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Altered expression of microRNA-30a-3p in papillary thyroid cancer and its association with clinicopathological characteristics

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Abstract: A growing number of studies suggest a tumor suppressive role and potential prognostic signif-icance of miR-30a-3p in different types of cancer. However, relatively few studies have focused on this microRNA in neoplastic thyroid lesions, including papillary thyroid cancer (PTC). The aim of our study was to shed more light on the potential involvement and clinical relevance of miR-30a-3p in this type of cancer. We examined the expression levels of this microRNA in 42 pairs of PTCs and matched non-tumor thyroid tissues using quantitative RT-PCR. We analyzed their association with clinical and histopathological parameters. The results revealed that miR-30a-3p was significant-ly downregulated in the majority of PTC tissues compared to corresponding non-tumor tissues. Moreover, decreased expression of miR-30a-3p was associated with advanced clinical stage, pres-ence of multiple tumor foci and capsular invasion, suggesting a role in aggressive disease. Although the role of this microRNA and its prognostic utility remain to be elucidated, the presented data sug-gest that downregulated expression of miR-30a-3p indicates poorer prognosis in PTC patients, war-ranting further investigations.

Keywords: miR-30a-3p; papillary thyroid cancer (PTC); thyroid cancer; expression, advanced stage

INTRODUCTION

Papillary thyroid cancer (PTC) is the most prevalent type of thyroid malignancies, accounting for 75-85% of all thyroid cancer cases. Over the last decades, the incidence of thyroid cancer has been increasing all over the world, and this increase is mainly due to an increase in PTC incidence [1,2]. Risk stratification of PTC patients is currently based on clinicopathological characteristics such as age, gender, tumor size, extra-thyroidal spread, nodal metastases, distant metastases and clinical stage. Although this approach is reliable in predicting the mortality risk, it has proved to be uncertain in predicting the risk of recurrence, which occurs more often than death from PTC, so it is more clinically relevant [3,4]. Therefore, the stratification of PTC patients into low- and high-risk prognostic groups still needs to be improved in order to accurately tailor management. Better understanding of molecular mechanisms of PTC occurrence and progression may be helpful in the identification of novel diagnostic and prognostic biomarkers as well as potential therapeutic targets for the treatment of more aggressive and/or advanced PTC cases.

A number of studies in the past two decades have demonstrated the importance of microRNAs (miRNAs or miRs) in cancer biology. MiRNAs are small, non-coding RNAs (consisting of ~ 22 nucleotides) involved in the posttranscriptional regulation of gene expression. MiRNAs can modulate target gene expression negatively by binding to the 3'-untranslated region of mRNAs, leading to their degradation or inhibition of translation [5]. Aberrant expression of different microRNAs has

been associated with processes such as tumor growth, invasion, angiogenesis, metastasis and immune evasion. Depending on their target genes, microRNAs can function as oncogenes or tumor suppressors [5,6].

One of the microRNAs with potential tumor suppressor activity that is frequently found to be deregulated and associated with aggressive tumor features and progression in various human cancers is miR-30a-3p. So far, downregulated expression of this member of the miR-30 family has been reported in lung cancer [7-10], breast cancer [11-13], colorectal cancer [14], bladder cancer [15], ovarian cancer [16], endometrial cancer [17], hepatocellular carcinoma [18], hematologic malignancies [19] and others.

The aim of this study was to investigate the expression levels of miR-30a-3p in papillary thyroid cancer and corresponding non-tumor thyroid tissue, and to evaluate the association with clinical and pathological parameters in order to determine the potential significance of miR-30a-3p in PTC malignancy. To our knowledge, this is one of only a few studies of miR-30a-3p expression in this type of cancer.

MATERIALS AND METHODS

Patients and clinicopathological characteristics

Tissue samples (paired tumor and corresponding non-tumor thyroid tissues) were obtained from 42 patients with PTC, following surgical excision at the Center for Endocrine Surgery, Clinical Center of Serbia, between 2012 and 2015. Samples were snap-frozen in liquid nitrogen and stored at -70°C until RNA extraction. Clinical and histopathological parameters are shown in Supplementary Table S1. All patients included in the study gave their informed consent. The study was approved by the Ethics Committee of Clinical Center of Serbia.

