European Journal of Translational Myology

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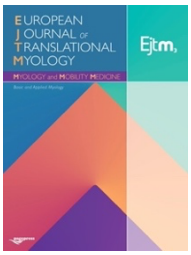

pISSN: 2037-7452 eISSN: 2037-7460

https://www.pagepressjournals.org/index.php/bam/index

Eur J Transl Myol 2024 [Online ahead of print]

To cite this Article:

He H, Hao D, Tian L, et al. **Research on the expression of Mir-218-2 in the serum of patients with papillary thyroid cancer and its clinical significance.** *Eur J Trans Myol* doi: 10.4081/ejtm.2024.12678

Research on the expression of Mir-218-2 in the serum of patients with papillary thyroid cancer and its clinical significance

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Abstract

Papillary thyroid carcinoma is an epithelial malignancy with follicular cell differentiation and sets of defined nuclear features and appearance of an irregular solid mass. The main objective of our study is to research on the expression of miR-218-2 in the serum of patients with papillary thyroid cancer and its clinical significance. Our study involved patients with thyroid nodules were divided into a capitate cancer group (N = 100) and a benign nodule group (N =100). Lastly, 50 cases of healthy individuals were used as controls. The total sample size was 250. All cases were clinically diagnosed and underwent histopathological examinations at the Tonglu County Hospital of Traditional Chinese Medicine between January 2023 and January 2024. Quantitative RT-PCR was used to assess the expression levels of miR-218-2 and its host gene SLIT3 in normal and cancer thyroid tissues. We found that 45% of tumour sizes were less than 1 cm with 90% of tumours did not infiltrate the glandular capsule, implying a favourable prognosis. Lastly, 85% of tumours were well differentiated with about 75% showing no metastasis while 60% of TNM stage were classified as stage I. Also, miR-218-2 and its host gene SLIT3 are significantly down-regulated in papillary thyroid carcinoma. The inhibitory effects of miR-218-2 act in synergy with its host gene SLIT3 to alter the rates of cell invasion, cell migration and cell proliferation. Our findings have clinical significance on the involvement of miR-218-2 and SLIT3. There exists a functional relationship between host genes and intronic miRNAs in the tumorigenesis of thyroid cancers.

Key words: papillary thyroid carcinoma, miR-218-2, SLIT3, PDGFRA and PLCG1.

Thyroid gland is situated at the base of the throat adjacent to the trachea. It is usually in the shape of a butterfly with the left and right lobes connected by the isthmus. Thyroid cancer can be either differentiated or medullary.[1] Differentiated thyroid tumours are usually cured whether they are papillary or follicular based thyroid cancers. In contrast, Censi *et al.*[2] suggested that medullary thyroid cancer involves the neuroendocrine tumours found in thyroid C cells that produces calcitonin responsible for maintaining healthy calcium levels in the blood. Hajeer *et al.*[3] showed that papillary thyroid carcinoma constitutes about 1% of all malignancies in the world and 80% of all thyroid cancer cases.[4] Papillary thyroid carcinoma has an annual prevalence of 20,000 cases and forms the 8th major cancer in women with a higher occurrence in those aged less than 25 years. However, an individual can contract papillary thyroid carcinoma at any age, most cases are usually reported in patients of below 40 years.[5] The risk factors for papillary thyroid carcinoma involves exposure to radioactive substances and genetic history of thyroid cancer. Several studies have reported good prognosis of papillary thyroid carcinoma with effective treatment and recovery in cases of early detection and diagnosis [5]. More than 20% of patients of papillary thyroid cancer involves lymph nodes during diagnosis, however, compared to other malignancies involving the lymph nodes implies a poor prognosis which is contrary to thyroid cancers where the lymph nodes have no effects on survival.[5] Lymph nodes alter the rate of recurrence of thyroid cancers without affecting prognosis.

Papillary thyroid carcinoma is an epithelial malignancy with follicular cell differentiation and sets of defined nuclear features and appearance of an irregular solid mass.[6] It is a commonly occurring thyroid neoplasm with a good prognosis. One of the key features of papillary thyroid carcinoma is the capacity to invade surrounding lymphatics leading to 10% metastasis upon the initial diagnosis.[6] Ito *et al.*[7] found that papillary thyroid carcinoma is a histopathological subtype of thyroid cancer that has led to more than 90% of all thyroid cancer cases. The technological

advancements in surgery and radiotherapy have not completely improved the rates of relapse and recurrent metastasis in papillary thyroid carcinoma patients with a 10% to 15% prevalence of relapse and distant metastases occurring among papillary thyroid carcinoma patients. These rates of relapse and distant metastasis is an indication of poor response to conventional therapies and adverse clinical outcomes.[4] As a result of this, the molecular mechanisms associated with the growth and development of papillary thyroid carcinoma constitute a significant element of research that requires further analysis for effective and efficient therapeutic techniques.[7]

