTC and control samples were considered as not-expressed miRNAs. During the process, we maintained the seeding concentration at 650 pM to ensure optimal sequencing depth and quality.

3.4.2 Data processing for miRNA expression profile assessment and correlation analysis

The quality check of the raw reads was performed via FastQC v0.11.7 and MultiQC. Forward and reverse reads were merged via PEAR v0.9.11 and then quality trimmed with Trim Galore v0.6.10. The quality threshold was set to 30, and only reads between 18 and 30 bp in length were used for further analysis based on the literature. One sample pair was removed from the analysis due to insufficient sequencing yield compared to the other samples. Based on recommendations, Bowtie1 v1.3.1 was used for the alignment of the reads to the miRBase v22.1 *H. sapiens* miRNA database with the following parameters: *-n 0 -l 8 --best --strata -m 1 –no-unal*. Read counts were calculated for each miRNA using SAMtools v1.14.

The expression levels of miRNAs can span several orders of magnitude, making the direct comparison of raw data challenging and less informative. To address this, we presented our data using logarithmic values, specifically $\log_2$ fold change (FC) for expression levels and $-\log_{10}P$ for p-values. We applied a threshold for marked differential expression with a minimum $\log_2$ FC of ±1 and the Benjamini–Hochberg-corrected p-value less than or equal to 0.05. By using a logarithmic transformation, we were able to mitigate the impact of extreme values, create a symmetrical view of up- and downregulation (both of which can be relevant), and make our results more visible. We applied PCA as a statistical tool in miRNA analysis as well.

Statistical analysis of the read counts was performed in the R v4.2.1 programming environment. Differential miRNA expression was calculated with the DESeq2 package. The ComplexHeatmap, EnhancedVolcano, and ggplot2 packages were used for the data visualization. The network graph was constructed using Python’s NetworkX library.

To highlight the potential biological and clinical implications of miRNA dysregulation in PTC, we performed a comprehensive KEGG pathway enrichment analysis using the miEAA analysis server, leveraging the miRPathDB database; in addition, we utilized the Gene Ontology (GO) framework to systematically categorize the functions of genes influenced by the differentially expressed miRNAs. The GO Resource is a collaborative bioinformatics tool that provides consistent descriptions of gene products across databases and species. It encompasses structured networks of defined

terms that represent gene product properties, covering biological processes, cellular components, and molecular functions. miEAA v2.1 was used for an over-representation analysis on the miRNAs with adjusted p-values less than or equal to 0.05. The analysis was performed on the Gene Ontology and KEGG terms available in the miRPathDB database via the miEAA analysis server. Only terms with at least 10 genes were surveyed, and they were considered significant if the FDR-corrected p-value was less than 0.01.

3.5. Literature review

The literature search was conducted up to 31 October 2024 using NCBI's PubMed database to identify disease associations and biological interactions related to gene fusions and miRNAs, ensuring the inclusion of the most recent studies available in our work. Our goal was to select peer-reviewed articles that contained relevant information about the specific gene fusions and miRNAs we were studying, particularly in the context of human diseases.

A variety of synonymous search terms were simultaneously employed to gather all the required information from the existing literature. These search terms included *"papillary thyroid carcinoma"*, *"PTC"*, and *"thyroid carcinoma"*. In the case of fusion mutation analysis, search terms also included *"fusion mutation"*, and *"gene fusion"* as well as the names of individual genes; while in the case of miRNA expression investigation, we searched for *"miRNA"*, *"microRNA"* and specifically the studied miRNAs.

Our miRNA expression study utilized the miRBase v22.1 *H. sapiens* miRNA database for human miRNA sequence and annotation retrieval.

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4. Results

4.1. Descriptive statistics and clinicopathological correlations related to certain gene fusions in PTC

4.1.1 Prevalence and distribution of fusion mutations in PTC

The sequencing data revealed the distribution of the fusion mutations of interest within the PTC cohort, providing a comprehensive insight into the molecular patterns underlying disease development, as illustrated in **Figure 2**.

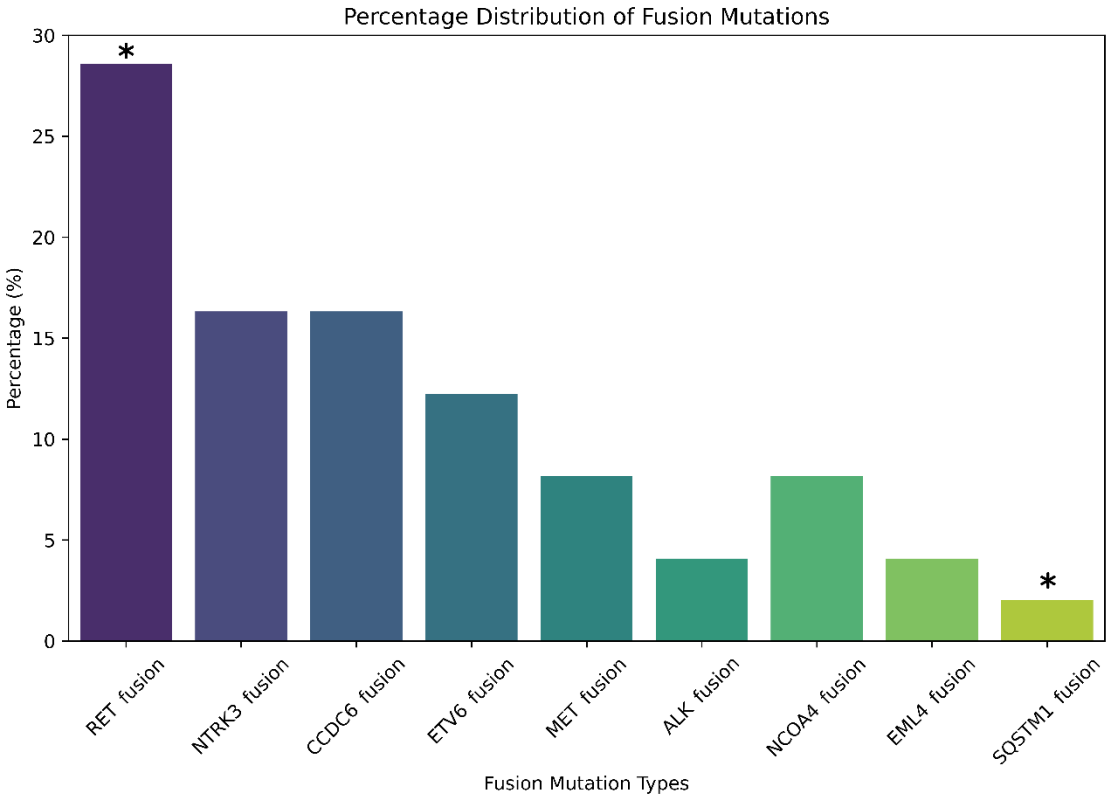

Figure 2. Calculated relative distribution of partner genes associated with detected driver fusion mutations ($n = 27$) in the PTC cohort without representing fusion non-carrier cases ($n = 73$). The relative frequency of occurrence was significantly different (Chi-square test) between *RET* and *SQSTM1* fusions ($p = 0.026$) as marked (*) on the plot. The most frequently identified fusion genes were *RET* (28.57%) and *NTRK3* (16.33%) and their common gene partners *CCDC6* and *ETV6*, respectively.

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Fusion mutations were identified in 27% of the analyzed samples, affecting nine distinct genes. The distribution of these mutations revealed the following frequencies: *RET* (28.57%), *NTRK3* (16.33%), *CCDC6* (16.33%), *ETV6* (12.24%), *MET* (8.16%), *ALK* (4.08%), *NCOA4* (8.16%), *EML4* (4.08%), and *SQSTM1* (2.04%). The most notable malignancies associated with these fusion genes, supported by sufficient data from the literature, are summarized in **Table 3**. (69–72).

Table 3. The most relevant malignancies and their associations with fusion genes identified in our study.

Fusion Gene	Cancer Type
RET	thyroid carcinoma, salivary intraductal carcinoma
NTRK3	breast carcinoma, fibrosarcoma
CCDC6	thyroid carcinoma
ETV6	acute lymphoblastic leukemia, breast carcinoma, fibrosarcoma
MET	NSCLC
ALK	anaplastic large T-cell lymphoma
NCOA4	prostate cancer, salivary intraductal carcinoma
EML4	lung cancer

4.1.2 Determination of correlations between mixed scale-type variables related to the fusion mutation status and clinicopathological data

Deploying PCA (**Figure 3**) with *d*-correlation allowed for the simultaneous analysis of multiple variable types, including nominal, ordinal, interval, and ratio-scale data. (73). Each point on the PCA plot is labeled to represent specific clinicopathological variables or fusion mutation statuses. This approach enables the visualization of larger groups of variables and their distribution relative to one another. On the **left side of Axis 1**, therapy-related variables are distinctly clustered with prognostic variables and those associated with poorer clinical outcomes, highlighting their interrelationships (7). In contrast, all fusion mutation-related variables are positioned on the **far-right side of Axis 1**, with many encircled by multiple clinicopathological variables. The opposite localization of gene fusions from therapy-related and prognostic variables suggests a negative correlation between these groups. Notably, common fusion partner genes, such as *RET*/*CCDC6* and *NTRK3*/*ETV6*, clustered closely together, reflecting their expected co-occurrence. (16,17,19,22).

The presence of a fusion mutation within PTC tissue may be associated with a prior diagnosis of Hashimoto's disease or endometriosis, as well as a family history of thyroid diseases. In the cases of *RET*, *CCDC6*, *MET*, *EML4*, and *ALK*, a similar clustering was

observed with each other and the type of thyroidectomy performed (near-total thyroidectomy, lobectomy, partial surgery with or without completion), the indication of radioiodine (RAI) therapy, a smaller than 1 cm tumor size in diameter during preoperative diagnostics with imaging techniques, a medical history of Hashimoto's disease and hypothyroidism, as well as a family history of any thyroid-related disease. Furthermore, clustering suggests a link between *SQSTM1* fusion and a medical history of thyroid/parathyroid adenoma, and features such as multifocality and sidedness on histology; links were also found between *NCOA4* fusion and sex, histological features of two-sidedness, multifocality and microcarcinoma character, a medical history of goiter, and, interestingly, obstructive pulmonary disease. Interestingly, no correlation was observed between the investigated fusion mutation types and the patient's age, clinical staging (as per the 8th edition of the AJCC), or a history of malignancies other than thyroid cancer, based on the *d*-correlation method. (74).