RNA extraction and reverse transcription

Total RNA extraction from tumor and non-tumor thyroid tissues was performed using TRI Reagent Solution (Ambion, Foster City, CA) according to manufacturer's instructions, following homogenization in liquid nitrogen. RNA quantification was done on NanoDrop™ 1000 (Thermo Fisher Scientific, USA). Next, 10 ng of total RNA was reverse transcribed using a TaqMan Micro-RNA Reverse Transcription Kit and specific stem-loop RT primer from TaqMan miR-30a-3p Assay (ID 000416), according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). Small nuclear RNA (nRNA) RNU6B (Assay ID 001093) was used as an endogenous control.

Quantitative PCR (qPCR)

RT-qPCR was performed on a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). PCR reactions were carried out in a 20-μL reaction volume using the TaqMan Universal Master Mix, No Amperase UNG (Applied Biosystems, Warrington, UK), specific TaqMan miR-30a-3p assay (ID 000416) or small nRNA RNU6B assay (ID 001093), which was used for normalization. The cycling conditions were as follows: 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C.

Ct values were calculated using Applied Biosystems 7500 system SDS software. The relative quantity of target microRNA in each tumor sample (T) was expressed as the fold change relative to the corresponding non-tumor tissue sample (N) and normalized to the internal reference RNU6B according to the $2^{-\Delta\Delta Ct}$ method, following the equation:

$$T/N=2^{-\Delta\Delta Ct},\ \Delta\Delta Ct=\Delta Ct(T)-\Delta Ct(N),$$
$$\Delta Ct=Ct_{target}-Ct_{endogenous\ control.}$$

In order to compare the relative expression level between tumor and non-tumor tissue, the relative quantity of target in each sample was expressed as fold change relative to the calibrator (1x sample, a sample with the lowest expression) following the equation:

$$RQ_{sample}=2^{-\Delta\Delta Ct},\ \Delta\Delta Ct=\Delta Ct_{(sample)}-\Delta Ct_{(calibrator)},\Delta Ct=Ct_{target}-Ct_{endogenous\ control.}$$

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5.01 (GraphPad Software). Nonparametric tests, Mann-Whitney U test and Spearman's correlation test, were used for the analysis of the association of miR-30a-3p expression levels with clinical and histopathological parameters, since a non-Gaussian distribution of the expression values was detected. The significance of the

difference between pairs of tumor and non-tumor tissues was evaluated by the Wilcoxon matched-pairs test. Classification of the fold change values between tumor and corresponding non-tumor thyroid tissues was based on the following criteria: fold change >1 as "increased"; fold change <1 as "decreased". P≤0.05 represented statistical significance, P<0.1 represented a statistical trend.

RESULTS

The expression level of miR-30a-3p in PTC and non-tumor thyroid tissues

The overall expression level of miR-30a-3p in tumor and non-tumor thyroid samples is shown in Fig. 1A. There was a significant difference in miR-30a-3p expression between tumor and corresponding non-tumor thyroid tissues (P=0.002). Most of the tumor samples (33/42; 79%) showed a significantly decreased level of miR-30a-3p compared to matched non-tumor samples (P<0.0001), as shown in Fig. 1B.