Micro-ribonucleic acid (miRNAs) consists of small non-coding RNAs with a mean length of 22 nucleotides. miRNAs are mainly transcribed from Deoxyribonucleic Acid (DNA) sequences into primary miRNAs (pri-miRNAs) before undergoing processing into precursor miRNAs and maturity into miRNAs. In most cases, miRNA undergo significant interactions with the 3' untranslated regions (3' UTRs) of their corresponding target messenger-RNA (mRNA) to suppress their expression.[8] In contrast, a study by Broughton *et al.*[9] reported significant interactions between miRNA and other regions such as the 5' UTR, gene promoters and coding sequences. Additionally, miRNAs have been found to regulate and activate the expression of genes under specific circumstances.[10] miRNAs have been established as critical regulators of several pathological and biological processes involved in cell migration, cell proliferation, cell invasion, tumorigenesis and apoptosis.[11] miRNAs can be classified as either tumour suppressors or oncogenes depending on the types of cancers.[12] Previous studies[13-15] have shown that a dysregulation of the miRNAs (for example, the miR-217, miR-199a-3p, miR-21) increases the progression and pathogenesis of thyroid cancers.

According to Lu *et al*.,[16] miR-218 is a miRNA that is typically transcribed from the dual loci situated on the chromosome 4p15.31 (miR-218-1) and chromosome 5q35.1 (miR-218-2) and is significantly downregulated in numerous cancers linked with cell invasion. Moreover, previous studies[17,18] have demonstrated that miR-218 has several anticancer properties. Guan *et al.*[19] showed that down-regulation of miR-218 and the associated host gene SLIT3 (Slit Guidance Ligand 3) increases the rate of cell invasion, cell proliferation and cell migration in thyroid cancer. Despite all this research into miR-218, its regulatory mechanisms through which it achieves the biological functions in thyroid cancer cells are unclear and not fully elucidated.

The secretion of SLIT glycoproteins such as SLIT1, SLIT2 and SLIT3 into the regulation of the cell environment through the mediation of the roundabout receptors.[20] Recent studies have suggested that the promoters of SLIT-roundabout pathway of genes are commonly hypermethylated in in several cancers proposing that SLITs are crucial candidates of tumour suppression genes.[21,22] In breast cancer, loss of SLIT2 and SLIT3 contributes to hyperplastic changes in cells via the Cxcr4 and Sdf1 signalling.[21] Furthermore, SLIT3 has been implicated in the suppression of cellular migration of melanoma cells by inhibiting the activator of protein 1.[20] SLIT2 and SLIT3 genes have been found to encode miR-218-1 and miR-218-2 situated on their introns.[23] Similar studies[24-26] have demonstrated that the down-regulation of miR-218 involves numerous types of cancer and malignant phenotypes. There has been limited studies investigating the expression of mature miR-218 in papillary thyroid carcinoma. Tetzlaff *et al.*[27] suggested that a comparison of the expression of miR-218 in papillary thyroid carcinoma compared to multinodular goiter is always down-regulated. Cahill *et al.*[28] found that two papillary thyroid carcinoma cell lines consisting of a RET mutation revealed extremely low levels of miR-218 compared to normal cell lines. However, the expression of miR-218-1 and miR-218-2 and host genes (SLIT2 and SLIT3) coupled with their functional importance in papillary thyroid cancer remains unclear and unknown.

The rationale of our study is to expound on the existing studies and investigate the expression of miR-218-2 in the serum of patients with papillary thyroid cancer and its clinical significance. We propose a reduction in the expression of miR-218-2 and the host gene SLIT3 in thyroid cancer. Our main objective is to research on the expression of mir-218-2 in the serum of patients with papillary thyroid cancer and its clinical significance.

Materials and Methods

Participants and study design

Our prospective cohort study involved 200 cases of thyroid surgeries who had been clinically diagnosed and underwent histopathological examinations at Tonglu County Hospital of Traditional Chinese Medicine between January 2023 and January 2024. Patients with thyroid

nodules were divided into a capitate cancer group (N = 100) and a benign nodule group (N =100). Lastly, 50 cases of healthy individuals were used as controls. The total sample size was 250.

All participants provided informed consent and ethical approval was obtained from the Institutional Review Board. All data were mainly used for research purposes and all personal identifiers were eliminated from the data.

Eligibility criteria

Our study involved any patient with thyroid nodules who underwent thyroid surgery in our hospital. The final diagnosis of these patients was determined based on the postoperative pathological results. The inclusion criteria involved patients with patients with papillary thyroid cancer, benign thyroid nodules and normal tissues. None of the normal controls had any other benign or malignant tumor diseases or blood system-related diseases in which the body's major organs malfunction, such as the liver, kidneys, lungs. Also, all patients with a complete clinical data were included. The exclusion criteria involved cases accompanied by nodular goiter; accompanied by other malignant tumors; with internal division, urinary diseases; Pregnant or lactating women.

