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The significant increase of miR-140-5P in papillary thyroid cancer samples

Haniyeh Nikkhah [a,b], Maryam Vafaei [a,c], Ehsan Farashahi-Yazd [a,*], Mohammad Hasan Sheikhha [b], Jamal Jafari-Nudoshan [d]

[a] Stem Cell Biology Research Center, Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
[b] Department of Genetics, Faculty of Medicine, International Campus, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
[c] Department of Genetics, Faculty of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
[d] Department of Surgery, Faculty of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

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ABSTRACT

Background and objective: Epi-miRNAs as a class of miRNA target genes involved in the epigenetic pathway like *DNMTs* and *HDACs*. They can play a role as a proto-oncogene or tumor suppressor. The present study evaluates the expression pattern of miR-140-5P, as a commonly mentioned tumor suppressor that targets *DNMT1* and *HDAC4* genes, in papillary thyroid cancer (PTC).
Methods: 32 tissue and normal tumor margins samples of PTC were collected. The relative expression of miR-140-5P, *DNMT1*, and *HDAC4* genes were evaluated and compared between these two groups.
Results: The results revealed no significant change in the relative expression of *DNMT1* and *HDAC4* in PTC samples. However, the expression of miR-140-5P, contrary to our hypothesis, significantly increased in the tumor group compared to the control.
Conclusion: According to the common role of miR-140-5P as a tumor suppressor gene in different groups of cancer, the expression increasing of it seems not to be acceptable. However, considering the increased expression of miR-140-5P as only reason for declining this role is also not sufficient.

1. Introduction

Thyroid cancer as the most common type of endocrine cancer has increased dramatically in recent decades (Jemal et al., 2011), but its significant increase is still lower than other high prevalence cancers, such as breast, lung, and colon cancer (Siegel et al., 2017). However, the elevated chance of survival in patients diagnosed with this type of cancer through successful surgery and treatment could be the underlying reason for being limited research in the cellular and molecular pathology of this particular cancer.

From a histological and cellular pathology viewpoint, thyroid cancer generally falls into two categories of follicular and para-follicular cell origins. Furthermore, follicular cancer which is more prevalent can be divided into three groups, namely, Well-differentiated (WDTC), Poorly differentiated (PDTC), and Anaplastic thyroid cancer (ATC) (Schlumberger, 2007). WDTC group includes thyroid follicular and papillary thyroid carcinoma (PTC), the most common type among all types of thyroid cancer. Most often, advanced forms of thyroid cancer, include PDTC and ATC, results from WDTC progression (Acquaviva et al., 2018).

The onset and progression of thyroid cancer, in its most common form, i.e. PTCs, like other cancers, are basically affected by genetic and environmental factors. The most frequent genetic factors that interfere with the development and progression of this group of cancers are V600E point mutations in the BRAF oncogene (in about two-thirds of PTC patients) (Xing, 2013; Xing et al., 2013; Choi et al., 2014) and different type of mutations in RAS isoforms, includes HRAS, NRAS, and KRAS (Liu et al., 2009). These mutant proteins can have a significant impact by activating the MAPK signaling pathway in the tumorigenesis process (*Cell*, 2014). In addition to these genetic changes, another set of

Abbreviations: PTC, papillary thyroid cancer; miRNA, micro RNA; Epi miRNA, epigenetic micro RNA; lncRNA, long noncoding RNA; WDTC, well-differentiated thyroid cancer; ATC, anaplastic thyroid cancer; DNMT1, DNA methyltransferase; HDAC4, histone deacetylases 4; MAPK, mitogen activated kinase-like protein; IGFBP1, insulin-like growth factor binding protein 1; TGFB1, transforming growth factor beta 1; WWP2, WW domain-containing E3 ubiquitin-protein ligase 2; SOX9, SRY-box transcription factor 9.