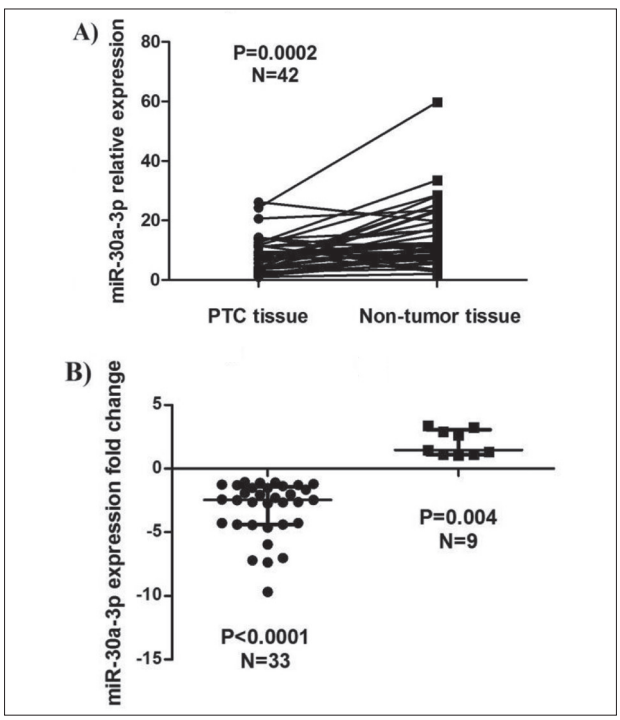

Fig. 1. The expression level of miR-30a-3p in PTC and non-tumor thyroid tissues. **A**– miR-30a-3p expression is deregulated in PTC tissues compared to matched non-tumor thyroid tissues. Paired PTC and corresponding non-tumor tissues are connected with lines. **B**– 78.6% (33/42) of PTCs had significantly decreased expression of miR-30a-3p; 21.4% (9/42) had an increased expression in PTC compared to matched non-tumor thyroid tissue. The dots represent patients with the decreased miR-30a-3p expression in tumor tissue while squares represent patients with an increased expression. Statistical analyses were performed by Wilcoxon matched pairs test and P<0.05 was considered statistically significant.

Relationship between miR-30a-3p expression and clinical and pathological characteristics

The expression levels of miR-30a-3p in PTC with respect to standard clinicopathological parameters are presented in Table 1. A significant association

Table 1. The association of miR-30a-3p expression level with clinicopathological parameters in PTC patients.

Clinicopathological parameters	miR-30a-3p relative expression	P value
Gender		
Male (n=10)	0.572 (0.141–0.959)	0.585
Female (n=32)	0.507 (0.270–0.934)	
Age	Corr.Coef. -0.233	0.142
≤45 (n=26)	0.660 (0.397–1.043)	0.096*
>45 (n=15)	0.378 (0.228–0.726)	
Tumor size (cm)	Corr.Coef. -0.033	0.834
Histovariant		
Classic (n=17)	0.404 (0.225–0.660)	0.099*
Follicular+other (n=19)	0.778 (0.234–1.109)	
Tumor focality		
Multifocal (n=13)	0.376 (0.179–0.610)	**0.024**
Monofocal (n=28)	0.660 (0.386–1.004)	
pT category		
pT1+2 (n=21)	0.600 (0.305–1.101)	0.352
pT3+4 (n=21)	0.488 (0.231–0.801)	
Lymph node metastases		
Yes (n=11)	0.494 (0.378–0.798)	0.841
No (n=31)	0.600 (0.228–1.022)	
Capsular invasion		
Yes (n=22)	0.392 (0.204–0.736)	**0.029**
No (n=20)	0.801 (0.408–1.075)	
Vascular invasion		
Yes (n=15)	0.600 (0.228–0.883)	0.989
No (n=26)	0.504 (0.340–0.968)	
Extrathyroidal spread		
Yes (n=9)	0.381 (0.188–0.691)	0.191
No (n=32)	0.632 (0.269–1.004)	
Clinical stage	Corr.Coef. -0.471	**0.002**
I+II (n=35)	0.657 (0.376–1.022)	**0.011**
III (n=7)	0.364 (0.139–0.381)	

The data are presented as the median with interquartile range and analyzed by Man-Whitney U test or Spearman's correlation test. Significant P values are shown in bold and the statistical trend is marked with an asterisk. miR-30a-3p relative expression represents the expression fold change in tumor tissue compared to matched non-tumor tissue.

of decreased miR-30a-3p was found with advanced clinical stage (P=0.011), which was also supported by the presence of a significant negative correlation between miR-30a-3p relative expression levels and stage (P=0.002, r=-0.471), obtained by Spearman's correlation test. Lower miR-30a-3p levels were also significantly associated with the presence of multiple tumor foci (P=0.024) and with capsular invasion (P=0.029). No other significant associations were detected.

DISCUSSION

MicroRNAs have been proposed by numerous studies as promising diagnostic, prognostic, therapeutic and surveillance tools for human cancers because of their critical roles in various biological processes during tumor development and progression [20-25].