Clinical data collection

The following data were collected: patient's gender, age, tumor size, whether glandular capsule infiltration occurs, information such as degree of differentiation, number of lymph node metastases, and TNM staging. Follow-up tests were conducted from the date of surgery and all patients were reviewed once a month in the first 3 months after surgery; from 3 to 12 months, reviewed once every 3 months; after 1 year, re-examination every 6 months while recording the patient's recurrence status, including in situ restoration, recurrence, contralateral thyroid recurrence, cervical lymph node recurrence, and distant metastasis. Cases of relapse were defined as those where the patient has undergone cervical ultrasound and whole-body scan to

find tumour nodules and when thyroid hormone subsides or high levels of thyroid stimulating hormone and presence of serum thyroid proteins or thyroid particles.

Preparation of plasma samples

All selected candidates, regardless of group assignment, were approached by trained personnel who used a coagulation tube to draw a 5 mL peripheral venous blood sample from the patient's elbow vein. For the patients in the papillary thyroid cancer group (who underwent thyroid surgery), the same blood collection method was employed. After the 5 mL blood sample was extracted, the technicians carefully inverted the collection tube and gently mixed it for 4-5 seconds several times. This ensured proper mixing of the blood with the anticoagulant within the tube. Following this, the tubes were placed upright at room temperature, typically for around an hour, to allow the blood to completely clot. Once the blood had coagulated, the samples were centrifuged at 3,000 rpm (with a centrifugal radius of 10 cm) for 10 minutes. This process separated the serum, the liquid component of the blood, from the red and white blood cells. The collected serum was then carefully transferred, 100 microliters per tube, into separate cryovials. These aliquots were then stored in a -80°C freezer for future analysis.

Real time polymerase chain reaction (RT-PCR)

Initially, we carefully measured out 750 microliters of Trizol reagent (Invitrogen, USA) and added it directly to the serum. The mixture was then incubated at a low temperature for 5 minutes to ensure proper interaction. Next, 200 microliters of pre-cooled chloroform were added, and the mixture was thoroughly mixed again. After another 10-minute incubation, centrifuge the mixture at 12,000 rpm for 10 minutes with a centrifugal radius of 8 cm and isolated the RNA-containing supernatant in a new sterile tube. To further purify the RNA, an equal volume of isopropyl alcohol was added to the supernatant, mixed well, and allowed to sit at room temperature for 3 minutes. Another centrifugation step at 12,000 rpm for 10 minutes (with the same centrifugal radius) followed.

The remaining RNA pellet was then washed with 1 mL of 75% ethanol and after another brief centrifugation step (2 minutes) at 4°C, and allowing the RNA pellet to air dry completely. Finally, 10 microliters of DEPC water were added to the tube to re-suspend the purified RNA. Then, a micronucleic acid protein concentration analyzer (Thermo Fisher Scientific) was used to determine the concentration and purity of the isolated total RNA.With the purified RNA, reverse transcription occurred into complementary DNA (cDNA). The TransScript II All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (One-Step gDNA Removal) kit (Beijing Quanshi Jin Biotechnology Co., Ltd.) was used. The reaction mixture typically included 1 microgram of total RNA, 4 microliters of the TransScript II SuperMix, and 1 microliter of gDNA Remover in a final volume of 20 microliters. The reaction conditions involved an initial incubation at 50°C for 15 minutes, followed by a heat inactivation step at 85°C for 5 seconds.

The subsequent step involved quantitative detection of miR-218-2 expression using the SYBR green dye method. An internal reference gene, U6, was used for normalization purposes. The RT-PCR reaction system typically contained 25 microliters of the 2xTransStart Top Green qPCR SuperMix, 0.2 microliters each of the forward and reverse primers specific for miR-218-2, 10 microliters of the cDNA template, and enough nuclease-free water to make up the final volume of 50 microliters. The thermal cycling program consisted of an initial pre-denaturation step at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds, and extension/fluorescence signal collection at 72°C for 30 seconds.

We used specific primers designed for miR-218-2: a forward primer sequence of 5'-AAGCACCGCGGAAAGCACCGT-3' and a reverse primer sequence of 5'-TAATACGACTCACTATAGGG-3'. Similarly, primers for the U6 reference gene were employed: a forward primer sequence of 5'-CTCGCTTCGGCAGCACA-3' and a reverse primer sequence of 5'-AACGCTTCACGAATTTGCGT-3'. Finally, we employed the 2-ΔΔCt method to calculate the relative expression levels of miR-218-2 compared to the U6 reference gene.