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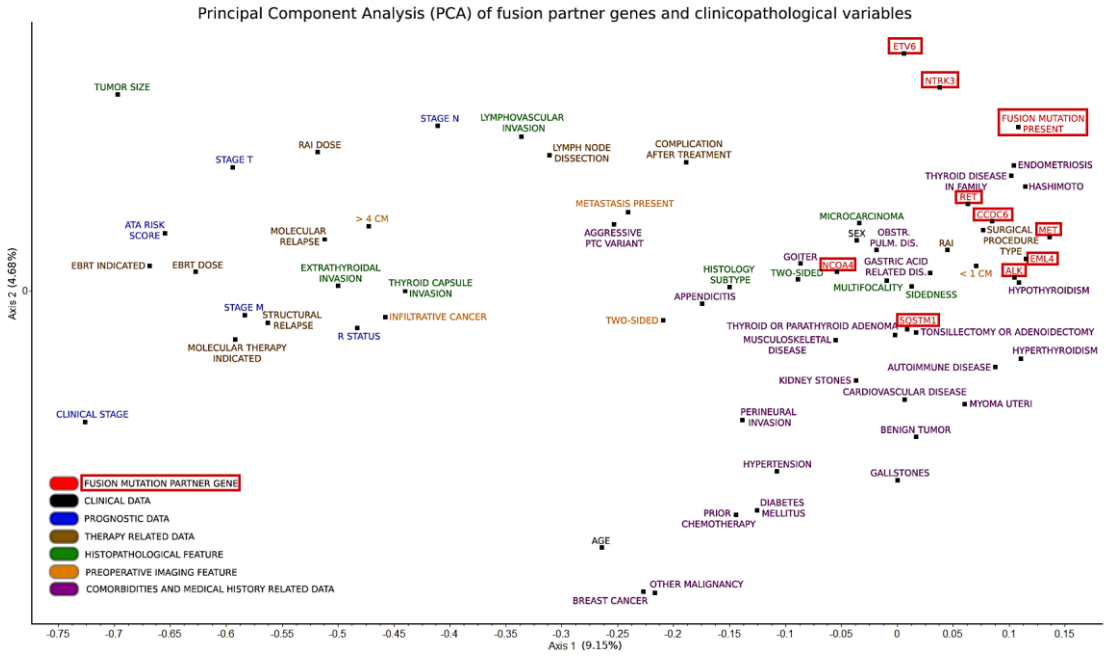

Figure 3. PCA of fusion genes and clinicopathological variables using *d*-correlation for mixed scale types. Black dots (**variable positions**) are labeled and color-coded (**bottom left corner**) to reflect the grouping of different individual variables into larger categories. Variables related to gene fusion status are indicated with **red rectangles**. It is well demonstrated that most fusion mutation-related variables tended to cluster with specific clinicopathological variables (**middle right side**). Therapy-related and prognostics-related variables (**middle left side**), however, correlated negatively with gene fusions.

4.1.3 Finding associations between NTRK3 and ETV6 fusions and clinicopathological variables with more in-depth analyses

Although it is true that *NTRK3* and *ETV6* fusions seemingly did not cluster well with clinicopathological variables via *d*-correlation, we hypothesized that it could be due to the more comprehensive rather than detail-oriented nature of this method compared to conventional statistics. While *d*-correlation is highly effective for analyzing large, multivariate datasets, employing more traditional statistical methods can be advantageous when focusing on a smaller number of variables. To further explore the relationship of these two fusion mutations to other variables, additional analyses were conducted using conventional statistical approaches. This deeper investigation uncovered further potential associations.

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To assess the associations between fusion mutation status and binary clinicopathological variables, a Chi-square test was initially performed to identify significant links ($p < 0.05$). For variables demonstrating significant associations, further analysis was conducted using a logistic regression model to evaluate the strength and direction of the relationships. Both *NTRK3* and *ETV6* fusion mutations exhibited a strong positive correlation with Hashimoto's thyroiditis, with an approximately 13-fold and 21-fold increased likelihood of co-occurrence, respectively, compared to other cases in the cohort. Additionally, *ETV6* fusion was significantly associated with a history of endometriosis, with a more than 15-fold increased likelihood of co-occurrence, as illustrated in **Figure 4**.

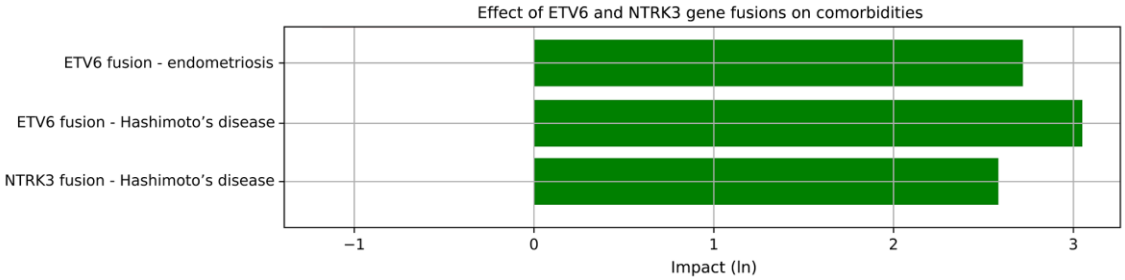

Figure 4. This horizontal bar chart displays the impact of carrying *ETV6* and/or *NTRK3* gene fusions on having certain comorbidities. The values are derived from a logistic regression analysis of the *ETV6* and *NTRK3* gene fusion partners (independent variables) and those binary/nominal-type clinicopathological features (dependent variables) that were associated with these fusions in a significant manner ($p < 0.05$). The length of the bars depends on the strength of the associations relative to other variable constellations in the cohort. All links represented are above the 0-value threshold on the **x-axis**, indicating that the directions of all the correlations are positive.

Statistical analyses were also conducted for non-binary/nominal variables to explore potential associations with the presence of *NTRK3* or *ETV6* fusions. These non-binary/nominal variables examined included the histological subtype of PTC, the localization of cancer within the thyroid, and the type of thyroidectomy performed. Similar to the analysis of binary variables, a Chi-square test was conducted to evaluate the significance of associations with non-binary variables. Among the variables examined, only the type of thyroidectomy demonstrated significant associations with

NTRK3 and *ETV6* fusions. The type of PTC surgery was categorized into three groups: primary (near-)total thyroidectomy, not-total thyroidectomy (lobectomy, partial resection), and secondary total thyroidectomy (completion after a not-total thyroidectomy).

To determine which surgical procedure is the most strongly predicted by the presence of the two tested fusions, we applied a multinomial logistic regression model. A baseline surgical category (not-total thyroidectomy) was defined, and coefficients were calculated to assess how changes in the mutation status influenced the likelihood of other surgical procedure categories relative to the baseline (**Figure 5**). In both *NTRK3* and *ETV6* fusion-positive PTC patient groups, total thyroidectomy was the most commonly performed surgical procedure. Interestingly, in fusion-positive groups, more patients underwent secondary completion surgery than primary total thyroidectomy as an initial procedure. *NTRK3* and *ETV6* gene fusions were associated with an approximately 15–33% increased likelihood of requiring a total thyroidectomy at some point during the course of patient care, with the need for secondary completion surgery being particularly pronounced. These results highlight the potential of preoperative genetic testing, at least for carriers of these mutations, to optimize surgical planning in PTC and reduce the need for unnecessary repeat surgeries.

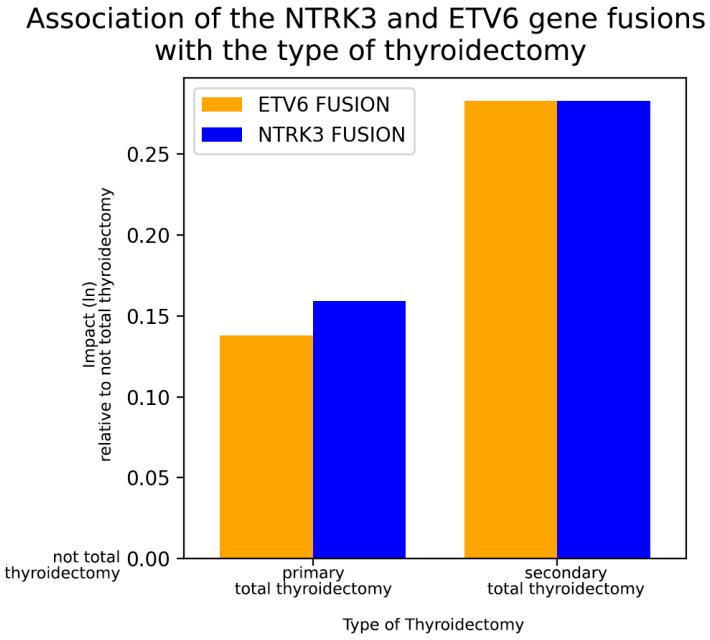

Figure 5. Using Chi-square test ($p < 0.05$), the type of surgical procedure performed was found to be significantly different when *NTRK3* and/or *ETV6* fusions occurred compared

to those cases without these fusions. This vertical bar chart of these two significant fusions, generated by applying multinomial logistic regression, illustrates the potential impact of the *NTRK3* (**blue column**) and the *ETV6* (**orange column**) fusion genes on surgical decision-making across three different categories: primary total thyroidectomy, not-total thyroidectomy (usually lobectomy), and secondary total thyroidectomy (completion of a not-total thyroidectomy). The likelihoods of the indications for total thyroidectomies (primary or secondary) are represented relative to not-total thyroidectomies (with a baseline value of 0). PTC patients with both *NTRK3* and/or *ETV6* fusion mutations underwent total thyroidectomies more frequently than not-total thyroidectomies. The number of *NTRK3* and/or *ETV6* fusion-positive patients who needed a secondary completion surgery was greater than the number of those with primary total thyroidectomy increasing the risks related to the repeated procedures.

4.1.4 Search for further links between fusion mutation status and ratio-scale or ordinal types of clinicopathological variables

The ratio-scale variables were initially tested for normality. Among these, only one variable (age at diagnosis) was found to follow a normal distribution, while the remaining ratio-type variables did not exhibit normality.

