Analysis of plasma exosomal miRNAs in papillary thyroid carcinoma with or without lymph node metastasis

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Research Article

Keywords: Papillary thyroid carcinoma (PTC), Exosomes, Plasma-derived exosomal miRNA, Lymph node metastasis (LNM), Early detection

Posted Date: May 30th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2982939/v1

Abstract

Objective: Lymph node metastasis (LNM) is one of the difficulties in the treatment of papillary thyroid carcinoma (PTC). To improve the early detection of PTC LNM and search for novel biomarkers, we carried out plasma-derived exosomal microRNAs studies to identify candidate differentially expressed (DE) exosome-derived miRNAs (Ex- miRNAs) as diagnostic biomarkers in several plasma samples from LNM subjects and patients without LNM.

Methods: From January 2022 to October 2022, 10 plasma samples from patients with thyroid papillary carcinoma diagnosed pathologically after surgery were involved. Among them, 6 cases had LNM. Plasma from 4 individuals with no LNM was collected as a control. We extracted exosomes by ultracentrifugation, and Ex-miRNAs were profiled using small RNA next-generation sequencing followed by differential expression analysis. Based on unsupervised hierarchical clustering, the exosomal miRNAs expression profile in patients with LNMwas significantly different from the control group. **Results**: The results indicated that the levels of exosomal hsa-miR-1226-5p were significantly lower in the LNM group compared with nonmetastatic controls (*p-value*<0.05), whereas hsa-miR-145-5p, hsa-miR-1226-3p, hsa-miR-4745-5p, hsa-miR-673,5-5p, and hsa-miR-6821-5p were significantly higher in the LNM cases than in lymph node-negative group(*p-value* 0.05). Ex-miRNAs cargo was profiled with microarrays followed by bioinformatics analyses. Their predicted target genes involved several cancer-related pathways, including Rap1 signaling pathway, cAMP signaling pathway, Calcium signaling pathway, Hippo signaling pathway, and ECM-receptor interaction (*p-value*<0.05).

Conclusions: Patients with LNM represent unique plasma exosomal miRNA profiles. Some abnormally expressed Ex-miRNAs can be identified as potential biomarkers for predicting PTC LNM.

INTRODUCTION

Thyroid cancer (TC) is the most common type of endocrine malignant tumor, new cases of which rank seventh in female malignant tumors in the world[100]. Papillary thyroid carcinoma (PTC) accounts for 85 ~ 90% of TC. Although the prognosis for patients with well-differentiated subtypes is good, 25% of TC patients develop recurrence and metastasis. Some PTC types are highly aggressive. When tumor cells lose the Iodine accumulating ability during the process of metastasis, they progressed to radioactive iodine-refractive differentiated thyroid cancer (RAIR-DTC). The mean survival time of RAIR-DTC patients was 3 5 years, and the 10-year survival rate was 10%. Except for surgery, radioactive iodine (RAI) treatment, and TSH suppression therapy, efficient therapeutic options for metastatic papillary carcinoma remain limited. Confronted with drug resistance dilemmas, clinical applications and indications of targeted medicine are relatively limited. Therefore, there is an urgent need to study the mechanism of PTC invasion and metastasis.

Exosomes are one kind of extracellular vesicles released by most types of cells into the circulation and present in various body fluids. They encapsulate biomolecules nucleic acid, lipid and proteins, and

participate in biological information transmission. CD47 protein help to maintain their integrity in circulation with more inelasticity and carcinogenic targeting than lipid[1]. MicroRNAs (miRNAs) in circulating exosomes remain stable under appropriate storage conditions. It is the most abundant type in the RNA cargo of exosomes. It remains undetermined whether tumor-associated miRNAs in peripheral circulation result from tumor cell secretion or tumor cell lysis and apoptosis. MiRNAs modulate physiological, pathological, and developmental cellular processes by repressing mRNA translation. It combines with target mRNA3 '-UTR or ORF and mediated gene silencing post-transcriptionally. Ex-miRNAs are widely involved in tumor initiation, growth, progression, and drug resistance. Exosomes containing miRNA contribute to forming an active feedback circuit between tumor cells and the extracellular matrix, eventually promoting the formation of the malignant phenotype of tumor cells. Ex-miRNAs have become malignant tumor research highlights in recent years.

Tumor-derived exosomes are involved in tissue invasion, angiogenesis, immune escape, and metastasis, which are related to gene transcription regulation or change of pre-metastatic niche. Tumor-derived exosomes are a major driving factor of the tumor-induced pre-metastatic niche. Liquid biopsy-derived exosomal microRNAs have the potential to be promising biomarkers for early diagnosis, prognostic evaluation, and recurrence monitoring[2]. For differentiated thyroid cancer (DTC), cancer cells can secrete exosomes to promote the pre-metastatic niche formation[3]. Exosomes from different PTC cell lines can show the function of inhibiting intracellular MAPK signal pathway[4]. Migration and invasion of PTC cells were increased when miR423-5p mimics in exosomes were overexpressed, and silencing Ex-miR423-5p might represent a potential against PTC[5]. According to exosomal miR-21-5p/TGFBI and miR-21-5p/COL4A1 regulatory pathway, hypoxic papillary thyroid cancer cells increase angiogenesis[6].