Statistical analysis

All statistical analyses were conducted in GraphPad Prism version 9.5.1 with all values representing at least three triplicates in means ± SD. Spearman rank correlation coefficients were calculated between study variables while a student's t-test was used to compare differences between normal and tumour tissues. A $p < .05$ was considered statistically significant.

Results

Preliminary analysis

The study population included 250 participants (Table 1): 100 with papillary thyroid cancer (70% female, average age 45.2 years old), 100 with benign nodules (65% female, average age 50.7 years old), and 50 healthy controls (60% female, average age 48.5 years old). There were slight gender skews favouring females in all groups, with the benign nodule group having the highest average age.

In Table 2, analysing papillary thyroid cancer characteristics within the study group (n=100), tumour sizes were fairly evenly distributed with 45% measuring less than 1 cm, 30% between 1-2 cm, and 25% exceeding 2 cm. In terms of local invasion, 80% of tumours hadn't infiltrated the glandular capsule, while 20% had. The majority (85%) were well-differentiated, with the remaining 15% being poorly differentiated. Lymph node involvement also varied, with 75% showing no metastasis, 15% having 1-3 affected nodes, and 10% exceeding 3 positive nodes. Finally, the distribution of TNM stages indicated a favourable prognosis with 60% classified as stage I, 30% as stage II, and only 10% at stage III/IV.

Analysis of miR-218-2 expression via RT-PCR revealed a ΔCt value of 4.3 in the papillary thyroid cancer group compared to the U6 reference gene (Table 3). After normalization to the healthy control group, the $2^{-\Delta\Delta Ct}$ value was 0.43, indicating a 2.6-fold increase in relative miR-218-2 expression in papillary thyroid cancer compared to healthy controls. Conversely, the benign nodule group displayed a ΔCt of 5.1, resulting in a negative $2^{-\Delta\Delta Ct}$ value (-0.4) and a relative expression of 0.4, suggesting lower miR-218-2 expression compared to the normal control group.

In Table 4, in situ recurrence shows microscopic evidence of cancer cells detected in the thyroid bed after surgery, but no evidence of spread beyond the original location locoregional recurrence shows reappearance of cancer in the thyroid bed or nearby lymph nodes. distant metastasis represents the spread of cancer to distant organs such as lungs, bones, or brain. lastly, the total recurrence rate gives the percentage of patients who experienced any type of recurrence during the follow-up period.

Down-regulation of miR-218-2 and its host gene SLIT3

Our study analysed the expression of miR-218-2 and its role in the growth and development of papillary thyroid cancer. In Figure 1A, there was a statistically significant down-regulation of miR-218 in malignant lesions compared to normal tissues. In Figure 1B & 2A, statistical analysis of transcriptional expression levels of SLIT3, SLIT2, pre-miR-218-1, and pre-miR-218-2 were statistically significantly positively correlated. Therefore, the initial levels of miR-218-1 and miR-218-2 undergo a combined transcription with the host genes. Our findings shows that miR-218 is an intragenic miRNA that is basically coded by 2 genes (miR-218-1 (intron 15 of SLIT2), and miR-218-2 (intron 14 of SLIT3)). Thus, the down-regulation of miR-218 in thyroid cancer is due to a reduction in 1 or both of these genes. Additionally, these intronic miRNAs undergo a combined transcription with their host gene mRNAs.

Our analysis of the down-regulation of miR-218 in papillary thyroid cancer as a result of the reduction in 1 or either of the 2 genes (miR-218-1n & miR-218-2) showed that the mature expression levels of miR-218 were significantly and positively correlated with the initial levels of miR-218-2 and not the initial levels of miR-218-1 (see Figure 2B and 3A). Furthermore, the comparison between papillary thyroid cancer tissues and normal thyroid tissues showed that the expression levels of SLIT3 was significantly reduced (see Figure 3B).

Discussion

Our findings showed that miR-218-2 and its corresponding host gene (SLIT3) are significantly down-regulated in papillary thyroid cancer. Furthermore, there was a synergistic combined inhibition effect of SLIT3 and miR-218-2 on the rates of cell invasion, and cell proliferation in papillary thyroid cancer. After normalization to the healthy control group, the 2-ΔΔCt value was 0.43, indicating a 2.6-fold increase in relative miR-218-2 expression in papillary thyroid cancer compared to healthy controls. Conversely, the benign nodule group displayed a ΔCt of 5.1, resulting in a negative 2-ΔΔCt value (-0.4) and a relative expression of 0.4, suggesting lower miR-218-2 expression compared to the normal control group. We found that in local invasion, 80% of tumours had not infiltrated the glandular capsule while 20% had infiltrated the glandular capsule. 45 % of tumours were classified as microcarcinomas measuring less than 1 cm. In the degree of differentiation, 85% of the tumours were well differentiated with 15% being poorly differentiated.