Following the normality test, we assessed the homogeneity of variances for fusion-related variables that occurred at least six times or more within the cohort. Next, significant differences in mean age between fusion-positive and fusion-negative patients were determined by applying an independent two-sample t-test for most of the fusion-positive cases and a Welch's t-test in the case of *ETV6* fusion due to potential violation of the homogeneity of variances assumption. A marked difference in the mean age was observed between *NTRK3*/*ETV6* fusion-positive and fusion-negative cases. These patients were much younger when diagnosed with PTC (mean age: 35.4 and 32.0 years, respectively) than those without any gene fusions (mean age: 47.8 years), as illustrated in **Figure 6**. In addition, patients' age with a generally positive status for fusion mutations (mean age: 39.0 years) showed a significant difference from the fusion-negative patients' age, suggesting a similar impact on the time of onset of PTC by other, less frequently occurring, therefore not individually included, fusion mutations as well.

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Significant associations could not be detected with ordinal variables (clinical stages based on AJCC 8th edition, TNM stages, R stage, and ATA risk score) or with not-normally distributed ratio-scale variables (tumor size under microscope, cumulative radioiodine dose, cumulative external beam radiation therapy dose).

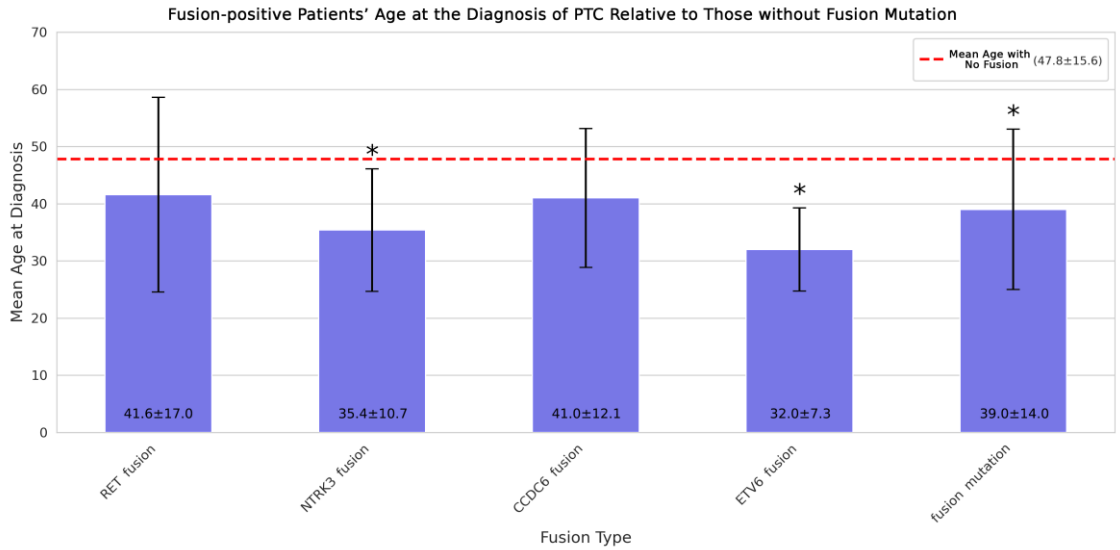

Figure 6. This bar chart illustrates a comparative analysis of the mean age at the diagnosis of PTC between patients carrying those gene fusions occurring at least 6 times in the cohort compared to the mean age of those patients not carrying any gene fusions. The height of the bars along the **y-axis** represents the mean age of the patients carrying gene fusions. The specific genes are indicated under the corresponding columns with the last column representing an overall positive status for any studied gene fusions (including those mutations with minimal occurrence as well). The **red dashed line** marks the mean age of the fusion-negative patients. All evaluated fusion mutations were associated with a younger age at the time of diagnosis than the age of patients without any gene fusions, with *NTRK3*, *ETV6*, and general fusion-positive status being significant as marked (*) on the plot. Data are presented as mean ± standard deviation (SD).

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4.2. The potential role of miRNAs in the development of PTC and their associations with certain clinical scenarios and biological conditions

4.2.1 Identification of individual miRNAs associated with PTC

The descriptive analysis of miRNA expression profiles in PTC patients provided a detailed landscape of miRNA dysregulation of the disease. **Table 4** presents the miRNAs with significant expression dysregulation identified through our sequencing data.

Table 4. The magnitude and significance of the expression deviations between tumor and non-tumor tissue samples corresponding to the "top 30 miRNAs". The $\log_2$ FC indicates the average expression level changes in the listed miRNAs. The standard error (SE) reflects the variability of the $\log_2$ FC estimates. Statistical significance was assessed using FDR-corrected p-values, with significance set at $p < 0.05$.

miRNA	$\log_2$ FC	SE	FDR-Corrected p
hsa-miR-21-5p	1.227	0.130	3.969×10^{-19}
hsa-miR-21-3p	1.364	0.163	3.926×10^{-15}
hsa-miR-31-3p	1.004	0.164	3.018×10^{-8}
hsa-miR-34a-5p	1.084	0.120	1.999×10^{-17}
hsa-miR-187-3p	1.005	0.176	2.520×10^{-7}
hsa-miR-221-5p	1.560	0.144	4.208×10^{-25}
hsa-miR-221-3p	1.866	0.153	8.969×10^{-32}
hsa-miR-222-5p	1.035	0.353	0.01644
hsa-miR-222-3p	1.591	0.135	6.766×10^{-30}
hsa-miR-137-3p	1.175	0.175	9.120×10^{-10}
hsa-miR-375-3p	1.823	0.178	1.694×10^{-22}
hsa-miR-376a-5p	1.475	0.381	8.764×10^{-4}
hsa-miR-431-5p	1.189	0.209	2.520×10^{-7}
hsa-miR-511-3p	1.003	0.201	8.883×10^{-6}
hsa-miR-146b-5p	3.345	0.202	1.294×10^{-58}
hsa-miR-146b-3p	3.507	0.245	4.798×10^{-44}
hsa-miR-508-3p	1.017	0.159	5.819×10^{-9}
hsa-miR-510-5p	1.147	0.415	0.0251
hsa-miR-514a-5p	1.229	0.350	0.0031
hsa-miR-556-5p	1.333	0.312	1.913×10^{-4}
hsa-miR-551b-5p	2.166	0.589	0.0017
hsa-miR-551b-3p	5.884	0.797	1.006×10^{-11}
hsa-miR-147b-3p	1.351	0.267	6.713×10^{-6}
hsa-miR-1277-5p	1.064	0.415	0.0405
hsa-miR-514b-5p	1.245	0.278	9.230×10^{-5}
hsa-miR-4695-3p	1.034	0.389	0.0317
hsa-miR-9983-3p	1.479	0.253	1.247×10^{-7}
hsa-miR-204-3p	−1.175	0.170	2.675×10^{-10}
hsa-miR-206	−2.273	0.488	4.060×10^{-5}
hsa-miR-873-3p	−1.316	0.197	9.781×10^{-10}

Subsequent analysis following miRNA isolation revealed significant differences in the expression of 30 individual miRNAs. Of these, 27 were over-represented in PTC compared to healthy thyroid tissue, including miR-551b-3p, miR-146b-3p, miR-9983-3p, miR-221-3p, miR-222-3p, and miR-375-3p. Conversely, three miRNAs were found to be

under-represented in PTC, namely miR-206, miR-873-3p, and miR-204-3p. As outlined above, the results include not only statistical significance but also individual FC values for each miRNA. The "top 20 miRNAs" with the highest statistical significance are illustrated in **Figure 7**. miR-551b-3p, miR-146b-3p, miR-146b-5p, miR-221-3p, miR-375-3p, miR-873-3p, and miR-204-3p are the most noteworthy. Notably, 582 of the studied miRNA types were found to have no detectable expression in thyroid tissue.

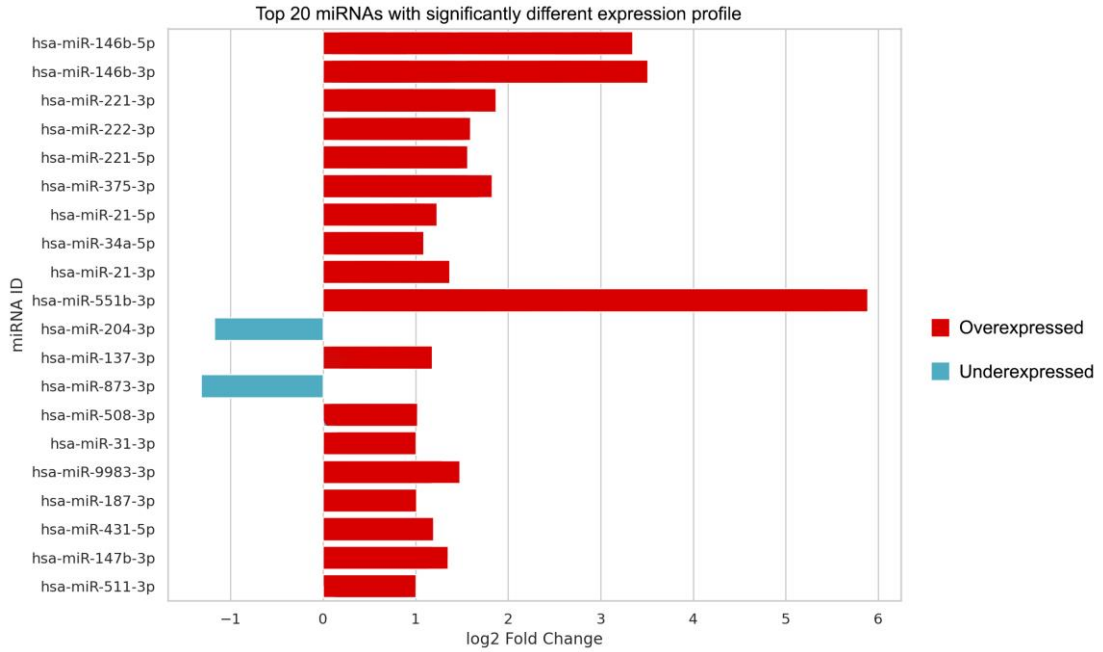

Figure 7. The bars of this chart present the $\log_2$ FC of the top 20 miRNAs (**vertical axis**) selected based on their significantly different expression profiles between cancer and control groups. Bars that extend to the right of the zero line (**red**) show overexpression of the particular miRNA in the tumor tissue, while those to the left (**blue**) indicate underexpression.