So far, studies of PTC-derived exosomes are always focused on biomarkers for the preoperative diagnosis of PTC as well as for predicting tumor recurrence. At present, several differential Ex- miRNAs have been isolated from the plasma of PTC patients for preoperatively differential diagnosis of thyroid benign nodules from malignant thyroid nodules[7–10], such as miR-16-2-3p, miR-25-3p, miR-130a-3p, miR-182-5p, miR-223-5p, miR-451, miR-346, and miR-34a-5p. MiR-181a from hypoxic PTC-secreted exosomes inhibits DACT2 by downregulating MLL3 and leads to YAP-VEGF-mediated angiogenesis[11]. Other studies show that PTC-derived exosomal miRNA-29a, miRNA-146b, miRNA-222, miRNA-148a-3p, miR-485-3p, miR-4433a-5p, miR-6774-3p, and miR-6879-5p can be regarded as biomarkers for the recurrence and metastasis of DTC. Combined use of TgAb, and Tg significantly enhanced the sensitivity and specificity of recurrence and metastasis[12–16]. Therefore, Ex- miRNAs have shown the potential to be efficient biomarkers for the diagnosis, screening, and monitoring of PTC lymph node metastasis. However, such studies on Ex- miRNAs are rare, especially in PTC.

Lack of effective tools for early diagnosis and improved risk stratification may lead to unseasonable identification of high-risk patients and more aggressive surgical treatment. There is an urgent need to develop sensitive and specific new non-invasive and sensitive biomarkers for PTC lymph node metastasis, which allow for an earlier diagnosis of lymph node metastasis. We determined that hsa-miR-

1226-5p levels in exosomes from PTC patients with lymph node metastasis tend to be lower than in exosomes from an individual without lymph node metastasis. On the contrary, the level of expression of hsa-miR-145-5p, hsa-miR-1226-3p, hsa-miR-4745-5p, hsa-miR-6735-5p, and hsa-miR-6821-5p present the opposite trend. Indeed, it is therefore conceivable that exosomes contain lymph node metastasis specific genes LIAT1, FLJ35746, UNC89, and G(gamma)13, which could be used to establish a PTC-specific test using exosomes.

The liquid biopsy using circulating exosomal genetic materials provides new insights for the diagnosis of PTC disease recurrence and helps to determine the scope of lymph node dissection and provide potential therapeutic targets for LNM[17]. How to illustrate the mechanism still needs future investigation. We need to examine the functional effects of differentially-expressed exosomal miRNA. Next, we will conduct further experiments to extract plasma exosomes from patients with lymph node metastasis and non metastatic before and after surgery, compare the preoperative and postoperative levels of miRNA in the exosomes, and further study the effect of radical thyroidectomy and radical lymph node dissection on the abnormal expressed Ex-miRNA.

MATERIALS AND METHODS

Patient Samples

10 patients with PTC with available clinical information including gender, age, stage, and baseline plasma samples were enrolled in this study in the 960th Hospital of People's Liberation Army from January 2022 to October 2022. Patients subjected to any medical treatment before sample collection or those with immune, metabolic or blood-related diseases were excluded. Pre-surgery plasma samples of PTC patients were collected on the day before the operation. All patients underwent either unilateral lobectomy or bilateral total thyroidectomy + central neck dissection or lateral cervical lymph node dissection. The diagnosis of PTC with lymph node metastasis required cytopathological confirmation or postoperative pathological results, which were independently confirmed by two pathologists. PTC tumor stage was classified according to the TNM classification established by the American Joint Commission on Cancer (AJCC; 2018, 8th edition). Before subject enrollment, the Clinical Research Ethics Committee (CREC) of 960th Hospital of People's Liberation Army approved this study protocol (Ethical batch No.: 2021 Scientific Research Ethics No. 129) and each participant signed informed consent in accordance with the Declaration of Helsinki.

Peripheral blood was collected into ethylenediaminetetraacetic acid (EDTA) -coated tubes with blood sampling needles prior to surgery from each patient regularly in the early morning. Specimens were gently mixed and promptly placed at room temperature or in a refrigerator (4℃) and then rapidly processed by centrifugation in the lab in an hour. 8 ~ 10mL whole blood was drawn from each patient. Finally, six control subjects and six experimental subjects were enrolled.

Sample preparation

6 samples from PTC patients with lymph node metastasis and 4 control samples were collected for exosome purification and exosomal miRNA profiling. The whole blood is centrifuged at 1900×g for 10 min at 4℃. Carefully absorb the supernatant and discard the remaining 500μL. The obtained plasma was centrifuged again at 3000×g for 15min at 4℃. Carefully absorb the plasma, and pay attention not to touch the sediment at the bottom and side. Plasma supernatants were collected and stored at -80℃ until analysis.

Isolation of Exosomes

The exosomes from plasma samples were extracted and purified by ultracentrifugation, and the extracted exosomes were used for electron microscopy and particle size detection. The plasma samples were thawed on ice and centrifuged at 2000×g for 30 min at 4°C for preliminary selection. The collected supernatant was then subjected to ultra-high-speed centrifugation at 10,000×g for 45 min at 4°C to remove large vesicles. Centrifugal supernatant filtered through 0.45μm micromembrane filtration was then centrifuged at 100000×g for 70 min at 4℃. The residue containing exosome was resuspended in 10 mL phosphate-buffered saline (PBS) and then centrifuged by the above method. The invalid portion was wiped off and the deposition was resuspended in 200μL precooled PBS, 20μL of which for electron microscopy observation, 10μL for particle size detection, and the remaining exosomes samples are stored at − 80℃ for long-term usage.

Transmission Electron Microscopy (TEM)

We used standard electron microscopy (EM) to detect exosome purity. We verified the exosomes from plasma as small double-leaflet membrane particles (30-150nm) by transmission electron microscopy (TEM). The microphotographs were obtained by an HT7700 transmission electron microscope (Hitachi, Japan). 10 μL exosomes suspended in PBS were dropped on copper-coated grids for 1 min. After staining with uranyl acetate for 1 min, grids were dried at room temperature and visualized using a Hitachi HT-7700 transmission electron microscope.

