PAX8-PPARγ Oncogene in Follicular Thyroid Tumors: RT-PCR and Immunohistochemical Analyses

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A B S T R A C T

US-guided fine needle aspiration cytology is currently the best diagnostic tool for thyroid nodules. However, it is not sensitive and specific enough for differentiating between benign and malignant follicular tumors. A potentially useful marker for this differentiation is the PAX8-PPARγ rearrangement, identified in follicular thyroid carcinomas, but not in follicular adenomas or other types of thyroid tumors. The aim of this research was to determine the clinical significance of the PAX8-PPARγ oncogene in diagnostics follicular thyroid tumors. The study included 62 patients with follicular or Hürthle cell tumors. Gene expression was determined by reverse transcription-polymerase chain reaction (RT-PCR) from paraffin embedded tissues, and PCR products were checked using the agarose gel electrophoresis. The immunohistochemical analysis was performed on archive paraffin embedded tissues with the monoclonal PPARγ antibody. The statistical analysis has indicated that neither the expression of PAX8-PPARγ mRNA, nor the immunohystochemical analysis with the PPARγ antibody correlate with the patohystological diagnosis. The oncogene PAX8-PPARγ has not met the expectations as a reliable tumor marker for differentiation between benign and malignant thyroid tumors, which makes the only reliable histological criteria – capsular and vascular invasion.

Key words: *follicular thyroid tumors, PAX8-PPARγ rearrangement, RT-PCR, immunohistochemistry*

Introduction

According to the 2004 World Health Organization classification of thyroid tumors, they occur as carcinoma, adenomas and related tumors, and other thyroid tumors[1].

Whereas for most thyroid tumors there are clear cytological criteria, the boundaries between adenomatoid nodule, a follicular adenoma and well differentiated follicular carcinoma are not cytologically well defined. It is therefore necessary to find clinically reliable tumor markers which would make this differentiation possible and thus reduce the number of unnecessarily surgeries[2–5].

The most common mutations of follicular carcinomas include RAS mutations and PAX8-PPARγ rearrangement, chromosomal translocation between the thyroid transcription factor PAX8 and the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ). PAX8-PPARγ was, et first, thought to be restricted to FCa (Kroll et al., 2000)[6], but other groups have detected the expresion of PAX8-PPARγ gene not only in FCa but also in follicular thyroid adenomas[7–13]. In the present study, we report the analysis of PAX8-PPARγ gene in a series of follicular thyroid tumors, as well as immunohistochemical analysis with PPARγ antibody.

Methods

The research included 62 patients with cytologically diagnosed follicular or Hürthle cell tumors, histologically verified as follicular or Hürthle cell adenomas or carcinomas.
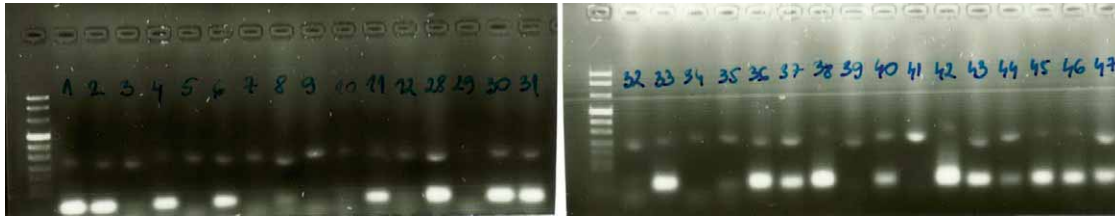
Fig. 1. RT-PCR analysis of thyroid tumors. 362 bp › presence of exons 7, 8 and 9 of the PAX8 gene; 68 bp › fusion between PAX8 exon 7 and exon 1 of PPARγ.