The volcano plot (**Figure 8**) provides a clear visualization of the differential miRNA expression, with the extent of expression deviation ($\log_2$ FC) (**horizontal axis**) and the strength of statistical significance ($-\log_{10}$P) (**vertical axis**). miRNAs of particular interest – those that are both statistically significant and exhibit FC above the defined threshold (as detailed in **Table 4**) – are highlighted as **red** dots in the volcano plot. These are located in the upper left quadrant (indicating underexpression) and the upper right quadrant (indicating overexpression) of the figure.

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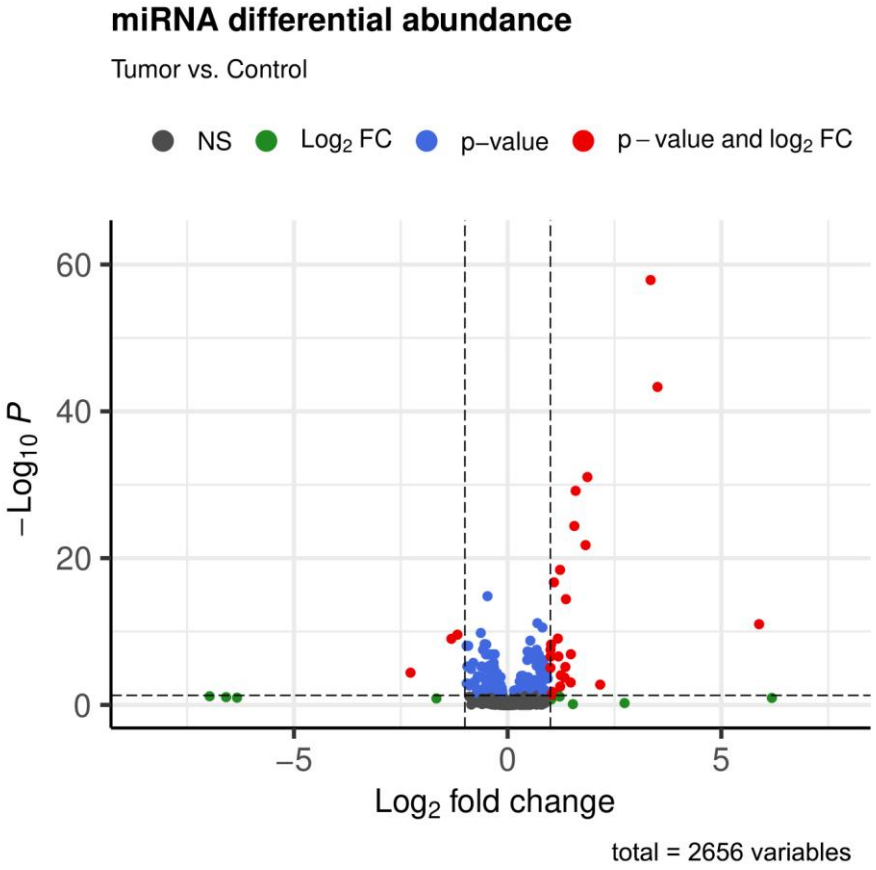

Figure 8. This volcano plot illustrates the different expressions of the miRNAs. On the **horizontal axis**, the $\log_2$ FC is represented, highlighting the magnitude of expression deviations. The **vertical axis** represents the negative logarithm of the p-value ($-\log_{10}$P), reflecting the statistical significance of the expression change related to each miRNA. Dots positioned above the horizontal threshold line (**blue** and **red**) represent miRNAs that meet the significance criterion. Dots located to the right or left of the vertical threshold lines (**red**) indicate miRNAs with not only high levels of statistical significance but also substantial overexpression or underexpression, respectively. Dots below the horizontal threshold line represent miRNAs with large fold changes that are not statistically significant (**green**) or miRNAs that do not meet any of the defined threshold criteria (**gray**).

Differential expression in relation to the 30 "top miRNAs" clustered together based on their strong significance can also be clearly seen in **Figure 9** (**C** and **D**). Notably, the cancerous tissue samples (**red group**, **A**) exhibit pronounced overexpression or

underexpression of the “top miRNAs” compared to the healthy controls (**blue group**, **A**). Expression deviations of each “top miRNA” across all samples are represented using Z-score color codes (**B**). In the heatmap, **red cells** indicate miRNA overexpression, while **blue cells** denote underexpression.

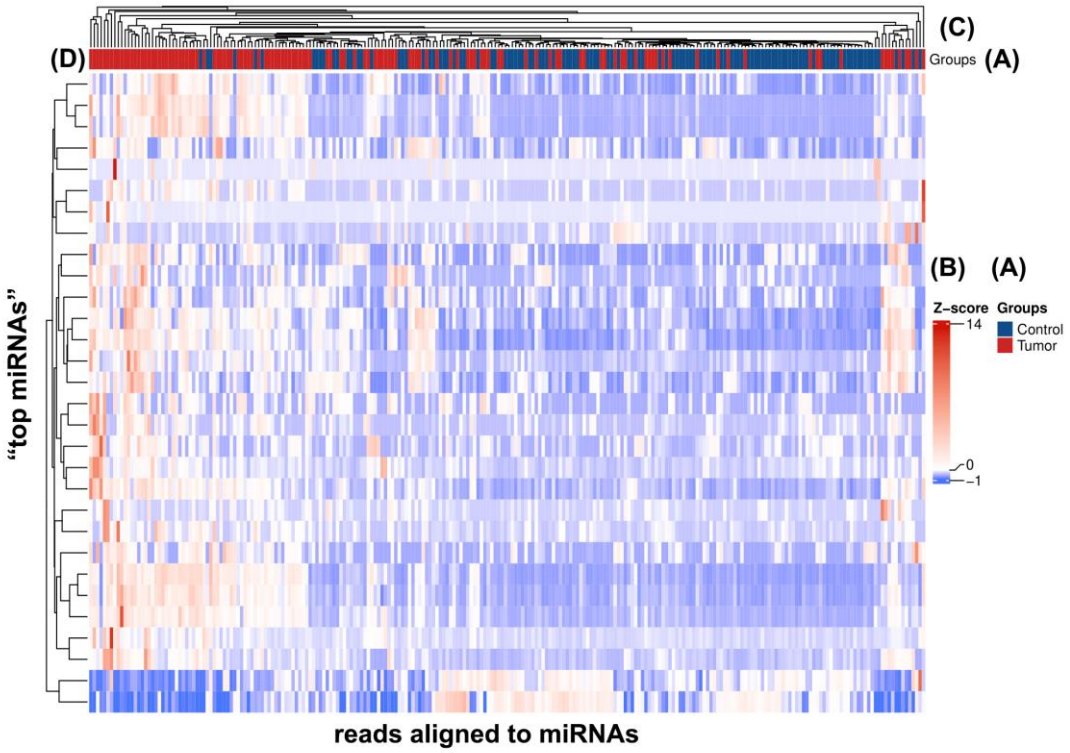

Figure 9. In this heatmap, the **rows** correspond to the “top miRNAs” (n = 30) of the miRNA expression study, selected based on their significantly different expression levels between tumor (**red**) and control (**blue**) groups categorized by histopathological characteristics (**A**). Each **column** represents one tissue sample (n = 236) subjected to molecular analysis. The color intensity within each cell reflects the Z-score derived from the normalized number of reads aligned to significant “top miRNAs”, with more red shades indicating higher expression and more blue indicating a lower expression pattern of the particular miRNA of the row (**B**). Hierarchical clustering is applied to both “top miRNAs” and samples of the two groups, as shown by the black branches, grouping similar expression profiles together. The **vertical dendrogram** (black lines on the vertical axis) illustrates the hierarchical clustering of “top miRNAs”, categorizing them based on the similarity in their expression patterns across all samples, while the **horizontal**

dendrogram (black branches on the horizontal axis) represents the hierarchical clustering of samples, highlighting that the samples with similar miRNA expression profiles tend to fall into the same (either control or tumor) group (**C**, **D**).

A comparative analysis of miRNA expression between the conventional and follicular subtypes within the PTC tumor cohort revealed no significant differences in the expression of the "top miRNAs". Further analysis of other subtypes was not conducted due to their low frequency in the study population.

4.2.2 Evidence for a general difference in miRNA expression patterns between tumor-containing and tumor-free thyroid tissue samples

Through PCA analysis of miRNA expression profiles, we identified distinct patterns that clearly differentiate between control and tumor samples. The PCA plot (**Figure 10**) (**A**) includes all evaluated miRNAs and demonstrates that the first two principal components (PC1 and PC2) account for a substantial portion of the variance within the dataset (44.27% and 17.78%, respectively). The scatterplot of control samples (**red**) and tumor samples (**blue**) along the axes reveals a discernible, though partially overlapping, distribution, suggesting a subtle relationship between miRNA expression patterns and tumor status. However, when the analysis is refined to focus exclusively on miRNAs with significant expression differences, PCA reveals a much clearer distinction between the control and tumor groups, as demonstrated by the plot (**Figure 10**) (**B**). In this refined analysis, PC1 alone captures a remarkable 86.07% of the variance, highlighting its strong explanatory power in distinguishing between the control and tumor groups. Here, the separation between the red and blue dots is much more pronounced, indicating that the miRNAs identified as significant could serve as robust biomarkers for PTC. PCA proves to be a valuable tool for visualizing the extensive dataset, effectively presenting the distinct miRNA expression landscapes between PTC and healthy thyroid tissue in a clear and transparent manner.