Sample size analysis

According to the manufacturer's instructions, nanoparticle tracking analysis (NTA) for exosomes was performed with Flow NanoAnalyzer model type N30 (NanoFCM Inc., Xiamen, China) to determine the granular concentration and size distribution of exosomes. The isolated exosomes were diluted with PBS at 1:3 dilution. The exosome sample can be loaded after the instrument performance testing with the standard sample is qualified.

Exosomal-miRNA Isolation and Analyses

According to the manufacturer's protocol, total RNA was extracted using an exoEasy Maxi Kit (QIAGEN, Turnberry Lane, Valencia, CA, USA). Quantitation and integrity of total RNA were assessed by Nanodrop 2000 (Thermo Fisher Scientific Inc., USA) and RNA Agilent 2100 Bioanalyzer (Agilent Technology, USA), respectively. NEBNext Small RNA Library Prep Set for Illumina kit (Cat. No. NEB#E7330S, NEB, USA) was used for the small RNA library construction following the manufacturer's recommendations. Briefly, the

adapter-ligated RNA was reverse transcribed to cDNA and then performed PCR amplification. The PCR products ranging from 140–160 bp were isolated and purified as small RNA libraries by agarose gel electrophoresis. Finally, the libraries were sequenced using the Illumina Novaseq 6000 platform after assessment by the Agilent Bioanalyzer 2100 system and generated 150 bp paired-end reads.

Small RNA sequencing experimental method

The basic reads were converted into raw sequence data by Base Calling. Low-quality reads such as the reads with poly (A) and 5’ primer contaminants were removed. The clean reads were obtained when the reads without 3’ adapter and insert tag, and those shorter than 15 nt or longer than 41 nt from the raw data were filtered.

Gene Targets Prediction and Bioinformatics

The length distribution of the clean sequences in the reference genome was determined. Then the sequences were aligned and subjected to the Bowtie search against Rfam v.10.1(http://www.sanger.ac.uk/software/Rfam). Cis-reg, rRNA, snRNA, scRNA, tRNA, and other RNAs were annotated and filtered. The mature miRNAs were identified by aligning against cDNA sequence, Repbase database of the species repeat sequence, and miRbase v22 database (http://www.mirbase.org/) according to Bowtie software, and the expression patterns in different samples were analyzed. DE miRNAs were calculated and filtered with the threshold of q value < 0.05 and FC>2 or FC<0.5. For the experiment with biological replicates, q value was calculated with the DEG algorithm in the R package. The targets of differentially expressed miRNAs were predicted by using the software Miranda, with the parameter as follows: $S \geq 150$, $\Delta G \leq -30$ kcal/mol, and demand strict 5' seed pairing. GO enrichment and KEGG pathway enrichment analysis differentially rent expressed miRNA-target-Gene were respectively performed using R based on the hypergeometric distribution. The small RNA sequencing and analysis were conducted by OE Biotech Co., Ltd. (Shanghai, China). Materials and methods are detailed in supplementary methods.

Statistical Analysis

Independent experiments were repeated 4 times. All data are expressed as means ± standard deviation. We use the Student’s t-test, the nonparametric Mann-Whitney test, and Kruskal-Wallis test to determine the difference in the levels of each miRNA between the two groups. All analyses were conducted using SPSS software (version 21.0; SPPS Inc., Chicago, IL), A two-side $p < 0.05$ was considered statistically significant.

RESULTS

Patient Characteristics

10 patients were enrolled in the exosomal miRNA differential expression analysis and were divided into lymph node metastasis group and control group without lymph node metastasis. The median age at

diagnosis is 43.5, ranging from 28 to 76. The average age (45.9 ± 14.24742), sex (male 3, female 7), and BMI (body mass index) (25.3693 ± 3.43253).

Postoperative pathology showed that 6 patients are classic PTC with tall cell features (PTC-TCF), and had severe lymph node metastasis with *BRAF*^{V600E} gene mutations, among which 3 patients are tall cell variant of papillary thyroid carcinoma (TCV-PTC) (30% or more of tumor cells have the 2:1 or 3:1 height to width ratio). The number of lymph node metastasis was (23.1667 ± 13.54129). 4 patients had no lymph node metastasis.

Because the blood samples were taken before the operation, the bias of the operational methods after the blood sampling was not excluded. All patients had operated with total thyroidectomy. Among them, 2 patients underwent bilateral cervical lymph node dissection, and 8 patients underwent unilateral cervical lymph node dissection. Table 1 shows the baseline clinicopathological characteristics of the enrolled patients.

Exosome Characterization and Quantifications

Quantification of absolute exosome counts in plasma was performed using high-sensitivity flow cytometry for nanoparticle analysis. Negative stain electron microscopy showed the exosomes in the typically shaped morphology of the extracellular vehicle in Fig. 1a. The results showed that the diameter of the lymph node metastasis group and control group without lymph node metastasis exosomes ranged from 30 TO 150nm. The measured diameter of the exosomes may be related to surface adherence, fixation, and desiccation. Taking C1 as an example, the total number of particles detected is 3858. The ratio of 30-150nm particles to total particles is 98.32%, and the average particle size concentration is 83.59nm (Fig. 1b). The number of particles detected in the sample is 3907, and the dilution ratio is 15:1. The final concentration of the sample is 2.09E + 9 particles/mL (Fig. 1c).