* Corresponding author at: Yazd Reproductive Sciences Institute, Bou-Ali alley, Timsar-Fallahi Avenue, Safaeieh district, Yazd Postal code/P.O. Box: 8916877391, Iran.
E-mail address: ehsanfarashahi@ssu.ac.ir (E. Farashahi-Yazd).

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changes called epigenetic changes can also affect tumor progression (Cao et al., 2018).

Epigenetic changes include DNA methylation (Singal and Ginder, 1999), Histone modifications (Falkenberg and Johnstone, 2014) and Non-coding RNA function; microRNAs (miRNAs) and long noncoding RNA (lnRNAs) (Esteller, 2011). The DNA Methyl Transferases (DNMTs), Histone Acetyl Transferases (HATs) and Histone Deacetylases (HDACs) gene products affects the transcription (Fardi et al., 2018) while miRNAs and lnRNAs affects translation of different genes in many studies of cancers (Macfarlane and Murphy, 2010; Schmitt and Chang, 2016). They, all, influence the level of the products of their targets and can affect the development of tumors positively or negatively (Xing, 2005; Alvarez-Nunez et al., 2006).

Among them, non-coding RNAs are more recent, and the focus of many studies on cancer, particularly in the last two decades, has been dedicated to them (Fabbri et al., 2007). Some miRNAs besides their independent inhibitory function on gene translation of different genes, interfere with the gene transcription of DNMTs and HDACs indirectly (Suzuki et al., 2012). From this group, the recognized ones such as mir-152 (Yang et al., 2017; Zhang et al., 2018a), mir-29a (Zhang and Xu, 2016; Takata et al., 2013), mir-140 can be mentioned that target the genes include *DNMT1*, *DNMT3A*, *DNMT3B*, and *HDAC4*. Among them, miR-140-5p has been also introduced as a possible tumor suppressor gene in different types of cancers and its expression level, in parallel with cancer development, gradually decreased (Zhai et al., 2015; Lu et al., 2017; Zhou et al., 2019; Li and Wang, 2020). This decrease can cause the increase of expression level of mir-140-p's gene targets, like *DNMT1* and *HDAC4*. Although limited studies have been performed on the expression of this miR in thyroid cancerous tissues (Qianqian Yu et al., 2021), no study has been performed to investigate the association of the expression of it with its potential gene targets (*DNMT1* and *HDAC*) in PTC, up to now. Therefore, in response to this question, the present study aimed to find the relationship between the expression level of miR-140-5P and the potential inhibitory effect on *DNMT1* and *HDAC4* mRNA levels, acting as an Epi-miR, in thyroid tumor tissue in compare to non-cancerous tumor margin.

2. Materials and methods

2.1. Demographic and pathologic characteristics of patients

Human thyroid carcinoma tissue and adjacent normal tissue (32 pairs) were obtained after collecting consent forms from the patients undergoing thyroid surgery at Mortaz Hospital, Shahid Rahnemoon Hospital, and Mojibian Hospital of Yazd, with the approval of hospital pathologist. Samples were transferred to −80 °C freezer in <1 h. There was no preoperative treatment for any of the patients, and the patients under 19 were excluded from the study. Clinical and metastatic data on lymph node status were collected from patient pathological data. The sample population includes 28 women and 4 men with the average age of 39.29 (±3.53). This study was approved by the Ethics Committee of Yazd University of Medical Sciences (Code: IR.SSU.MDICINE.REGREC.1396.70).

2.2. RNA extraction, cDNA synthesis and qPCR

The miRNAs were isolated using High Pure miRNA Isolation Kit (Roche, Basel, Switzerland) according to the protocol. The miRNAs cDNA synthesis was done by poly A tail method using BONmiR High Sensitivity Kit (Bonyakhteh, Iran). Total RNA was extracted manually using TRIzol solution (Invitrogen, USA). Total RNA cDNA synthesis was performed using Revert Aid First Strand cDNA Synthesis kit (Thermo Scientific, USA) according to the protocol.