Gene expression was determined by reverse transcription-polymerase chain reaction (RT-PCR). Total mRNA was isolated from paraffin embedded tissues using High Pure RNA Paraffin Kit (Roche Applied Science, EU) and translated into cDNA by reverse transcriptase PCR (Superscript II RT, Invitrogen, EU). PAX8-PPARγ rearrangements were detected by PCR using one reverse (PAX8--R) and three forward pairs of primers (PAX8-7, PAX8-8 and PAX8-9). The nucleotide sequence of the primers was based on the mRNA sequences deposited in GenBank. Human hypoxanthine phosphorybosyl-transferase 1 (hHPRT1) was used as a referent housekeeping gene. Products of PCR reactions were separated by 1% agarose gel electrophoresis and analyzed. A 362-bp amplification band corresponds to the presence of exons 7, 8 and 9 of the PAX8 gene, a 173-bp band corresponds to PAX8 exons 7 and 9 and a 68-bp band is the result of the fusion between PAX8 exson 7 and exon 1 of PPARγ.

Immunohistochemistry was performed on formalin--fixed paraffin-embedded sections attached to silanized slides. Microwave antigen retrieval was performed at 95 °C for 30 minutes in 10-mM citrate buffer (pH 6.0). Slides were incubated with PPARγ monoclonal antibody E8 (SC-7273P, 1:100, Santa Cruz, CA, USA) for 60 minutes on a DAKO Autostainer. The Universal DAKO LSAB--Plus Kit detection system (avidin-biotin complex) and DAB chromogen (DAKO) were used according to manufacturer's protocol for immune complex detection. Sections were counterstained with Mayer's haematoxylin, dehydrated and mounted. A tumor with PAX8-PPARγ rearrangement detected by RT-PCR was used as a positive control. Nuclear staining was scored as none (0), weak (1+), moderate (2+), or strong (3+)

The statistical analysis was conducted using the SPSS 9.0, and included the analysis of categorical variables (2x2 and RxC contingency tables), as well as the analysis of correlation and variance[14–16].

Results

Detection of the PAX8-PPARγ rearrangement

RT-PCR method has proven the expression of PAX8--PPARγ mRNA in 33% of follicular carcinomas, 14% of Hürthle cell carcinoma, but also in 33% of follicular and 19% of Hürthle cell adenoma (Figure 1). Therefore, neither individually nor globally (steam comparison of individual categories) there is no differences in expression

PAX8-PPARγ mRNA among the analyzed histologic categories p=0.631, Freeman-Halton exact test, Table 1). Furthermore, there is no difference in expression between different cytological categories (p=0.281, Fisher exact test), or between adenomas and carcinomas (p= 0.177, Fisher exact test).

TABLE 1
ANALYSIS OF PAX8-PPARγ mRNA EXSPRESSION IN PARAFFIN EMBEDDED TISSUES

PAX8-PPARγ	FAd	FCa	HAd	HCa
–	9	11	11	10
+	7	7	4	3

p=0.631, Freeman-Halton exact test; FAd – follicular adenoma, FCa – follicular carcinoma, HAd – Hürthle adenoma, HCa – Hürthle carcinoma

Immunohistochemistry

Immunohistochemical staining with PPARγ antibodies revealed medium and strong positive results in 3/16 follicular adenomas (18%), 3/18 follicular carcinomas (17%), 1/15 Hürthle cell adenomas (7%), 2/13 Hürthle cell carcinomas (15%) (Figure 2). Contingency table analysis has shown that there are no differences in PAX8--PPARγ expression, neither globally nor in individually--paired comparisons of individual categories, between the analyzed histological (global p=0.911, Freeman-Halton exact test, Table 2) and cytological categories (global p=0.926, Freeman-Halton exact test).
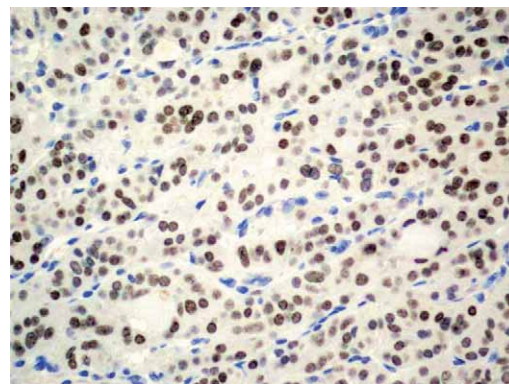

Fig. 2. Immunohistochemical staining with PPARγ antibodie; strong nuclear staining (3+).