Liu *et al.*[29] demonstrated that intronic sequences contains several functional regulatory elements besides biological functions. Examination of the intragenic miRNA consists of 408 human intronic miRNAs and through bioinformatic analyses, the expression levels of intronic miRNAs was significantly and largely correlated with transcription of host genes and miRNAs obtained from a similar transcript. The miR-218 is an intronic miRNA that has been reported to undergo down-regulation in numerous types of cancers and linked with tumorigenesis of several cancers.[24-26] Our findings were similar with Venkataraman *et al.*,[30] who postulated that miR-218 was down-regulated in cancers such as medulloblastoma and its differential expression is involved in several phenotypes of cancer. In a study by Kinoshita *et al.*,[31] the presence of miR-218 was found to suppress the rates of cell invasion, cell proliferation and cell migration in head and neck squamous cell carcinoma by targeting the laminin-332. Alajez *et al.*[32] found that the levels of miR-218 inhibited the growth and development of tumours in nasopharyngeal carcinoma by targeting the regulation of the SLIT2-ROBO1 pathway and survivin.

In the context of Papillary Thyroid Cancer (PTC), the analysis of miR-218-2 expression through RT-PCR yielded intriguing results. When compared to the healthy control group, the papillary thyroid cancer group exhibited a 2.6-fold increase in relative miR-218-2 expression after normalization. This is numerically represented by a 2-ΔΔCt value of 0.43. Conversely, the benign nodule group displayed a lower relative expression of miR-218-2 compared to the healthy

controls. This is indicated by a negative 2-ΔΔCt value (-0.4) and a relative expression of 0.4, which suggests miR-218-2 might be downregulated in benign nodules. These findings point towards a potential link between miR-218-2 and PTC. While the underlying mechanisms are yet to be fully understood, miR-218-2 might play a role in the development of PTC. Future studies could explore the functional role of miR-218-2 in PTC and determine if it can serve as a biomarker for PTC diagnosis or prognosis. Additionally, PTC is likely a complex disease influenced by multiple factors beyond a single microRNA.

The downregulation of miR-218-2 and SLIT3 in papillary thyroid cancer might not solely be driven by transcriptional or genetic alterations, but also by epigenetic modifications.[33] These molecular mechanisms act as chemical switches on the DNA and its associated proteins regulating gene expression without changing the actual DNA sequence. In the context of papillary thyroid cancer, the downregulation of miR-218-2 and SLIT3 suggests a potential link to epigenetic alterations. DNA methylation involves the addition of methyl groups to the DNA molecule, often occurring at CpG sites in the promoter regions of genes. This modification can result in the repression of gene transcription by impeding the binding of transcription factors to the affected regions. Similarly, histone modifications which encompass changes in the structure and function of histone proteins, contribute to the epigenetic regulation of gene expression. Acetylation, methylation, and phosphorylation of histones can influence chromatin structure, either promoting or inhibiting transcriptional activity. In the case of papillary thyroid cancer, these histone modifications may contribute to the observed downregulation of miR-218-2 and SLIT3. We found 45% of tumours to be less than 1 cm indicating a significant proportion (almost half) of the tumours were classified as microcarcinomas, which are very small tumours less than 1 cm in diameter. Papillary thyroid microcarcinomas are generally associated with a favourable prognosis implying that patients with microcarcinomas have a high chance of successful treatment and a lower risk of recurrence compared to those with larger tumours. Moreover, the small size of these tumours suggests they might have been detected at an early stage. Early detection of cancer is crucial for successful treatment and improved patient outcomes. Early-stage cancers are often less aggressive and easier to treat compared to more advanced stages. Lastly, the favourable prognosis be beneficial for patients as it minimizes the potential side effects associated with more extensive cancer treatments like surgery or radiation therapy. Our findings showed that 80% of tumours hadn't infiltrated the glandular capsule which

is a positive finding, as capsular invasion indicates a higher risk of the cancer spreading beyond the thyroid gland. The high percentage of non-invasive tumours suggest a potentially good prognosis for this group. The thyroid gland is surrounded by a thin connective tissue layer called the capsule, thus, when a papillary thyroid cancer remains confined to the thyroid gland and hasn't breached the capsule, it's considered non-invasive. Conversely, if the cancer cells infiltrate the capsule, it's termed capsular invasion. This finding can influence treatment decisions. For patients with non-invasive tumours, less aggressive surgical procedures like a lobectomy (removal of one thyroid lobe) might be considered. The favourable prognosis associated with non-invasive tumours can also impact post-surgical management. Decisions regarding the use of radioactive iodine ablation therapy might be tailored based on the individual's risk profile.