Plasma Exosomal miRNA Signatures Discriminated Between nmPTC and mPTC

Exosomes and exosomal miRNA in a limited volume of plasma are qualified to establish small RNA libraries (Fig. S1). The miRNA sequencing was then performed in plasma-derived exosomes from 6 PTC patients with LNM and 4 control subjects. On average, approximately 24.005 million reads were generated in each library (Fig. 2a). 113 miRNAs were differentially expressed between H group and C group. Further analysis revealed that there were 88 miRNAs with significantly up-regulated expression and 25 miRNAs with significantly down-regulated expression. The top 10 upregulated and top 10 downregulated miRNAs are listed in table 2. Hierarchical clustering analysis of 10 candidate miRNAs roughly divided plasma samples into two distinct groups: control group without lymph node metastasis and the lymph node metastasis group (C vs H). Heatmap clustering for DE miRNAs in patients with metastasis and nonmetastasis illustrates miRNA expression patterns (light blue, reduced miRNA expression in exosomes; dark red, increased miRNA expression) (Fig. 2b). We use gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways to predict target gene (Fig. S2).

The Volcano plot displayed DE miRNAs between the plasma exosome of the H group and C group. Small RNA sequencing was performed in plasma exosomes of 6 H patients and 4 C controls. There were 113 differentially expressed with *p*-value < 0.05 (i.e., above the dotted line). After screening the target genes of the above diagnostic miRNAs, we performed to investigate the potential functions involved in PTC by KEGG pathway enrichment analysis. Among the 20 significant pathways (FDR < 0.05), most of them are cancer-related, such as Rap1 signaling pathway, cAMP signaling pathway, Calcium signaling pathway, Hippo signaling pathway, and ECM-receptor interaction signaling pathways (Table 3). The other pathways are related to endocytosis and proteoglycans in cancer, whereas aldosterone synthesis and secretion, insulin secretion, and glycosaminoglycan biosynthesis-keratan sulfate are known to be associated with the disorder of endocrine system. These results suggested that these miRNAs can not only serve as diagnostic biomarkers but also are potentially involved in various steps in other endocrine malignancies.

We identified 113 DE miRNAs in the plasma samples from PTC patients, among which 88 were upregulated and 25 downregulated miRNAs. Some of these top regulated miRNAs were highly related to cancer pathways. Among them, hsa-miR-146a has been well studied in malignancy and is related to different signaling pathways viz. TNF-α, NF-κB and MEK-1/2, and JNK-1/2 are involved in various cancers. Importantly, all 6 PTC cases exhibited high levels of exosomal hsa-miR-145-5p, hsa-miR-1226-3p, hsa-miR-4745-5p, hsa-miR-6735-5p, and hsa-miR-6821-5p. Meanwhile, the top regulated miRNAs which were downregulated in patients with PTC have not been reported in different cancer types. Further studies are needed to prove the findings of hsa-miR-1226-3p, hsa-miR-4745-5p, hsa-miR-6735-5p, and hsa-miR-6821-5p related to PTC.

Next, we will assess the relationship between clinicopathologic characteristics and three diagnostic Ex-miRNAs in PTC patients.

DISCUSSION

As the most common endocrine malignancy, incidence rates of TC have increased annually. PTC is most often insidious in onset and its clinical presentation is not obvious and with non-specific symptoms. Therefore, PTC tends to cause misdiagnosis or missed diagnosis. The evaluation ability of preoperative imaging examination, such as active US surveillance of the thyroid and neck lymph nodes, is limited.

Aggressive histological subtypes such as hobnail, tall-cell, solid, columnar-cell variants and diffuse sclerosing, and a rapid Tg level doubling time mean a higher frequency of lymph node metastasis. Recurrent TC is divided into local recurrence and regional recurrence, which include central compartment recurrence (primary or nodal recurrence), lateral neck recurrence (nodal lesions), and distant recurrence. Lymph node metastasis determines the scope of surgery. Total thyroidectomy is regarded as the standard surgical treatment. However, lobectomy alone does not reduce OS. Due to the lack of prospective randomized studies, it is very difficult to regard the management of recurrent/persistent lymph node

metastases. Removing the nodes in areas of vital structures may help to prevent de novo distant metastases, but it has not been proven[18].

Neck high-resolution ultrasound and serum thyroglobulin (Tg) assays are the mainstays of PTC follow-up. Experienced ultrasound physicians can improve the detection rate of abnormal cervical lymph nodes. However, non-invasive imaging assessment of lymph node metastasis of TC is a substantial operator dependency. Despite the consistent prediction of malignancy progress, micrometastasis of lymph nodes may also be misdiagnosed. What's more, deep lymphonodus acoustically shadowed by air and bone may result in unsatisfactory visualization. Increasing Tg levels are highly suspicious for persistent/recurrent PTC. As a sensitive marker for the presence of thyrocytes, serum Tg cannot discriminate between malignant and normal cells, which results in false-positives in detectable values and high negative predictive values at undetectable levels. To avoid interference with Tg assays, it is necessary to assess serum TgAb concomitantly to avoid false-positive results. TSH suppression (serum level < 0.1 μU/mL) is recommended for PTC patients. Levothyroxine treatment inhibits the growth of PTC cells, and supplements thyroid hormones in the body. On the one hand, long-term low levels of TSH may aggravate the cardiac load and myocardial ischemia, and cause arrhythmia. On the other hand, it increases the incidence of osteoporosis (OP) in postmenopausal women.