Pathohistological diagnosis	N	Immuno histochemistry*			
		0	1	2	3
Follicular adenoma	16	12	1	1	2
Follicular carcinoma	18	12	3	1	2
Hürthle cell adenoma	15	13	1	0	1
Hürthle cell carcinoma	13	8	3	1	1

none (0), weak (1+), moderate (2+), strong (3+)

Moreover, adenomas and carcinomas do not differ concerning the PAX8-PPARγ immunopositivity (p= 0.501, Freeman-Halton exact test).

Discussion and Conclusion

This research included 62 patients with cytologically diagnosed follicular (34 patients) or Hürthle cell tumors (28 patients). Histologically, we have confirmed 16 follicular adenomas, 18 follicular carcinomas, 15 Hürthle cell adenomas and 13 Hürthle cell carcinomas.

As is well known, cytologic diagnosis has its limitations and the cytologycal criteria are unable to distinguish follicular lesions on smears.

Searching for a better diagnostic indicator and parameter which could be reliable in differentiating between malignant and non-malignant changes in the thyroid tissue, Kroll et al. in 2000. described the translocation of the PAX8 and PPARγ gene [t(2;3)(q13;p25)], chimeric oncogene PAX8-PPARγ, responsible for follicular oncogenesis. As it was found only in follicular carcinomas, and not in follicular adenomas, papillary carcinomas and nodular hyperplasia, the authors indicated it could be a potentially useful marker for distinguishing malignant and benign follicular tumors. Immunohistochemical staining with PPARγ antibodies showed that all tumors for which the presence of PAX8-PPARγ gene was found using RT-PCR, show strong and diffuse positivity of the nucleus to PPARγ[6].

Many authors have only partially confirmed the research results described above. In 2002 Nikoforova et al. found PAX8-PPARγ gene in 53% of follicular carcinomas, but also in 8% of follicular adenomas[8]. According to their research, follicular carcinomas in which this gene was isolated are highly invasive, as opposed to minimally invasive PAX8-PPARγ negative carcinomas. In 2002, Marques et al.[9] isolated PAX8-PPARγ in 56% carcinomas, but also in 13% follicular adenomas. This translocation was not found for papillary and anaplastic carcinomas, or in nodular hyperplasia, so the authors consider this marker to be specific for follicular tumors, but not reliable enough to differentiate between carcinomas and adenomas. Similar conclusion was made by Cheung et al. in 2003, who found the PAX8-PPARγ oncogene in 35% follicular carcinomas, and in as many as 55% of follicular adenomas[10].

Hibi et al. have analysed the expression of PAX8--PPARg gene in Japanese with nodular thyroid changes. They explain the negative findings in follicular adenomas and carcinomas, papillary carcinomas, nodular hyperplasia, and in normal thyroid tissue by a likely ethnic--based tendency for translocation[17].

The expression of PAX8-PPARγ gene results in the increased synthesis of PPARγ protein. In most studies cited above, immunohistochemical staining using PPARγ antibody was also conducted. In PAX8-PPARγ positive tumors was found strong positivity of the nucleus to PPARγ antibodies[6,18]. Light and medium positivity of the nucleus and cytoplasm is found in adenomas, non-tumour changes, and in normal thyroid tissue. In 2005 Sahin et al. analysed a group of 108 patients with hystologically determined follicular tumors and performed the immunohistochemical staining using PPARγ antibody in order to determine the value of immunohistochemistry as an alternative RT-PCR method to distinguisn between adenomas and carcinomas. They found the immunopositivity in 57% follicular carcinomas, 4% Hurthle cell carcinomas, and in 13% follicular adenomas, and concluded that this method is useful in distinguishing between these diseases. Moreover, according to the clinical findings they collected, they state that there is a better prognosis for patients with immunupositivity to PPARγ antibody[19]. Most authors state the opposite, stressing a more frequent vascular invastion and worse long-term prognosis for patients with PAX8-PPARγ[7,14,22].