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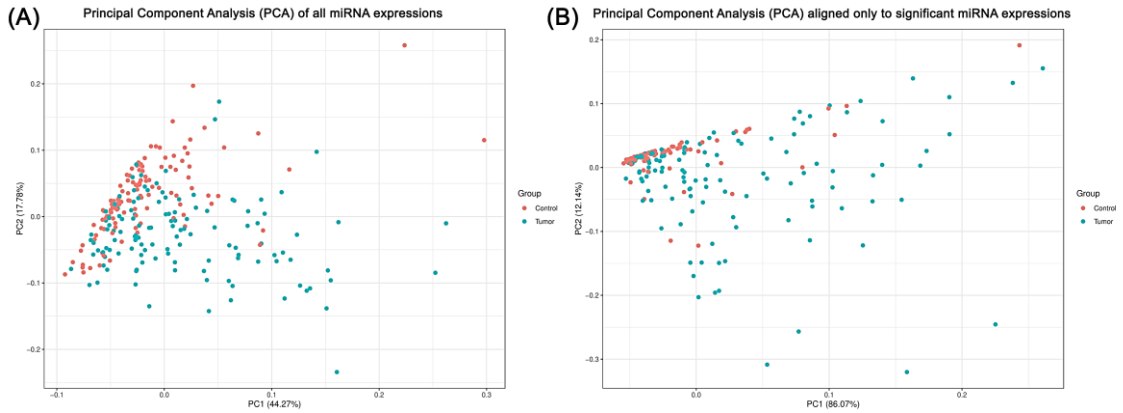

Figure 10. Comparative PCA of miRNA expressions in tumor and control samples. In plot (**A**), a PCA of all miRNA expressions tested is shown, with the **horizontal axis** representing principal component 1 (PC1), which accounts for 44.27% of the variance, and the **vertical axis** representing principal component 2 (PC2), accounting for 17.78% of the variance. Variables of the control group are marked in **red** and the tumor group in **blue**, indicating moderate separation along PC1, suggesting differential expression patterns between the two states. Plot (**B**) however displays a PCA focused exclusively on miRNA expressions found to be significant previously, with PC1 explaining a dominant 86.07% of the variance and PC2 accounting for 12.14%. Here, the separation between the two groups is more pronounced along PC1, indicating an explicit distinction in the expression profiles. The juxtaposition of these two plots highlights that specific miRNAs (marked as significant) contribute mostly to the molecular variance between the tumor and non-tumor conditions. The comparison illustrates the utility of focusing on significant miRNAs for a more targeted understanding of the molecular background of PTC.

4.2.3 Determination of associations between miRNA expressions and states of pre-selected clinicopathological variables

In our extensive analysis of miRNA expression data, we identified a total of 352 significant ($p < 0.05$) miRNA expression differences between different states of the examined clinicopathological variables – such as age, sex, ATA risk score, as well as TNM and AJCC (8^{th} edition) stages – of the study cohort. We investigated the miRNA expression deviations and their connections to clinicopathological variables in the cases of both tumor and adjacent healthy control tissues.

Notably, 36 of the miRNA expression deviations pertained to tumor samples, while the majority, 316, were related to control samples. Our analysis also revealed a quite balanced distribution in the context of the direction of miRNA expressions, with 165 links being caused by miRNA overexpression and 187 by underexpression. Furthermore, 31 of these "miRNA expression–state of variable" associations were highly intense, exhibiting extreme $\log_2$ FC values either above 10 or below -10, as labeled explicitly in **Figure 11**. Among these stronger associations, it is worth highlighting those miRNAs with the most prominent links to ATA risk: miR-6880-5p (direct, in tumor), miR-6753-5p (inverse, in tumor), miR-3648 (inverse, in control), and miR-6862-3p (inverse, in control); and those showing link to TNM score: miR-6753-5p (inverse, in tumor), miR-6805-5 (inverse, in control), miR-519c-3 (inverse, in control), and miR-6862-3p (inverse, in control). Note that downregulation of miR-6862-3p in healthy thyroid tissue adjacent to PTC is associated with greater ATA risk score, TNM score, and clinical stage as well.

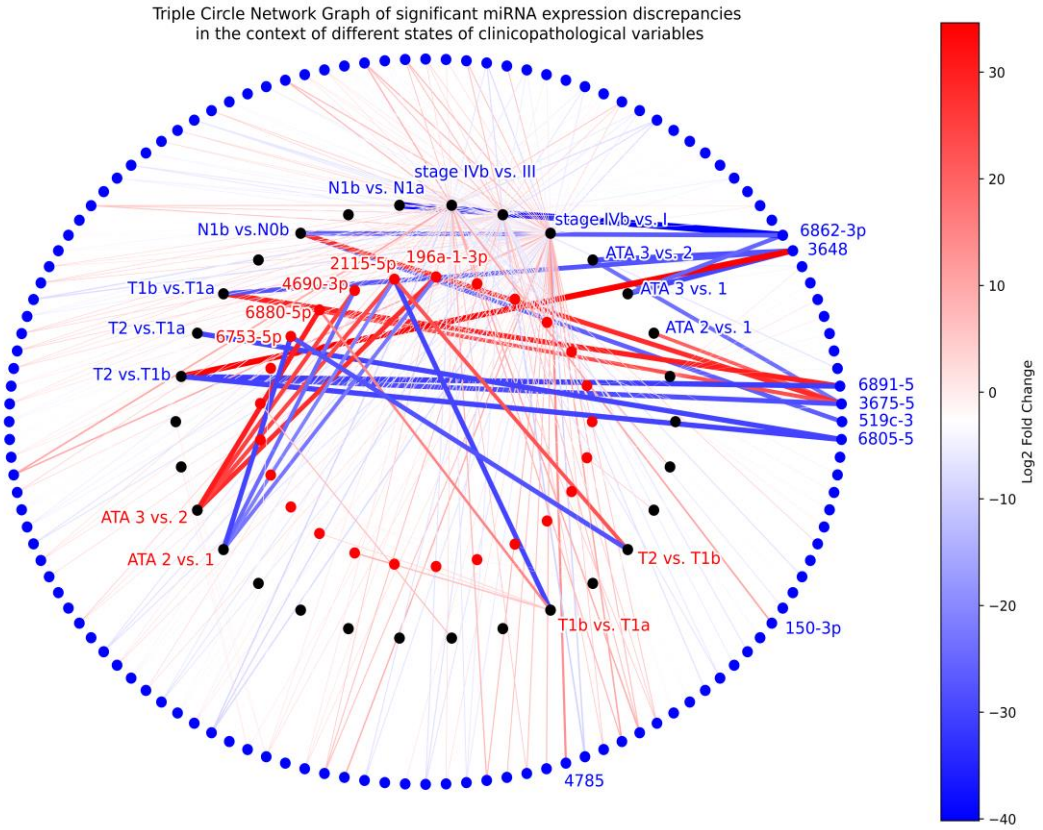

Figure 11. Triple circle network graph illustrating the most relevant miRNA expression differences between certain states of the examined clinicopathological variables such as age, sex, ATA risk, and stages (TNM and AJCC 8th edition) (**middle circle**, **black nodes**). Differentially expressed miRNAs within the control samples are represented as blue nodes (**outermost circle**), whereas they are indicated as red nodes in the context of tumor samples (**innermost circle**). All **red** and **blue** nodes represent a significant change ($p < 0.05$) in miRNA expression in relation to at least one clinicopathological variable. The significant associations are indicated by lines, with blue indicating negative changes and red indicating positive changes in miRNA expressions. The color gradient of the lines from **blue** to **red** represents the $\log_2$ FC of miRNA expressions, with **darker shades** representing greater expression differences and thus stronger links. To provide a clear and uncluttered visual representation of the network structure, the graph is devoid of any node labels related to associations with $\log_2$ FC values between 10 and −10.

4.2.4 A look for molecular similarities between other mapped miRNA expression patterns and PTC's own

The KEGG pathway (75,76) and Gene Ontology (77–79) analyses revealed a set of molecular and biological processes heavily enriched in conjunction with the significant miRNAs. The results suggest an underlying complex network of miRNA-mediated regulations that extends beyond the PTC pathogenesis. The enrichment of certain pathways, such as those related to cancer signaling, underscores the potential roles the investigated miRNAs may play in the development of PTC and probably thyroid cancer in general. It also highlights the functional consequences of miRNA dysregulation (**Figure 12**).

In addition, overlaps with pathways implicated in other diseases provide insights into a likely shared molecular origin of the pathogenesis and could be the basis of future research.

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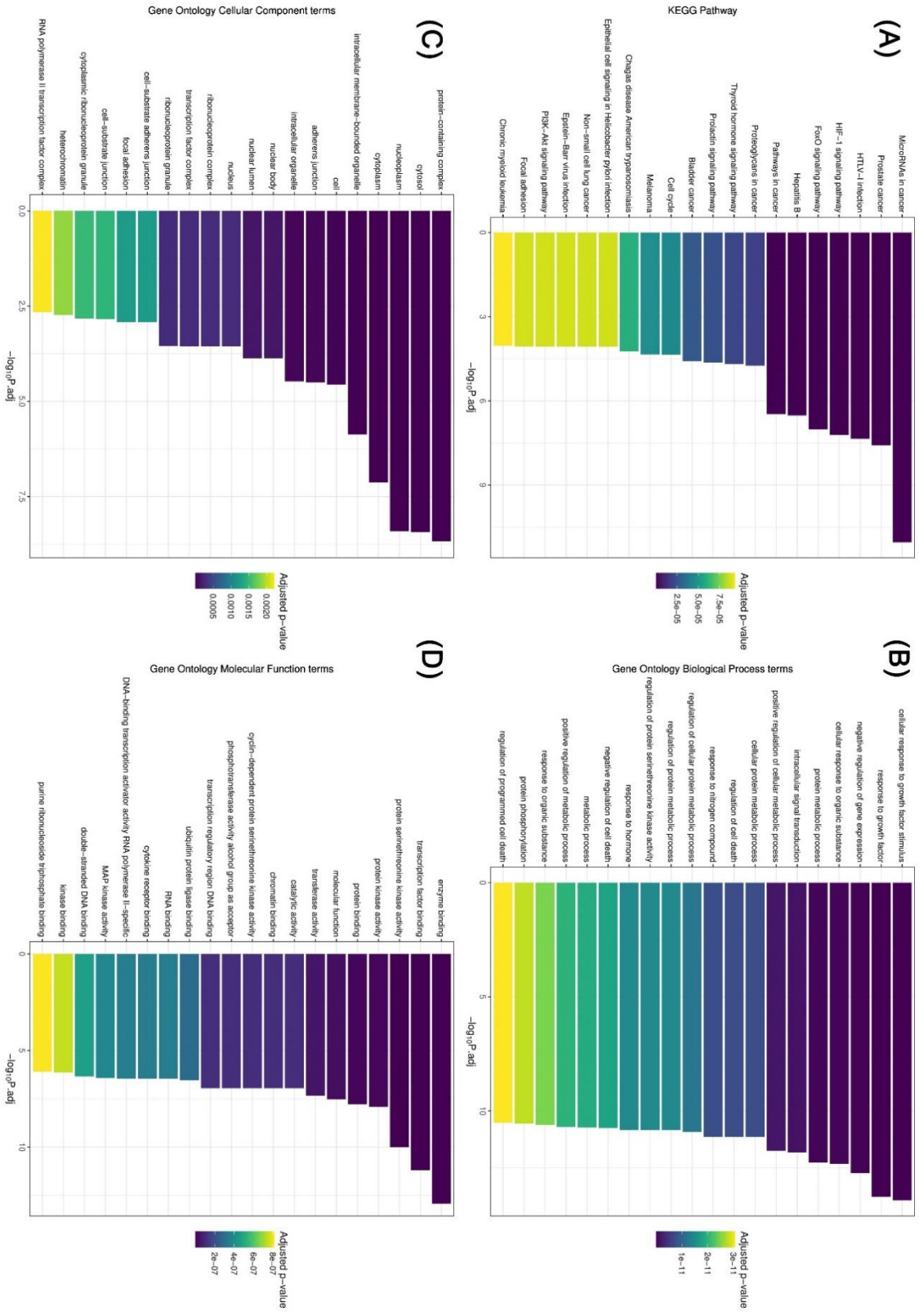