Radioactive iodine therapy can irradiate possible foci of neoplastic cells and then reduce the recurrence risk to achieve the purpose of treating persistent or recurrent diseases. Treatment with high RAI activities is recommended for patients with a high risk of recurrence, while the predetermined RAI dose should outweigh the risks associated with its administration, which include diminished QoL and adverse events (AEs)[19]. Nevertheless, treatments for recurrent and metastatic RAIR-DTC were limited. 4 One-third of PTC patients are RAI-refractory. RAI refractoriness occurs in around 60 70% of metastatic TCs. 10-year overall survival is < 50% in RAIR-DTC patients with distant metastasis. There are no curative drug treatments for RAI-R TC at present, At present, 2 multikinase inhibitors (MKI) (sorafenib and lenvatinib) are the first-line treatment of advanced RAIR-DTC patients. MKIs are accompanied by side effects, such as hypertension, diarrhea, weight loss, fatigue, anorexia/nausea, mucositis/stomatitis, proteinuria, skin rash, alopecia, and so on. Owing to resistance mechanisms, many targeted drugs have no significant impact on improving the overall survival rate for this disease. As associated with a longer progression-free survival time and improved local disease control, external beam radiotherapy (EBRT) can be considered in case of inoperable primary or locally recurrent TC and recurrent lymph node metastases after repetitive surgery. But it may cause radiation fibrosis and respiratory dysfunction[20]. Ultrasound-guided fine-needle aspiration cytology (FNAC) improved the accuracy in the preoperative diagnosis of suspicious LNM. The predictive value of FNAC is still limited in subjects with cytological features of the suspicious metastatic lymph node. FNAC is heavily dependent on the technical experience of the operators. Inadequate FNA sampling can lead to the failure in cytological diagnosis. Repeated aspiration can be dangerous because it may lead to an invasive wound. As an invasive method, it may increase the potential of metastasis and finally lead to poor survival and prognosis. The extracted small cells or tissues fail to monitor dynamic tumor progression or represent tumor heterogeneity. Early differential diagnosis of PTC with lymph node

metastasis is the focus of clinicians. Although the above preoperative assessments are helpful for lymph node metastasis, it is difficult to find micrometastasis. Accurate preoperative evaluation is crucial for the selection of surgical methods, especially for the scope of lymph node dissection. If the scope of lymphnode dissection is insufficient, metastatic lymph nodes will be omitted. On the other hand, preventive lymph node dissection may cause excessive diagnosis problems. To sum up, the treatment of TC faces many problems. It is necessary to study the clinical markers of metastatic TC.

In advanced thyroid cancer, some mutations in genes have been found with variable frequency, such as *BRAF*^{V600E}, *NRAS, RET*, *PIK3CA*, and *PTEN*[21]. The combination of V-Raf murine sarcoma viral oncogene homolog B1 (BRAF) V600E and telomerase reverse transcriptase (TERT) promoter mutations have a robust synergistic promoting effect on the aggressiveness of PTC[22]. The clinical value of some immunohistopathology markers such as Galcitonin, TG, CyclinD1, CK19, CD56, HMBE-1, p27, p21, and E-cadherin is limited and further studies are needed to support their usefulness. The utility of RNA sequencing and genomic alterations is a burgeoning field of advanced thyroid cancer. Recently, the use of liquid assays such as circulating tumor cells (CTCs), circulating tumor nucleic acids (ctDNA/ctNAs), extra chromosomal circular DNA (ecDNA), and tumor-derived extracellular vesicles have been reported[23].

Our study identified plasma-derived exosomal hsa-miR-1226-5p, hsa-miR-145-5p, hsa-miR-1226-3p, hsa-miR-4745-5p, hsa-miR-6735-5p and hsa-miR-6821-5p as valuable diagnostic biomarkers for the early detection of PTC LNM, which may lay a preclinical foundation for the inclusion of routine blood examinations. In the present study, we demonstrated that high exosomal levels of hsa-miR-145-5p, hsa-miR-1226-3p, hsa-miR-4745-5p, hsa-miR-6735-5p, and hsa-miR-6821-5p and low expression of hsa-miR-1226-5p differentiate PTC patients with lymph node metastasis from those normal control samples. This innovative experimental result may potentially change the landscape of clinical detection and therapy for patients with lymph node metastasis, and potentially give patients affected by 131I treatment options that can prolong survival. Such a combined plasma and exosome signature may lead to the development of specific and sensitive biomarkers for early PTC lymph node metastasis diagnosis and for monitoring postoperative disease recurrence. Future studies with a larger number of PTC samples are necessary to assess the true specificity and sensitivity of this Ex- miRNAs signature. How to manage individual patients with persistent nodal or suspected recurrent PTC is challenging. This study may help to minimize the recurrence rates and need for reoperation and sustain a disease-free status.

Next, we will continue to compare the levels of miRNAs and assess their utility as potential PTC biomarkers related to lymph node metastasis using exosomes extracted from the plasma of individuals with PTC performed following total thyroidectomy and radical cervical dissection.

Conclusions

Due to the limited number of PTC cases for difficult stratification in the present study, it is unlikely to establish a validation and training cohort. There is no detailed analysis of the potential relationship between the levels of plasma hsa-miR-1226-5p, hsa-miR-145-5p, hsa-miR-1226-3p, hsa-miR-4745-5p, hsa-

miR-6735-5p and hsa-miR-6821-5p and the clinicopathological features, and the lack of functional study of these miRNAs. Next, we need to furtherly apply droplet digital PCR (ddPCR) to verify these Ex- miRNAs candidates in an independent validation cohort (plasma samples, tumors, and adjacent normal tissues) in a new bigger population. The lymph node metastasis of the patients involved in our study is generally serious and ultrasound can detect abnormal lymph nodes. In addition, we did not classify TC subtypes. The above factors may cause bias in results. Many studies confirmed the high variability of the results that analyzed the Ex-miRNAs from different series of thyroid tumors[24]. It is necessary to establish standardization of the pre-analytical and analytical procedures to validate the potential role of exosomal miRNAs as biomarkers for the diagnosis and prognosis of lymphonode metastatic PTCs (mPTC).