In vitro studies have shown that miR-30a-3p is involved in the regulation of cell proliferation, angiogenesis, cell migration, invasiveness, epithelial-mesenchymal transition (EMT) and apoptosis [11,18,26,27]. Interestingly, one of the targets experimentally confirmed to be negatively regulated by miR-30a-3p is C-jun-amino-terminal kinase-interacting protein 4 (SPAG9), a scaffold protein that activates the mitogen-activated protein kinase (MAPK) signaling pathway in tumorigenesis, leading to cell proliferation and inhibition of apoptosis [28]. Constitutive activation of the MAPK signaling pathway is frequently found in PTC [29]. Pathway activation is caused by mutations in proto-oncogene B-Raf (*BRAF*) or one of the *RAS* family genes, or by *RET*/PTC rearrangements in 70% of cases [30]. Other PTC cases do not harbor any of these genetic alterations suggesting that some other mechanisms, including microRNAs might be involved.

To date, aberrant expression of miR-30a-3p has been detected in various types of cancer, but relatively few studies have investigated the biological role or the clinical relevance of this microRNA in neoplastic thyroid lesions including PTC [31,32].

To shed more light on the involvement of miR-30a-3p and its clinical relevance in PTC, we investigated the level of expression of this microRNA in matched tumor/non-tumor tissue samples and its association with clinical and pathological parameters. The results of the present study show that miR-30a-3p expression is significantly downregulated in PTC tissues compared to corresponding non-tumor thyroid tissues in the majority of analyzed samples, suggesting a potential involvement of this microRNA in PTC development and/or progression. This is in line with the results of other studies of miR-30a-3p in PTC. miR-30a-3p is significantly downregulated in PTC compared to adjacent non-tumor tissue and to benign goiter tissue [31]. Next-generation sequencing and expression analysis of miRNAs in PTC and contralateral normal thyroid tissue specimens revealed miR-30a-3p to be among the most abundantly downregulated miRNAs in PTC [32]. Significantly decreased level of miR-30a-3p in tumor tissue compared to corresponding non-tumor tissue was also found in lung carcinoma [7,8,10,26], esophageal carcinoma [27], gastric cancer [28], colorectal cancer [14] and liver cancer [18].

Our analysis showed that a decreased expression of miR-30a-3p was significantly associated with advanced clinical stage (according to the TNM staging system), the presence of multiple tumor foci and capsular invasion. We also detected a trend towards the association with classical papillary growth pattern and older age. Findings suggest that multiple foci in PTC represent intraglandular metastatic spread [33] and multifocality was associated with extrathyroidal spread, nodal metastases, advanced disease and recurrence [34,35]. Although classical and follicular histovariants of PTC are currently categorized into the same risk group [36,37], some evidence suggest that a classical histovariant is associated with higher risk of aggressive behavior and worse prognosis [38,39]. Also, according to the thyroid differentiation score determined in the Cancer Genome Atlas (TCGA) study of thyroid cancers, although both histologic variants are well differentiated, it was found that the classical histovariant is less differentiated than the follicular variant [40,41]. Considering all this, our results show that a decreased expression of miR-30a-3p is in correlation with more aggressive tumor features. These results are in accordance with the results of other studies of this microRNA in various cancers. A significant difference in the expression of miR-30a-3p in high invasive PTC (present lateral lymph node metastasis) compared to low invasive PTC (no lymph node metastasis), with a trend towards association with tumor multifocality was described [31]. In other types of cancer, miR-30a-3p downregulation was associated with advanced stage

and more aggressive tumor features such as tumor size, pT category, nodal metastases, TNM stage in lung cancer [8], with higher tumor grade and poorer differentiation in ovarian cancer [16,42], vascular invasion in hepatocellular carcinoma [18], distant metastases in lung and renal carcinoma [43,44] and shorter progression free or overall survival in many of them [8,11-13,28,42].

Although the data presented herein need to be confirmed in larger cohorts, they demonstrate that miR-30a-3p expression is deregulated in PTC and that the decreased expression of this microRNA is associated with more aggressive tumor features and advanced disease stage, which supports its potential usefulness in identifying PTC patients at higher risk for disease progression. The exact role of miR-30a-3p, its potential involvement in the regulation of the MAPK signaling pathway, as well as its utility as a diagnostic and/or prognostic tool, remain to be elucidated.

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Conflict of interest disclosure: The authors declare that they have no conflict of interest.

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Supplementary Material

The Supplementary Material is available at: http://serbiosoc.org.rs/NewUploads/Uploads/Todorovic%20et%20al_4688_Supplementary%20Table%20S1.pdf