Figure 12. KEGG and Gene Ontology (GO) enrichment analyses (ORA – over-representation analysis) based on statistically significant ($p \leq 0.05$) miRNAs of this study. Associations were found between the miRNA expression patterns in PTC marked as

significant and the molecular patterns of pathways (**A**) listed in the KEGG database as well as biological processes (**B**), cellular components (**C**), and molecular functions (**D**) listed in the GO database. Based on the strength of significance, the plot visualizes the "top 20 molecular patterns" of the KEGG and GO databases showing potential correlations with PTC. Each bar represents a pathway, a biological process, a cellular component, or a molecular function of these databases (**vertical axes**), with the length of the bar reflecting the significance level of the possible association with PTC as indicated by the $-\log_{10}$ of the adjusted p-value (p_{adj}) (**horizontal axes**). The color gradient conveys the p_{adj}, transitioning from **yellow** (less significant) to **dark purple** (more significant). The data suggest that these molecular patterns (**A–D**) may be influenced by the same miRNAs as the development and/or progression of PTC.

4.3. An outlook toward merely the clinicopathological links of the PTC cohort regardless of genetic alterations

Variables followed our clinical expectations in general: smaller PTCs underwent relapse fewer times than bigger ones, PTCs with more advanced histological features relapsed more frequently than their counterparts, etc. Interestingly, though, molecular therapies were usually indicated in a more advanced disease state of being after a relapse, showing thyroid capsule-, extrathyroidal-, and/or lymphovascular invasion, or with the need for external beam radiation therapy (EBRT) as represented in **Figure 13**. This observation greatly contrasts the fact that fusion mutations, which are frequent targets of these therapies, showed rather negative correlations with the same clinicopathological variables via the *d*-correlation method. This finding suggests that these patients might benefit from earlier molecular diagnostics – even in less advanced stages of the disease – during their medical management and, if feasible, the earlier initiation of molecular target therapies.

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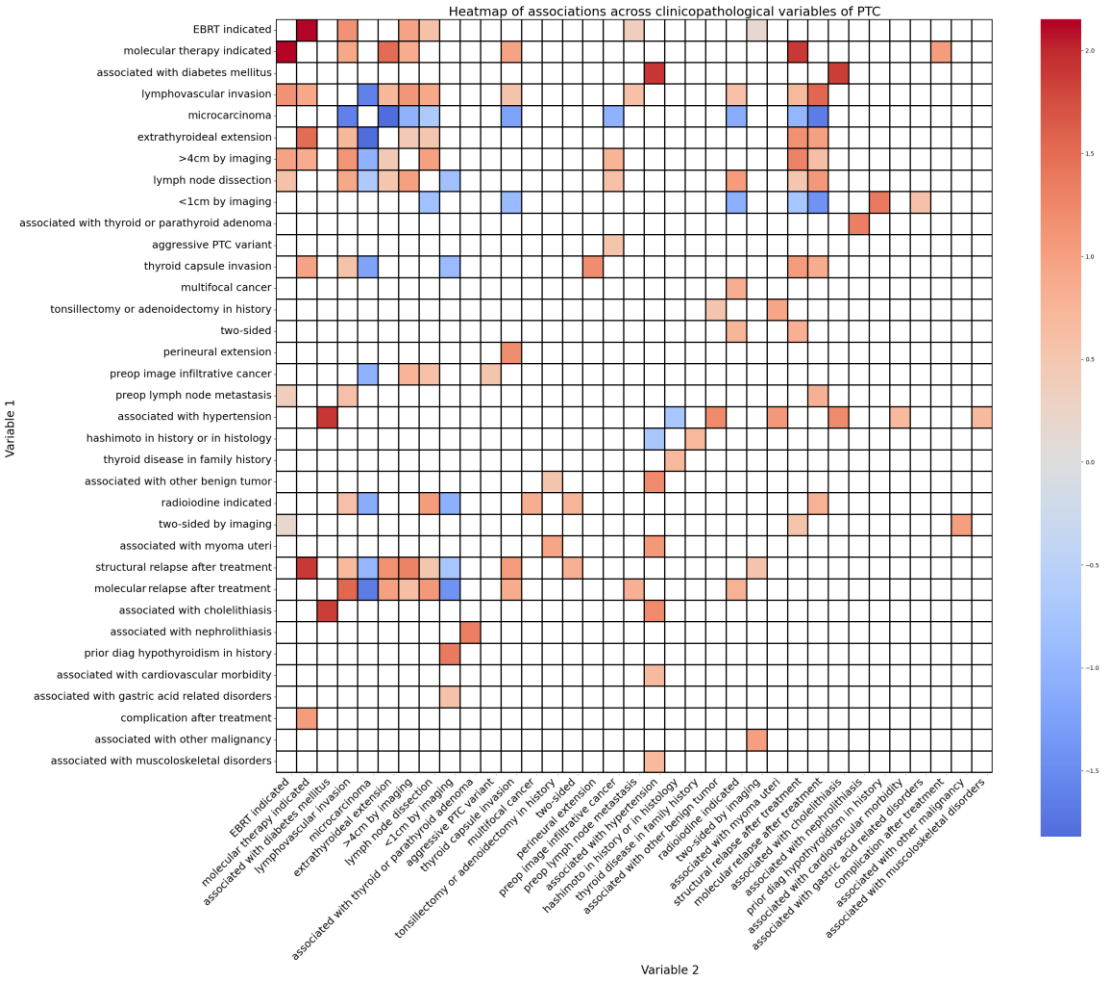

Figure 13. Heatmap listing significant associations between binary-type clinicopathological variables (**x-axis** and **y-axis**) of the PTC study cohort. The color scale illustrates the direction of the correlations ranging from strongly positive correlations (**red**) to strongly negative correlations (**blue**). Empty (**white**) cells mark no significant associations. Significant associations mostly tend to occur as clinically expected (e.g., strong positive correlation between lymphovascular invasion and lymph node dissection surgery). Medical indication of molecular therapies explicitly correlated with variables, such as relapse, thyroid capsule invasion, extrathyroidal extension, (lympho)vascular extension, or need for EBRT, usually related to a more advanced state of illness.

5. Discussion

Despite being a well-researched topic, the exact pathogenesis of PTC remains unknown, with most of the current, mutation-based models providing only a partial understanding of the disease (80). Our results not only enhance our understanding of PTC's molecular foundations but also illuminate the possibilities of gene fusions and miRNAs as novel therapeutic targets and biomarkers.

5.1. Underscoring the presence of relevant fusion mutations in a comprehensive Hungarian PTC cohort

In our comprehensive studies on PTC cohorts, we found that 27% of the PTC patients carried a fusion mutation within their tumor tissue. The frequent occurrence of fusion mutations highlights the relevance of screening for them more often in everyday clinical practice. This is amplified by the fact that the fusion proteins originating from the majority of the detected driver mutations, namely those of *RET*, *NTRK3*, *CCDC6* (when co-occurs with *RET*), and *MET*, can be effectively targeted with small tyrosine kinase inhibitors (TKIs) in thyroid cancer (24–26).

In the TCGA study, fusion mutations occurred in 15.3% of the cases. (19). Another study from 2017 detected fusion mutations in only 7.97% of the PTC cases (23). However, a recent study discovered fusion mutations in 29.86% of advanced DTC cases, which is quite similar to our results (21). Plus, the finding that *RET*-related fusion mutations are the most common in our fusion mutation study is consistent with the recent literature data (22).

5.2. Confirming and better understanding the role of individual miRNAs in PTC development

The role of miRNAs in the development of PTC is rather complex, but associations with many signaling molecules such as tumor protein p53 (TP53) (30), cyclin-dependent kinase inhibitor 1B (CDKN1B) (30) insulin-like growth factor binding protein 5 (IGFBP5) (81), transforming growth factor beta (TGF-β) (82), zinc and ring finger 3 (ZNRF3) (62), the RB1 gene (29), as well as serum thyroglobulin (Tg) (36) levels are assumed. So, it is not surprising that insights into non-conventional tumor formation caused by miRNA regulatory mechanisms on gene expression are necessary to be deepened for the improvement of cancer diagnostics and therapeutics (83,84). Therefore,

we also identified 30 miRNAs that showed significant up- or downregulation in PTC compared to healthy thyroid tissue. This was demonstrated via PCA which showed a clear separation of cancer versus healthy tissues based on their miRNA expression patterns. This not only reinforces the validity of the recognized “top miRNAs” as promising biomarkers but also suggests their utility in distinguishing between different stages or subtypes of PTC. The overlaps in PCA plots indicate a complex interplay of miRNAs, which may reflect the heterogeneity of the disease as well as the relevance of a pattern-based approach during the analysis instead of the evaluation of individual miRNA quantities.

In the widely known and robust TCGA study, 484 individual PTC cases were involved. However, even the TCGA study lacks clinicopathological aspects in contrast to our investigations. Moreover, for miRNA analysis, it applied only around half as many matched controls and examined miRNA types as we did.