Abbreviations

AEs, adverse events; Benign nodular goiters; BMI, body mass index; BRAF, V-Raf murine sarcoma viral oncogene homolog B1; CREC, Clinical Research Ethics Committee; CTCs, circulating tumor cell; ddPCR, droplet digital PCR DE miRNAs, differentially expressed; DGE, differential gene expression; DTC, differentiated thyroid cancer; EBRT, external beam radiotherapy; EDTA, ethylenediaminetetraacetic acid; Ex-miRNAs, exosome-derived miRNAs; FNAC, fine-needle aspiration cytology; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; LNM, Lymph node metastasis; MKI, multikinase inhibitors; mPTC, metastatic PTC; miRNAs, MicroRNAs; NG., benign nodular goiters; nmPTC, non-metastatic PTC; NTA, nanoparticle tracking analysis; OP, osteoporosis; PTC, papillary thyroid carcinoma; PTC-TCF, PTC with tall cell features; RAI, radioactive iodine; RAIR-DTC, radioactive iodine-refractive differentiated thyroid cancer; TC, thyroid cancer; TCV-PTC, tall cell variant of papillary thyroid carcinoma; TERT, telomerase reverse transcriptase; Tg, thyroglobulin.

Declarations

Acknowledgements

The authors thank Changrui Liu and Dan Wang for an outstanding collection of biospecimens in the present study. The authors are also greatly indebted to Yuan Cao and Chen Wang for providing necessary support and technical advice and discussion.

Ethics approval and consent to participate

All included patients gave their written informed consent in accordance with the Declaration of Helsinki. The study was approved by the Ethics Committee (the Clinical Research Ethics Committee of 960th Hospital of People's Liberation Army) (Ethical batch No.: 2021 Scientific Research Ethics No. 129).

Availability of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request.

Conflict of interest statement

The authors declare that they have no competing interests.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Funding

National Key Research and Development Program of China (Grant 2019YFC0119205);

Natural Science Foundation of Shandong Province (Grant ZR2021MH328);

Natural Science Foundation of Shandong Province (Grant ZR2022QH096);

Authors' contributions

QH contributed to the conception and design of the manuscript. XC wrote the first draft of the manuscript. XL did data analyses. YM collected plasma samples and clinical data. JZ critically and substantially revised several versions of the manuscript and incorporated new ideas. All authors have read and approved the final version of the manuscript to be published.

References

1. Kamerkar S, LeBleu VS, Sugimoto H, et al. Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. *Nature.* 2017;546(7659):498-503.
2. Rappa G, Puglisi C, Santos MF, Forte S, Memeo L, Lorico A. Extracellular Vesicles from Thyroid Carcinoma: The New Frontier of Liquid Biopsy. *Int J Mol Sci.* 2019;20(5).
3. Yang X, Zhang Y, Zhang Y, et al. The Key Role of Exosomes on the Pre-metastatic Niche Formation in Tumors. *Front Mol Biosci.* 2021;8:703640.
4. Agarwal K, Saji M, Lazaroff SM, Palmer AF, Ringel MD, Paulaitis ME. Analysis of exosome release as a cellular response to MAPK pathway inhibition. *Langmuir.* 2015;31(19):5440-5448.
5. Ye W, Deng X, Fan Y. Exosomal miRNA423-5p mediated oncogene activity in papillary thyroid carcinoma: a potential diagnostic and biological target for cancer therapy. *Neoplasma.* 2019;66(4):516-523.
6. Wu F, Li F, Lin X, et al. Exosomes increased angiogenesis in papillary thyroid cancer microenvironment. *Endocr Relat Cancer.* 2019;26(5):525-538.
7. Li M, Song Q, Li H, Lou Y, Wang L. Circulating miR-25-3p and miR-451a May Be Potential Biomarkers for the Diagnosis of Papillary Thyroid Carcinoma. *PLoS One.* 2015;10(7):e0132403.