Relative expression of miR-140-5Pin the normal and cancerous tissues was done using a mixture containing an appropriate concentration of specific primers. The sequences of miR-140-5P forward and its universal reverse primers were 5′-ACA GTG GTT TTA GCC TAT GGT-3′ and 5′-GAG CAG GGT CCG AGG T-3′ respectively. The reference gene was *U6* and its forward and reverse primer sequences were 5′-AGA TTT AAC AAA AAT TCG TCA C-3′ and 5′-GAG CAG GGT CCG AGG T-3′. qPCR was performed using a mixture containing a specific primer, cDNA synthesized from the sample, and SYBR Green premix (Real QPlus 2× Master Mix Green High ROX ™, Ampliqon, Denmark) in ABI step one machine (Applied Biosystems, Foster City, USA). The reaction conditions were as follows: The reaction solution was incubated for 15 min at 95 °C, then 40 cycles were repeated for 5 s at 95 °C, and repeated at 60 °C for 30 s. Relative expression of *HDAC4* (NM_006037.3), *DNMT1* (NM_001379.4), and *B2M* (NM_004048.4) and *GAPDH* (NM_001357943.2) genes (as reference genes) in PTC and normal margin tissues using an appropriate concentration of the specific primers are as follows; *HDAC4* forward primer 5′-TCC AAC GAG CTC CAA ACT CC-3′, reverse primer 5′-CAT CAG GCA TTC TAC CAG GGA G-3′; DNMT1 forward primer 5′-CAT CAG GCA TTC TAC CAG GGA G-3′, reverse primer 5′-CCT CAC AGA CGC CAC ATC G-3′ *GAPDH* forward primer 5′-CAA GAG CAC AAG AGG AAG AGA GAG-3′ *GAPDH* reverse primer 5′-TCT ACA TGG CAA CTG TGA GGA G-3′ *B2M* forward primer 5′-AGAT-GAGTATGCCTGCCCTG-3′ *B2M* reverse primer 5′-TGCGGCATCTT-CAAACCTC-3′.

qPCR was performed using SYBR Green premix (Ampliqon, Denmark) according to the protocol. qPCR was performed by ABI step one (Applied Biosystems, Foster City, USA). The following conditions were optimized: the reaction solution was incubated at 95 °C for 15 min, then 40 cycles were repeated at 60 °C for *DNMT1* and 65 °C for *HDAC4* gene for 20 s, 72 °C for 30 s.

2.3. Statistical analysis

The relative expression of miR-140-5Pand *DNMT1* and *HDAC4* genes were analyzed by SPSS software (version 16). Initially, Kolmogorov-Smirnov and Shapiro-Wilk tests were performed for data distribution. Then, according to the distribution of data, Wilcoxon or Paired Sample *t*-test was used to compare the data in the tumor and control groups. Differences in gene expression with invasive (lymph node metastasis) tumor status were evaluated using Mann-Whitney test. Pearson correlation coefficient test was used to examine the intensity and direction of the correlation between the variables.

3. Results

The transcription of miR-140-5P, *DNMT1* and *HADC4* were evaluated by RT-qPCR in 32 tissue samples from PTC patients and the 32 controls from tumor margins of the same subjects. Based on the data obtained, miR-140-5P shows increase in tumor samples compared to control samples (Fig. 1). According to the *t*-test, with a 95% confidence interval, the difference in miR-140-5Pexpression between the two groups was statistically significant (P-value < 0.05) (Fig. 1). Moreover, no statistically significant changes were observed in the expression of *DNMT1* and *HDAC4* genes in tumor versus control samples (Fig. 1). In these two genes, due to the non-normality of the obtained data, Wilcoxon test was used and (P-value > 0.05) were set respectively. Also, according to the results using Pearson test, no significant correlation was found between *DNMT1* and *HDAC4* gene with miR-140-5Pin the samples.