In this research we used the method of polymerase chain reaction with reverse transcription to determine the expression of PAX8-PPARγ mRNA from paraffin embedded tissues in 62 patients with cytologically and hystologically verified follicular or Hürthle cell carcinomas. RT-PCR method has proven the expression of PAX8--PPARγ mRNA in 33% of follicular carcinoma, 14% of Hürthle cell carcinoma, but also in 33% of follicular and 19% of Hürthle cell adenoma. Therefore, neither individually nor globally (steam comparison of individual categories) there is no differences in expression PAX8-PPARγ mRNA among the analyzed histological categories. Similarly, adenomas and carcinomas do not differ regarding the expression of mRNA. These findings support the results reported in the studies discussed above[9,10,23,24].

Immunohistochemical staining with PPARγ antibody in archive paraffin embedded tissues revealed medium and strong positivity in 18% follicular adenomas, 17% follicular carcinomas, 7% Hürthle cell adenomas, and in 15% Hürthle cell carcinomas. Contingency table analysis has shown that there are no differences in PAX8-PPARγ expression, neither globally nor in individually-paired comparisons of individual categories, between the analyzed histological and cytological categories. Moreover, adenomas and carcinomas do not differ concerning the PAX8-PPARγ immunopositivity.

The comparison of the PAX-PPARγ gene expression and immunohistochemical positivity showed no statistical significance.

This investigation has shown that the expression of PAX8-PPARγ mRNA, as well as the immunohistochemical positivity do not correlate with pathohistological diagnosis.

The aim of these study was to determine the value of the PAX8-PPARγ oncogene in differentiating between follicular adenoma and carcinoma. Its relation to follicular thyroid tumors is undoubtedly proven, but our results do not confirm the value of this oncogene as a reli-

able marker for distinguishing these tumors. Therefore, it is questionable whether all patients with cytologically diagnosed follicular tumors may avoid surgery.

Preliminary studies on the significance of PAX8-PPARg gene for differentiating between follicular tumors were conducted on a small number of patients. To confirm the significance of this oncogene as a prognostic factor for tyhroid tumors further studies and larger samples are required.

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ONKOGEN PAX8-PPARγ U FOLIKULARNIM TUMORIMA ŠTITNJAČE – RT-PCR I IMUNOHISTOKEMIJSKA ANALIZA

SAŽETAK

Ciljana citološka punkcija pod kontrolom ultrazvuka metoda je izbora u u dijagnostici čvorastih promjena, no nedovoljno osjetljiva i specifična za razlikovanje benignih i malignih folikularnih tumora. Potencijalno koristan marker za njihovu diferencijaciju je onkogen PAX8-PPARγ, identificiran kod folikularnog karcinoma, a ne kod folikularnog adenoma ni drugih tumora štitnjače. Cilj ovog istraživanja je određivanje kliničkog značaja PAX8-PPARγ gena u dijagnostici folikularnih tumora štitnjače. U istraživanje su uključena 62 ispitanika s folikularnim ili Hürthleovim tumorom. Ekspresija gena je određena metodom lančane reakcije polimeraze s obrnutom transkripcijom (RT-PCR) iz parafinskih rezova, a PCR produkti provjereni elektroforezom na agaroznom gelu. Na arhivskim parafinskim rezovima je izvršeno imunohistokemijsko bojanje monoklonalnim protutijelom PPARγ. Ekspresija PAX8-PPARγ mRNA, kao ni imunohistokemijska analiza PPARγ protutijelom ne korelira s patohistološkom dijagnozom. Onkogen PAX8-PPARγ nije opravdao očekivanja u smislu pouzdanog tumorskog markera koji bi omogućio diferencijaciju benignih i malignih tumora štitnjače, te je i dalje jedino pouzdan histološki kriterij – proboj kapsule i vaskularna.