8. Wang Z, Lv J, Zou X, et al. A three plasma microRNA signature for papillary thyroid carcinoma diagnosis in Chinese patients. *Gene.* 2019;693:37-45.
9. Liang M, Yu S, Tang S, et al. A Panel of Plasma Exosomal miRNAs as Potential Biomarkers for Differential Diagnosis of Thyroid Nodules. *Front Genet.* 2020;11:449.
10. Yin G, Kong W, Zheng S, et al. Exosomal miR-130a-3p promotes the progression of differentiated thyroid cancer by targeting insulin-like growth factor 1. *Oncol Lett.* 2021;21(4):283.
11. Wang Y, Cen A, Yang Y, et al. miR-181a, delivered by hypoxic PTC-secreted exosomes, inhibits DACT2 by downregulating MLL3, leading to YAP-VEGF-mediated angiogenesis. *Mol Ther Nucleic Acids.* 2021;24:610-621.
12. Lee JC, Zhao JT, Gundara J, Serpell J, Bach LA, Sidhu S. Papillary thyroid cancer-derived exosomes contain miRNA-146b and miRNA-222. *J Surg Res.* 2015;196(1):39-48.
13. Wen Q, Wang Y, Li X, Jin X, Wang G. Decreased serum exosomal miR-29a expression and its clinical significance in papillary thyroid carcinoma. *J Clin Lab Anal.* 2021;35(1):e23560.
14. Li S, Zhang S, Yang W, Li F, Long H. Diagnostic Value of Exosomal miR-148a-3p in the Serum of Patients with Differentiated Thyroid Cancer. *Clin Lab.* 2021;67(2).
15. Dai D, Tan Y, Guo L, Tang A, Zhao Y. Identification of exosomal miRNA biomarkers for diagnosis of papillary thyroid cancer by small RNA sequencing. *Eur J Endocrinol.* 2020;182(1):111-121.
16. Chen W, Li G, Li Z, Zhu J, Wei T, Lei J. Evaluation of plasma exosomal miRNAs as potential diagnostic biomarkers of lymph node metastasis in papillary thyroid carcinoma. *Endocrine.* 2022;75(3):846-855.
17. Khatami F, Tavangar SM. Liquid Biopsy in Thyroid Cancer: New Insight. *Int J Hematol Oncol Stem Cell Res.* 2018;12(3):235-248.
18. Tufano RP, Clayman G, Heller KS, et al. Management of recurrent/persistent nodal disease in patients with differentiated thyroid cancer: a critical review of the risks and benefits of surgical intervention versus active surveillance. *Thyroid.* 2015;25(1):15-27.
19. Filetti S, Durante C, Hartl D, et al. Thyroid cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-updagger. *Ann Oncol.* 2019;30(12):1856-1883.
20. Fugazzola L, Elisei R, Fuhrer D, et al. 2019 European Thyroid Association Guidelines for the Treatment and Follow-Up of Advanced Radioiodine-Refractory Thyroid Cancer. *Eur Thyroid J.* 2019;8(5):227-245.
21. Shonka DC, Jr., Ho A, Chintakuntlawar AV, et al. American Head and Neck Society Endocrine Surgery Section and International Thyroid Oncology Group consensus statement on mutational testing in thyroid cancer: Defining advanced thyroid cancer and its targeted treatment. *Head Neck.* 2022;44(6):1277-1300.
22. Geropoulos G, Psarras K, Papaioannou M, et al. Circulating microRNAs and Clinicopathological Findings of Papillary Thyroid Cancer: A Systematic Review. *In Vivo.* 2022;36(4):1551-1569.

23. Khatami F, Larijani B, Nasiri S, Tavangar SM. Liquid Biopsy as a Minimally Invasive Source of Thyroid Cancer Genetic and Epigenetic Alterations. *Int J Mol Cell Med.* 2019;8(Suppl1):19-29.
24. Capriglione F, Verrienti A, Celano M, et al. Analysis of serum microRNA in exosomal vehicles of papillary thyroid cancer. *Endocrine.* 2022;75(1):185-193.