Our analysis identified that maturity of miR-218 is encoded by miR-218-1 and miR-218-2 and their distinct locations within the introns of SLIT2 and SLIT3. Although, the expression levels of miR-218-1 and miR-218-2 has not been extensively examined in histological types of thyroid cancer. In the study, our findings have demonstrated that the expression of miR-218-2 and SLIT3 are downregulated and underexpressed in papillary thyroid cancer. Furthermore, restoring the expression levels of miR-218-2 and SLIT3 inhibits the rate of cell invasion, and cell proliferation of papillary thyroid cancer cells. These findings were similar to a previous study by Najafi-Shoushtari *et al.*,[34] who found a synergistic interaction effect between host genes and intronic miRNAs. Their findings showed that miR-33a/b and their corresponding sterol control element-binding protein host genes act in synergy to regulate the homeostasis of cholesterol. Similarly, a study by Van Rooij *et al.*[35] found that miR-208a/b controls the expression levels of myosin and performance of muscles in a synergistic effect with their corresponding Myh6/7 host genes. In the context of papillary thyroid cancer, the dynamic relationship between the expression levels of miR-218-2 and the host gene SLIT3 assumes a pivotal role in regulating fundamental cellular processes, particularly in terms of cell invasion and proliferation.[7] Simultaneously, the host gene SLIT3 plays a critical role in this regulatory network. SLIT3 is a member of the SLIT family of secreted proteins and is implicated in various cellular processes, including cell migration and axon guidance.[36] In the context of papillary thyroid cancer, the expression levels of SLIT3 become integral to the modulation of cell invasion and proliferation. Its interaction with miR-218-2 may further amplify or mitigate the impact of their combined influence on these cellular behaviours. Our analysis showed in lymph node involvement, 75% of cases showed no

metastasis. hence, it indicates the absence of cancer spread to the lymph nodes in a significant portion of the study group. the absence of lymph node involvement is associated with a better prognosis. Lymph nodes are part of the body's immune system and act as filters, trapping cancer cells that might spread from the tumour. The absence of cancer spread to the lymph nodes in such a large portion of the study group suggests a lower risk of distant metastasis (spread to other organs) and a more favourable prognosis. Patients without lymph node involvement typically have a better prognosis compared to those with positive lymph nodes. This can influence treatment decisions. For instance, individuals with no lymph node involvement might be candidates for less extensive surgical procedures or potentially require lower doses of radioactive iodine ablation therapy, minimizing potential side effects. Previous studies[37,38] have suggested that there are series of functionally related genes that act in coordination of biological functions in cancer. Shih & Holland[41] showed that after stimulation of the receptors, there are several cellular processes and corresponding signals that area activated. Therefore, regulating the functions of these receptors is based on a single specific regulatory effect. Certain miRNAs are capable of controlling several target genes that are synergistically significant in controlling cellular processes. A study by Mavrakis *et al*.[38] showed that miR-19 has been implicated in leukemogenesis by modulating the regulators of the signalling of phosphatidylinositol-3-OH kinase. According to Rheinheimer *et al.*,[44] miR-218-2 resides within an intron of the SLIT3 gene, implying, they're physically connected on the same DNA strand. Our study suggests their downregulation is "concomitant," implying a possible shared mechanism, but the exact process needs further investigation. One possibility is the targeting of distinct complementary pathways. miR-218-2, can directly silence genes like PDGFRA and PLCG1, disrupting their pro-cancerous signalling. Nian *et al.*[39] suggested that in papillary thyroid carcinoma, MCM3AP-AS1 & GLUT1 was up-regulated due to the synergistic effects of miR-218 in increasing the rate of cellular proliferation. MiR-218 is a tumour-suppressive miRNA that inhibits the growth and development of cancer by altering the behaviour of cancer cells including invasion and proliferation.[32,40] Down-regulation of miR-218 is significantly and positively correlated with the growth and development of papillary thyroid carcinoma.[19] Furthermore, the inhibitory effects of miR-218 increases the rate of cellular proliferation in papillary thyroid carcinoma. miR-218 is capable of forming strong base pairings with MCM3AP-AS1; however, lncRNAs are capable of sponging miRNAs and reducing their downstream effects on signalling cascades.[41] Previous studies have

reported that miR-218 targets the GLUT1 and limits the growth and development of bladder cancer.[42] In papillary thyroid carcinoma, elevated levels of GLUT1 increases the growth of cancer cells and poor prognosis.