4. Discussion

miR-140-5P can potentially acts as a tumor suppressor in thyroid cancer cells, like its role in other cancers, in two ways (Takata et al., 2013; Zou et al., 2019; Song et al., 2009; Liao et al., 2018; Nie et al., 2019). One way is direct targeting and inhibition of genes such as *IGFBP1*, *SMAD2/3*, *FGF9* and *TGFB* that activated in the ERK, MAPK, and TGF-β pathways which helps the inhibition of cancer cell

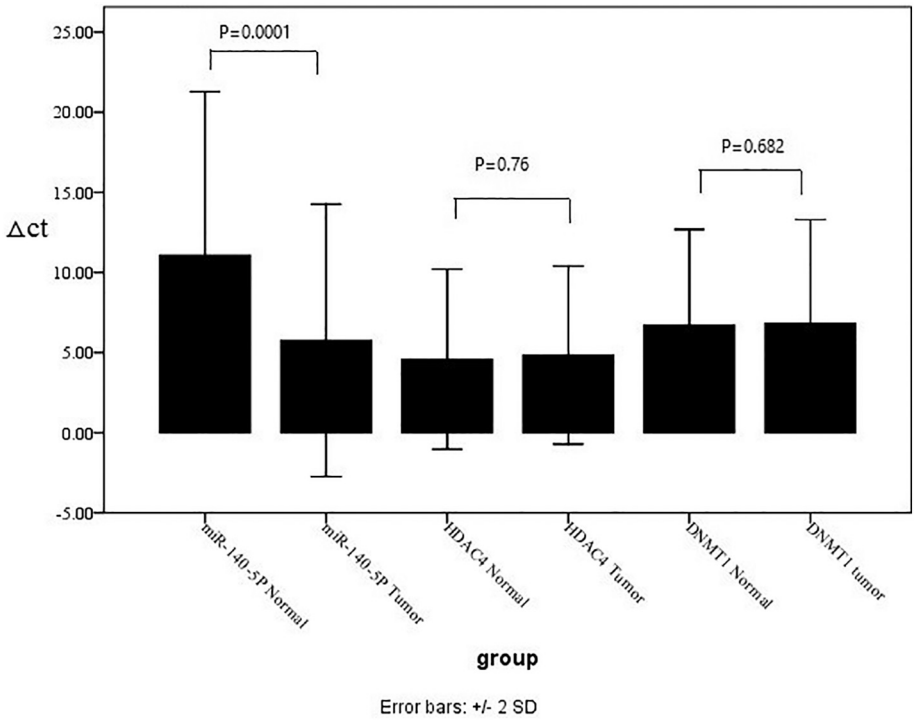

Fig. 1. The relative expression of miR-140-5Pshows increase in PTC tumor samples, but there are no statistically significant changes in the relative expression of *DNMT1* and *HDAC4* genes.

proliferation and migration (Lu et al., 2017; Lan et al., 2015; Jing et al., 2016; Fang et al., 2017). The other way is miR-140-5P indirect support miRwith maintaining the expression level of tumor suppressor genes via targeting *DNMT1* and *HDAC4* genes which consequently preventing of DNA methylation and histones deacetylation (Takata et al., 2013; Zhang et al., 2015). Our prediction for the results of miR-140-5P expression behavior was in favor of decreasing expression miRin tumor specimens but, in contrary to our prediction and other previous studies (Zhang et al., 2018b), the expression change was in favor of multiple-fold increaseit. Our prediction regarding the increased expression level of *DNMT1* (Zhang et al., 2018b) and *HDAC4* (Giaginis et al., 2014) in result of miR-140-5P decrease was also not realized and we did not observe any significant changes in the expression level of these genes.