Note that our miRNA expression study is the first to describe the association of PTC with the overexpression of miR-9983-3p, miR-4695-3p, miR-1277-5p, miR147b-3p, miR-511-3p, and miR-137-3p, although most of them have been previously mentioned in the context of other malignant diseases (85,86). In the case of miR-9983-3p and miR-147b-3p, however, there is very little historical evidence regarding their roles in any cancer. Interestingly, miR-551b-3p was overexpressed almost 60-fold in our cancer samples compared to adjacent healthy thyroid tissue, suggesting its prominent oncogenic role in PTC, unlike in other cancer types discussed in previous research. Indeed, previous papers have reported on the irregular expression of miR-551b-3p in PTC (87,88). It is noteworthy, however, that miR-551b-3p has been formerly recognized rather as a tumor suppressor in malignancies, such as gallbladder or gastric cancers (89,90). It is also worth mentioning that in these studies (87–90), the number of patients involved was much lower (n = 42–60) than in our own investigation. Based on these studies, miR-551b-3p was found to be underexpressed in these malignancies in contrast to our own results in PTC. This observation underscores the dynamic nature of miRNA function, wherein certain miRNAs may manifest either oncogenic or tumor-suppressive properties depending on the specific malignancy under consideration. In agreement with previous data, we also confirmed the overexpression of miR-21, miR-221, miR-222, and miR-146b, among others (58,91). Dysregulation of these miRNAs, recognized as among the most

established ones in thyroid cancer, continues to be implicated in the molecular landscape of PTC (59). miR-146b has been previously associated with epithelial–mesenchymal transformation and the rather invasive features of PTC (62).

In addition, not yet published associations between the miRNA pattern of PTC and other physiological events, biological processes, and diseases were revealed as well. For this, and to put our results into a broader context, we conducted an extensive enrichment analysis of miRNA expression profiles in PTC using both the KEGG pathway and GO term annotations. Our findings reveal a significant correlation between the dysregulated miRNAs and various biological pathways and processes that may contribute to the development of PTC and/or to the coincidence of comorbidities with similar molecular backgrounds. In this regard, among other diseases and gene functions, we were able to demonstrate remarkable similarities between the miRNA pattern of PTC and that of prostate cancer, HTLV-infection, HIF-1 signaling, negative regulation of gene expression, as well as cellular responses to growth factor stimulus, and to organic substances. Furthermore, based on our data comparison with GO cellular component database, miRNA dysregulation in PTC seems to be mostly influenced by the molecular changes of the protein-containing complexes and the cytosol, as well as enzyme binding and transcription factor binding mechanisms.

5.3. Emphasizing the promising aspects of the molecular diagnostics if integrated more explicitly into the everyday clinical practice

Although the initial detection of the disease as well as its treatment options are already quite advanced with low rates of recurrence and complications, further improvement in the follow-up of the patients could still be achieved (92). For example, the currently used circulating biomarkers in DTC diagnostics and surveillance like Tg levels have limitations, especially in the presence of Tg antibodies (TgAb), which can heavily compromise the accuracy of Tg measurements (93). This is why novel circulating biomarkers for diagnostics and surveillance are keenly researched (36,37), with thyroid stimulating hormone receptor (TSHR) mRNA, Tg mRNA, and certain miRNAs as candidates (93). mRNAs are inherently unstable molecules, and the sensitivity of circulating mRNAs is dependent on the timing of blood sampling; in addition, the specificity of Tg mRNA can easily be influenced by non-thyroid origins of the molecule

and/or technical difficulties of the measurement (93). This is not a problem for miRNAs however. In this sense, the inclusion of specific miRNA expressions in the risk assessment might be worth considering. Mainly because, in clinical practice, disease management is heavily dependent on risk assessments such as the ATA risk stratification system. Its latest version already takes the $BRAF^{V600E}$ mutation into account to estimate the chance of disease recurrence (7). However, it lacks the inclusion of other genetic alterations and miRNA expression deviations, both of which could largely alter the long-term outcome of each clinical setting. For example, our studies highlight that gene fusions correlate rather negatively with the ATA risk score and most of the classical metrics used to calculate it. Moreover, oncogenic cellular pathways related to gene mutations can be effectively targeted by TKIs. In fact, having a targetable mutation is clearly accompanied by the advantage that additional therapeutic options are available for the particular patient. Also, some miRNAs such as miR-146b, miR-203a, miR-204, miR-221, or miR-222 are already suggested to be potential prognostic indicators (36,61,94,95), although available data in this regard are still controversial (96). Interestingly, the latest studies on miRNAs suggest that underlying correlations with *BRAF* mutations themselves are possible as well (58,62). Given their potential, part of our research aimed to detect and analyze the expression levels of a wide range of miRNA types in PTC, both in tumor tissues as well as in their adjacent healthy-tissue counterparts from the same patient's thyroid. We established two subcohorts of identical size (tumor and control) and performed the same molecular analysis on each of them. With this approach, we were able to identify PTC-specific miRNAs expressed in significantly higher or lower amounts than in the control samples. This is consistent with previous findings in which miRNAs acted as promising diagnostic biomarkers distinguishing thyroid cancer from benign thyroid disease or healthy controls (36,37,61,63,97). By comparing the molecular dynamics within the same thyroid source tissue, we also had the opportunity to eliminate most of the biases related to patient selection and sample processing.

5.4. Finding those clinicopathological constellations in which molecular alterations are more frequent or relevant

As a crucial part of our studies, we analyzed molecular data (both miRNA expression and fusion mutation-related) in the context of everyday clinicopathological variables. Unlike in the fusion mutation study, in the case of the miRNA expression study, we

performed an analysis with the involvement of fewer clinicopathological variables; and evaluated associations between clinicopathological states of variables and the expression levels of the studied miRNAs. It is important to note that in the miRNA expression study, we were able to check the molecular profile and the relations to the clinicopathological variables of not only the PTC cancer tissues themselves but the adjacent, pathologically intact tissues as well. On the other hand, in the fusion mutation study, we only analyzed cancerous tissue, however, we more heavily focused on a broader understanding of the clinicopathological associations in certain molecular settings, requiring the involvement of more types of clinicopathological data.

We identified 352 significant "miRNA expression–state of variable" links, of which 31 were highly suggestive of being caused by underlying correlations between certain miRNA expression patterns and the presence of the different clinicopathological states such as those related to ATA risk (miR-6880-5p, miR-6753-5p, miR-3648, and miR-6862-3p), TNM score (miR-6753-5p, miR-6805-5, miR-519c-3, and miR-6862-3p), and clinical stage (miR-6862-3p). It is striking that miR-6862-3 underexpression in the healthy adjacent thyroid tissue was pronounced related to all these three above-mentioned variables. This underscores the notion that some miRNAs might have a role in the development of PTC, its clinical behavior, and the prognosis of the disease through direct or indirect effects (e.g., expression dysregulation facilitated by age or sex).

The association between clinicopathological features and fusion mutations has been previously studied in PTC. For instance, a meta-analysis showed that *NTRK3*-fused PTC cases had an increase in disease aggressiveness and a shorter PFS when compared to *NTRK1*-fused PTC cases (98). The possible associations between fusion mutations and RAI refractoriness have also been studied previously (21,99). Another study also reported that *RET*-rearranged tumors are more likely to have an advanced disease state compared to *BRAF*-mutant and *RAS*-mutant PTC cases (100). In our fusion mutation study, the most optimal way to illustrate the relation between a large number of measures and the gene fusion-related variables seemed to be the application of a PCA. However, as the study involved multiple variable types, comparing them simultaneously was a relatively complex task and required a special approach. Therefore, we decided to use a novel statistical framework, named *d*-correlation, which calculates the matrix correlation based on semi-matrices derived for all pairs of observations (73). *d*-correlations showed us that

the observed gene fusions mainly cluster with each other but also with some other clinicopathological variables. PTC patients with comorbidities like endometriosis or Hashimoto's thyroiditis or those with a family history of any thyroid disease have a greater chance of a positive fusion mutation status in general. Thyroid disease in the patient's family, Hashimoto's disease, and hypothyroidism, as well as the type of thyroidectomy, the need for indicating RAI, and smaller tumor size clustered quite well with gene fusions related to *RET*, *CCDC6*, *MET*, *EML4*, and *ALK*. Moreover, the patient's sex, comorbidities such as goiter and obstructive pulmonary disease, or histological features like microcarcinoma, two-sidedness, and multifocality tended to cluster with the *NCOA4* fusion gene. *SQSTM1* fusion also clustered well with multifocality and with a medical history of thyroid/parathyroid adenoma. Oddly, *d*-correlation did not reveal any marked associations with clinicopathological variables in the context of *NTRK3* and *ETV6* fusions, which mutations, on the other hand, clustered very well together. This is consistent with the observation that *NTRK3-ETV6* fusion pairs were particularly frequent in the cohort. Due to this discrepancy, we would have liked to investigate the correlation pattern of these two fusion genes separately, as well as apply more detailed, conventional statistical methods this time. As a result of these methods, we identified some additional associations both in the case of *NTRK3* and *ETV6*. PTC patients with a history of Hashimoto's disease showed a positive correlation of having *NTRK3* and/or *ETV6* fusion mutations as well. Additionally, *ETV6* positively correlated with the medical history of endometriosis. Moreover, total thyroidectomy was significantly more often indicated than not-total thyroidectomy in groups carrying *NTRK3* and/or *ETV6* fusions. Plus, compared to the number of primary total thyroidectomies, significantly more patients needed a secondary completion of an initially subtotal thyroidectomy with these mutation statuses. This means that *NTRK3* and *ETV6* fusions might be causally related to the extent of the tumor mass, and a primary total thyroidectomy might be more beneficial over a subtotal one for those patients having either *NTRK3* or *ETV6* fusion mutation within their PTC tissue. This approach could help prevent secondary surgeries and complications related to them. We also found that the patients' age at the time of diagnosis was much younger in fusion-positive PTC cases relative to those without any fusions. This observation was proven to be significant in the context of *NTRK3* and *ETV6* fusions and fusion mutation positivity in general, suggesting the importance of molecular diagnostics in younger-than-

average PTC patients. This finding, suggesting that fusion mutation frequency is age-dependent in PTC, is concordant with previous results in the literature (21,101). Additionally, fusion mutations are more commonly found in pediatric patients; moreover, these mutations are associated with a younger age in adults, as well as in pediatric PTC patients (21,101). It is important to note that the average age in our whole cohort was consistent with the literature data (102).