Conclusions

Our research on the expression of mir-218-2 in the serum of patients with papillary thyroid cancer and its clinical significance has showed that miR-218-2 and its corresponding host gene SLIT3 are significantly down-regulated in papillary thyroid carcinoma. Synergistically, the down-regulation of miR-218-2 enabled the increase in cell invasion and cell proliferation in papillary thyroid carcinoma. Furthermore, we found that 45% of tumour sizes were less than 1 cm with 90% of tumours did not infiltrate the glandular capsule, implying a favourable prognosis. Lastly, 85% of tumours were well differentiated with about 75% showing no metastasis while 60% of TNM stage were classified as stage I. Hence, restoring and preserving the expression levels of SLIT3 and miR-218-2 could have a beneficial therapeutic effect and favourable prognosis in papillary thyroid carcinoma. Further studies should examine the functional relationships between host genes and intronic miRNAs in papillary thyroid carcinoma.

List of abbreviations

miRNA: MicroRNA,PTC: Papillary Thyroid Carcinoma,SLIT3: Slit Guidance Ligand 3,

RT-PCR: Real-Time Polymerase Chain Reaction,UTR: Untranslated Region,TNM: Tumor,

Node, Metastasis,cDNA: Complementary DNA,DEPC: Diethyl Pyrocarbonate,

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Conflict of interest: the authors declare no potential conflict of interest, and all authors confirm accuracy.

Funding: Hangzhou Medical and Health Science and Technology Project, No. B20230443

Ethics approval: the Ethics Committee of Tonglu County Hospital of Traditional Chinese Medicine approved this study (No.2022a1809). The study is conformed with the Helsinki Declaration of 1964, as revised in 2013, concerning human and animal rights.

Informed consent: all patients participating in this study signed a written informed consent form for participating in this study.

Patient consent for publication: written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

Availability of data and materials: all data generated or analyzed during this study are included in this published article.

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Table 1. Demographics across the three groups.

Variable	Papillary Thyroid Cancer (n=100)	Benign Nodule (n=100)	Normal Control (n=50)
Gender (Female %)	70	65	60
Age (Mean ± SD)	45.2 ± 10.1	50.7 ± 11.4	48.5 ± 9.8

Table 2. Tumour characteristics (papillary thyroid cancer group only).

Feature	Frequency (%)
Tumour Size (cm)	
≤ 1	45 (45)

1-2	30 (30)
> 2	25 (25)
Glandular Capsule Infiltration	
Yes	20 (20)
No	80 (80)
Degree of Differentiation	
Well-differentiated	85 (85)
Poorly differentiated	15 (15)
Number of Lymph Node Metastases	
0	75 (75)
1-3	15 (15)
> 3	10 (10)
TNM Stage	
I	60 (60)
II	30 (30)
III/IV	10 (10)

Table 3. ΔCt values and relative miR-218-2 expression.

Group	ΔCt (miR-218-2 - U6)	2-ΔΔCt (Normalized to Normal Control)	Relative Expression (Fold Change)
Papillary Thyroid Cancer	4.3	0.43	2.6
Benign Nodule	5.1	-0.4	0.4
Normal Control	6	Reference (1)	1

Table 4. Recurrence status after thyroid surgery.

Follow-Up Period	In Situ Recurrence	Locoregional Recurrence	Distant Metastasis	Total Recurrence Rate (%)
0-3 Months	0	1	0	1
3-12 Months	1	2	0	3
1+ Years	2	3	1	6
Total	3	6	1	10

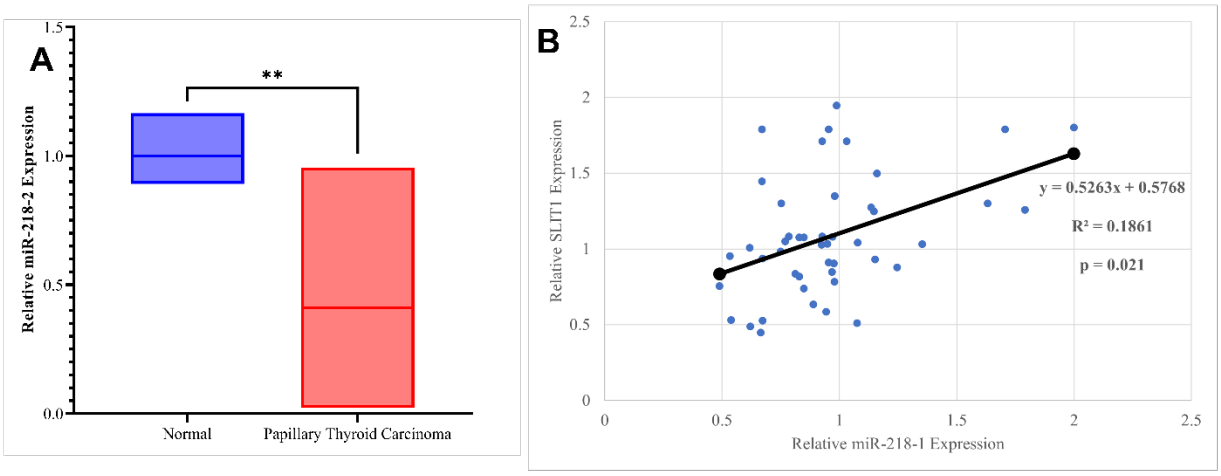

Figure 1. Analysis of the down-regulation of miR-218-2, miR-218-1 and SLIT1. The mature miR-218-2 expression levels were analysed in normal tissues and tissues of papillary thyroid carcinoma.