Our attempts to understand and explanation of the reasons of these unexpected results guided us to regulatory mechanism of miR-140 transcription. The transcription level of mir-140 is potentially regulated in two ways: one way is through its position within the *WWP2* gene (WW domain-containing E3 ubiquitin-protein ligase 2) which affected by the promoter of *WWP2* gene; and the other way is through its dedicated promoter in intron 10 (Li et al., 2018). *WWP2* gene is a member of the *NEDD4*-like protein family, which is recognized by the ubiquitin ligase activity (Liu et al., 2019; Yang et al., 2013). This gene is expressed higher than average in the thyroid tissues (Kai et al., 2014) and its proto-oncogenic role in other cancers such as liver (Su et al., 2016), lung (Yang et al., 2011), and ovarian cancer (Zhao et al., 2010) has also been reported. The *WWP2* gene promoter and the mir-140 gene-specific promoter are both affected by a common transcription factor, *SOX9* (SRY-box transcription factor 9) (Li et al., 2018; Bernassola et al., 2008; Uhlen et al., 2015), which plays an important role in controlling cell proliferation (Qin et al., 2016). Nevertheless, SOX9 has conflicting roles in a variety of malignancies. For instance, it has been reported as a tumor suppressor in melanoma and bladder cancers (Yang et al., 2016; Jung et al., 2014) while, in other groups, such as breast, glioma, and thyroid, it acts as a tumor-promoting agent (Yamashita et al., 2012; Miyaki et al., 2010; Lee and Saint-Jeannet, 2011).

Based on these findings, the significant increase in miR-140-5P could be justified in relation to thyroid progression. But given the well-known targets of miR-140-5P which are often included in proto-oncogenes group, the tumor-promoting role of this type of miR cannot be defended easily. To justify this paradoxical behavior of miR-140-5P, namely upup regulation of miR-140-5P along with its tumor suppressor functional mechanism, only the inhibitory simultaneous function of other existing and active molecular agents in PTC tumor cells can be referred to; factors that inhibit all miRNAs in general, or miR-140-5P in particular. In this respect, a class of long noncoding RNAs (lncRNAs) with sponge properties can be mentioned. Among this sponge group for miR-140-5P, we can mention *HOXA11-AS*, *SNGH16*, and *PVT1* All the three genes can play oncogenic roles in cancers by reducing the function of microRNAs, such as miR-140-5P (Huang and Guo, 2017; Wang et al., 2012; Yoon et al., 2014) and there are numerous reports have revealed a high expression of them in PTC (Li et al., 2014; Ebert and Sharp, 2010; Cui et al., 2017). Therefore, neutralizing the inhibitory and preventive effect of such high concentration of miR-140-5P in PTC tumor samples can be related to the high concentration of *HOXA11-AS*, *SNGH16*, and *PVT1* sponges in PTC cells. But what is clear is that the simultaneous study of the expression behavior of these genes along with miR-140-5P transcription can provide a clearer picture of miR-140-5P role in PTC.

5. Conclusion

In the end, it is necessary to mention a few points. The first one is that, given the lack of previous studies on the expression of this gene (miR-140-5P) in thyroid tumors, it is not still possible to provide a definite comment on its inhibitory or tumor-promoting role in thyroid tumors. The second point refers to the increased expression of miR-140-5P in PTC tissue, and no significant changes in the expression of *DNMT1* and *HDAC4* genes in the tumor group compared to the control group and the failure to fulfill the initial hypothesis that miR-140-5P is involved in thyroid cancer. The third point to be regarded is the hypothetical model proposed in relation to the inhibitory role of sponges, which necessarily requires rigorous experimental-laboratory studies and studies with a large population.

CRediT authorship contribution statement

Haniyeh Nikkhah: Conceptualization, Validation, Formal analysis, Data curation, Writing – original draft, Visualization. **Maryam Vafaei:** Conceptualization, Validation, Resources, Formal analysis. **Ehsan Farashahi Yazd:** Conceptualization, Methodology, Validation, Investigation, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Mohammad Hasan Sheikhha:** Methodology, Resources, Funding acquisition. **Jamal Jafari Nudoshan:** Methodology, Resources, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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