Tables

Tables 1-3 are not available with this version

Figures

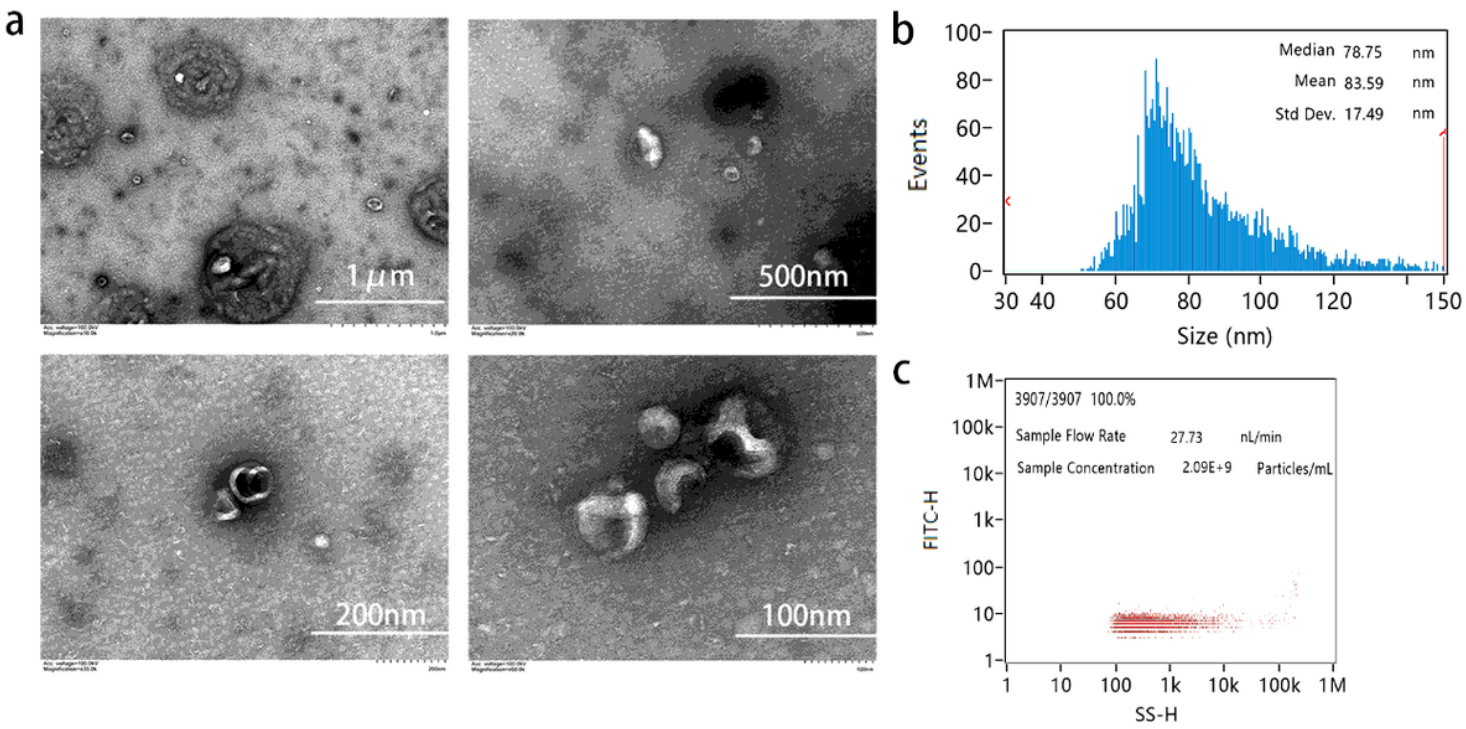

Figure 1

Electron microscope images for isolated exosomes and the size of these exosomes. **a** Representative TEM images of purifed exosomes from plasma. Scale bar, 1μm, 500 nm, 200 nm, 100 nm. **b**/**c** High sensitivity flow cytometry for nanoparticle analysis for plasma-derived exosomes.

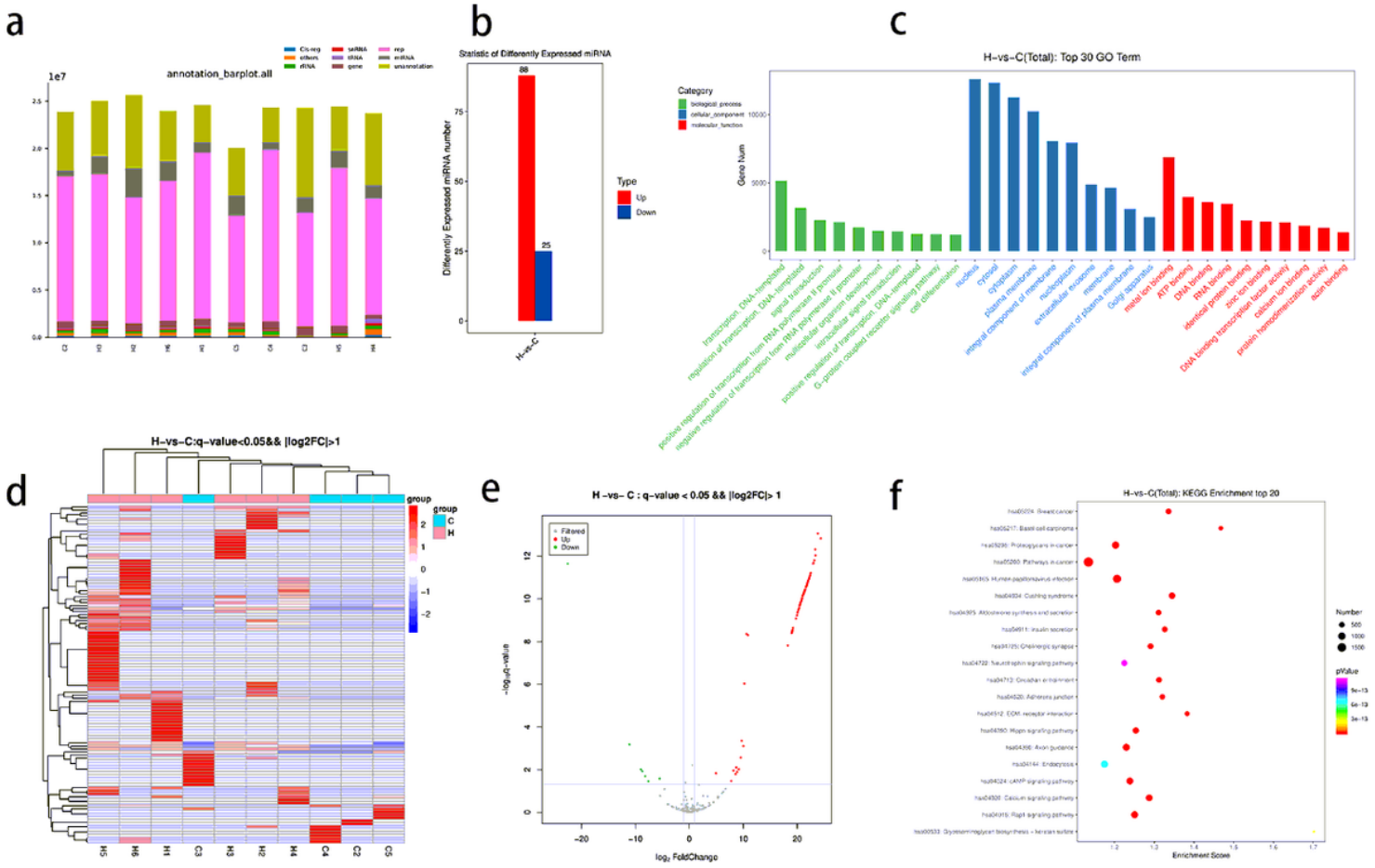

Figure 2

Plasma Ex-miRNAs profiling and bioinformatics analyses results. **a** Dysregulated miRNAs in plasma exosomes of nmPTC and mPTC patients. **b** Heatmap clustering for DE miRNAs is denoted by different colors. **c** 88 upregulated and 25 downgulated miRNAs were shown by bar chart. **d** GO enrichment analysis showed top 10 genes in three categories. **e** Similarities and dissimilarities in expression profiles was shown by hierarchical clustering in dendrograms. **f** KEGG enrichment analysis by bubble chart showed top20 genes.

Supplementary Files

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- SupplementaryMaterialforReview.docx