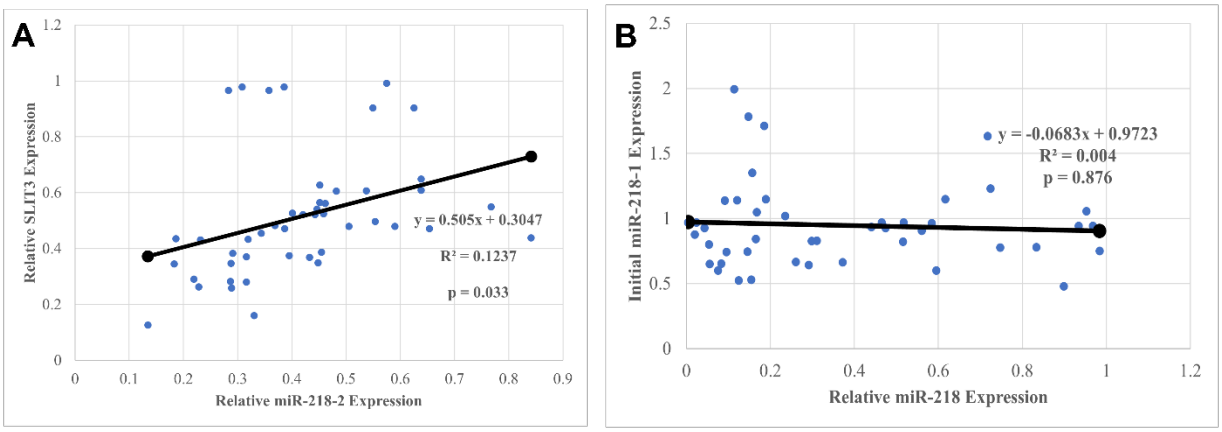

Figure 2. Analysis of the down-regulation of miR-218-2 and SLIT3. The mature miR-218-2 expression levels were analysed using RT-PCR in normal tissues and tissues of papillary thyroid carcinoma. There were significant positive correlations between SLIT2 and miR-218-1, between miR-218-2 and SLIT3.

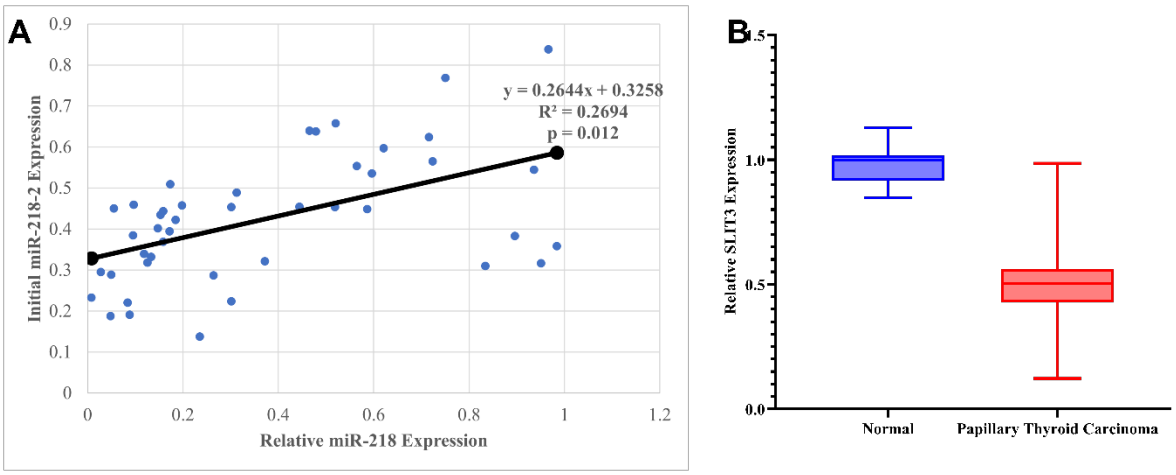

Figure 3. Analysis of the down-regulation of miR-218-2 and SLIT3. The mature miR-218-2 expression levels were analysed using RT-PCR in normal tissues and tissues of papillary thyroid carcinoma. There were significant positive correlations between SLIT2 and miR-218-1, between miR-218-2 and SLIT3. However, no significant correlations were found between the levels of miR-218-1 and miR-218.

Submitted: 19 May 2024

Accepted: 17 June 2024

Early access: 26 August 2024