As a side analysis on the fusion mutation cohort, we also evaluated the relation of clinicopathological variables relative to each other without taking fusion mutations themselves into account. In this respect, we analyzed 35 different clinicopathological variables commonly documented when managing PTC patients (7,74). Most of the associations discovered were unsurprising from a clinical point of view. However, the indication of molecular therapies showed a positive correlation with variables generally linked to a more advanced cancer, like the need for EBRT as well as the tendency of relapse, invasion of the thyroid capsule, the extrathyroidal space, or the surrounding small vessels. Despite this, advanced disease-related variables correlated rather negatively with the presence of targetable fusion mutations in our study via the *d*-correlation method. Contrary to the literature, this indicates that molecular therapies might have a role in earlier stages of the disease, and reserving them only for advanced scenarios might not benefit the patients overall since the molecular targets of most of these treatments prefer to occur in seemingly more peaceful PTCs (15).

6. Conclusions

Using Hungarian PTC cohorts, our studies provide valuable insights into the prevalence and distribution of fusion mutations as well as the differences in miRNA expression profiles related to the disease. Fusion mutations were identified in 27% of the cases, and our results provided strong evidence that the miRNA expressions differ in PTC-containing and non-tumorous areas of the thyroid gland by a significant margin. The most commonly affected driver genes were *RET* and *NTRK3*, however, only nine distinct types of fusion genes could have been detected. This emphasizes the relatively narrow mutation spectrum of PTC concerning fusion mutations. Note that our miRNA-related results originated from the largest dataset of this kind, including original molecular data, matched controls, and the most comprehensive set of analyzed miRNAs.

Our results suggest that the identified genetic alterations might play a significant role in the pathogenesis of the disease and contribute to the development of different clinicopathological states, underscoring the value of integrating genetic profiling into routine thyroid cancer diagnostics. Our investigations also highlighted fundamental similarities between the molecular patterns of other biological processes and that of PTC. Interestingly, links between the expression levels of some miRNAs and values related to disease advancement (ATA risk, TNM, and clinical stage) can be found not only when analyzing PTC tissue itself but for the histopathologically healthy adjacent tissue as well.

The mapped associations generated by the applied statistical methods (conventional statistics and PCA powered by the *d*-correlation) revealed potential causal links of molecular alterations with clinicopathological characteristics (73). For instance, *RET* and *CCDC6* fusions clustered with variables such as type of thyroidectomy, the need for RAI therapy, smaller tumor size, Hashimoto's disease, and hypothyroidism. *MET*, *EML4*, and *ALK* fusions also clustered with similar variables, along with the family history of thyroid diseases in general. *NCOA4* and *SQSTM1*, however, showed a quite different association pattern. *NCOA4* fusion was associated with patients' sex, multifocality, microcarcinoma character, medical history of goiter, and obstructive pulmonary disease, while *SQSTM1* fusion was linked with multifocality and medical history of thyroid or parathyroid adenoma. Further, the more conventional statistical analyses identified significant associations of *NTRK3* and *ETV6* fusions with Hashimoto's disease, plus in the case of *ETV6*, with endometriosis. Both *NTRK3* and

ETV6 fusions were more commonly associated with the need for total thyroidectomy or secondary completion surgeries, suggesting a causal relationship with the indication algorithms of the different surgery types and highlighting the potential benefits of preoperative genetic testing of this kind. Moreover, patients with fusion mutations were diagnosed at a significantly younger age, particularly those with *ETV6* fusions. This underlines the importance of early molecular diagnostics in younger-than-average PTC patients to fine-tune treatment decisions. Interestingly, analysis of clinicopathological–clinicopathological variable pairs raised the possibility that initiating molecular target therapies might be advantageous even in clinically less advanced stages, contrary to the actual clinical practice (15).

Most of the time, miRNAs labeled as significant were rather overexpressed in PTC cancer tissue; however, some of them showed a significantly reduced expression in PTC. Strangely, 582 miRNAs showed no expression in either tumor or control samples; however, these miRNAs could still turn out to be clinically significant in future studies of other types of thyroid cancer such as follicular, medullary, anaplastic, or even non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP). As shown in the cases of miR-221 and miR-222, miRNAs have an effect on the thyroid stroma not only when cancer develops but also in other diseases with more benign behavior, such as multinodular goiter (63). In previous studies, miR-222-3p expression in thyroid cancer was also associated with immune microenvironment regulation (103). The evidence of an underlying interplay of certain miRNAs with the tumor environment and immune cells is emerging, however, comprehensive, in-depth studies regarding the miRNA-immune axis in thyroid cancer are still needed. For instance, PD-L1, a predictive biomarker related to immune response and cancer immunotherapy, has recently been reported to be associated with PTC (104).

Beyond the traditional options, future treatments for PTC are likely to focus on personalized medicine based on the molecular profile of the tumor. This includes expanding the use of targeted therapies tailored to specific genetic mutations found in PTC or even exploiting the molecular pathways related to miRNAs. Additionally, further combining molecular therapies with standard treatments, such as RAI, may help overcome RAI resistance more frequently, potentially improving treatment efficacy and patient outcomes. Apart from therapy, our studies contribute to advanced diagnostics by

helping to understand the underlying connections between the genetic landscape of PTC involving clinicopathological metrics of everyday clinical practice as an extra layer of complexity. Based on our results, a more widespread use of molecular testing (for both gene fusions and miRNA expression discrepancies) could enhance diagnostic precision and optimize treatment plans for PTC. In addition, our data can serve as a foundation for further, clinically highly relevant research in the field. For example, it would be worth investigating the potential of all the PTC-related miRNAs of our study as possible liquid biopsy biomarkers resulting from the serum as it is already successfully presented with a few miRNAs in PTC as well as with other markers in other malignancies (38,105,106). This could help improve PTC diagnostics as a routine laboratory test and could also provide a joint molecular diagnostic methodology for parallel research on different cancer types.

To the best of our knowledge, no study in the literature has analyzed the associations between clinicopathological variables and fusion mutations or miRNA expressions in PTC in such a comprehensive manner. It should be noted, however, that our studies have limitations. First and foremost, we utilized tissue samples retrospectively from existing histological archives. Hotspot mutations and CNVs were not involved in this study. Additionally, individual cellular pathways related to the detected fusion mutations were not investigated due to the limited amount of samples in the tissue archives suitable for sufficient quality molecular processing. Plus, individual miRNA functions and their exact roles in molecular pathways were not investigated; we only compared expression deviations of each miRNA between cancerous and healthy thyroid tissues. Also, we did not consider miRNA relations to mutational data such as $BRAF^{V600E}$. We corrected our miRNA-related results for a limited amount of clinicopathological data, such as age, sex, ATA risk score, and stage, compared to our gene fusion-related dataset for which we analyzed many more additional variables as well. Besides, our studies focused on individual miRNA expression variations and did not investigate inter-miRNA interactions or the combined effects of the miRNAs on PTC development. In fact, we recognize that our study lacks functional validation experiments (in vivo and/or in vitro) which would be crucial for gaining a deeper understanding of the relation between PTC pathogenesis and the identified differentially expressed miRNAs.

In addition, our results would be more reliable if repeated measurements had been taken on the samples. Moreover, a replacement of FFPE tissue samples with fresh tissues could have improved the quality of sequencing. An even larger sample size could have contributed to validating the suspected but not significantly confirmable associations emerging during our studies. Furthermore, the statistical power to detect genetic alterations across less common PTC subtypes – such as oncocytic, columnar cell variant, etc. – was limited and could have also been improved with a larger cohort. However, this was mainly due to the relatively high prevalence of the conventional subtype and a relatively low occurrence of other histological variants, which is consistent with other population-level observations (107,108). In addition, we focused on a limited number of gene fusions, potentially missing the broader landscape of genetic alterations. Lastly, patient selection biases might have affected our results as all of our patients were Caucasians living in Eastern–Central Europe. Lastly, a later extension of our investigation to multiple centers would definitely help verify our conclusions by avoiding potential patient selection biases from the same geographical region.

7. Summary

PTC, the most common subtype of thyroid cancer, exhibits unique molecular and genetic alterations that are crucial to its pathogenesis and prognosis. Despite its generally favorable outcomes due to effective treatments such as thyroidectomy and RAI therapy, PTC often necessitates second-line therapies in cases where complete eradication of the tumor is challenging. Molecular profiling, particularly the identification of miRNA dysregulations and gene fusion mutations, has emerged as a promising possibility for refining diagnostic, prognostic, and therapeutic approaches to the disease.

In our studies, we employed advanced molecular diagnostics, mainly NGS, to comprehensively investigate miRNA expression patterns and fusion mutations and their relation to other clinically measurable variables in PTC. Through the analysis of 118 thyroid tissue sample pairs originating from PTC patients, we identified 30 significantly dysregulated miRNAs, including upregulated miRNAs such as miR-551b, miR-146b, miR-221, miR-222, and miR-375, as well as downregulated ones like miR-873 and miR-204. Pathway enrichment analyses revealed links between these miRNA deviations and critical biological processes, such as cellular responses to growth factor stimuli and HIF-1 signaling. Furthermore, our research uncovered 352 associations between certain miRNAs and clinicopathological variables, emphasizing their potential as biomarkers for PTC diagnosis, prognosis, and even therapeutic targeting.

In parallel, we analyzed fusion mutations in 100 different PTC samples applying NGS again. Fusion mutations were detected in 27% of cases, involving nine gene types, such as *RET*, and *NTRK3*. Notable associations were identified between certain fusions and clinicopathological factors. For instance, certain fusions were linked to the patients' age, the size of the tumor, the type of thyroid surgery, or other elements in the medical history.

Our findings provide a deeper understanding of the molecular mechanisms driving PTC, offer potential biomarkers in diagnostics and prognostics, and highlight the value of integrating molecular profiling into routine PTC management. Our data could benefit surgical planning and other therapeutic strategies. These results support the need for the earlier molecular profiling of PTC patients. Together, these approaches have the potential to contribute to personalized medicine and improve clinical outcomes overall.

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