Is the *PIK3CA* gene expression level in FNAB washouts equivalent to that in postoperative tissue specimens of papillary thyroid carcinoma?

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ORIGINAL ARTICLE

Abstract

INTRODUCTION: Papillary thyroid carcinoma (PTC) is the most common malignant tumor of the thyroid gland. The pathogenesis of PTC remains still mostly enigmatic, although PI3K/PTEN/AKT pathway has been proposed to play a role in development of PTC. Moreover, the significance of genetic analysis in the material from fine-needle aspiration biopsy (FNAB) in PTC patients has recently been demonstrated. Hereby, we present a study analyzing expression of *PIK3CA* in FNAB washouts of PTC and a comparison of the level of that expression with expression in postoperative PTC tissue. Furthermore, we have assessed correlation between tumor size, evaluated according to pTNM scale, and level of *PIK3CA* gene expression in postoperative PTC tissue.

METHODS: Total RNA was extracted by use of an RNeasy Micro Kit (Qiagen, Hilden, Germany) in FNAB material, and RNeasy Midi Kit (Qiagen, Hilden, Germany) in tissue material. The purity of total RNA was assessed by NanoDrop® ND-100 spectrophotometr. One hundred nanograms of total RNA were used in the first strand cDNA synthesis with TaqMan® Reverse Transcripton Reagents (Applied Biosystems, Branchburg, New Jersey, USA). The gene expression level of *PIK3CA* was analyzed by real-time PCR in the ABI PRISM ®7500 Sequence Detection System in the 21 (17 women, 4 men) FNAB and 20 (16 women, 4 men) postsurgical specimens of PTC. pTNM staging of PTC was assessed based on UICC classification.

RESULTS: Overexpression of *PIK3CA* was confirmed in FNAB washout specimens and in postoperative tissues of PTC, in comparison to macroscopically unchanged thyroid tissue. Furthermore, statistically significant differences in *PIK3CA* gene expression levels between both examined groups were not confirmed. Moreover, correlation between pTNM staging and level of *PIK3CA* gene expression in PTC samples was not found.

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CONCLUSION: The genetic analysis of overexpression of *PIK3CA* in FNAB washout specimens may be equivalent of postsurgical PTC tissue. A possibility of its future clinical application in FNAB specimens – adequate or undetermined for cytological analysis – awaits for evaluation. The level of expression of *PIK3CA* is independent of primary thyroid tumour size, evaluated according pTNM scale.

Abbreviations:

ATC   - anaplastic thyroid carcinoma
FNAB   - fine-needle aspiration biopsy
FTC   - follicular thyroid carcinoma
MAPK   - mitogen-activated protein kinase
PDTC   - poorly differentiated thyroid carcinoma
PI3K   - phosphatidylinositol-3 kinase
PIK3CA   - PI3K kinase
PTC   - papillary thyroid carcinoma
RT-PCR   - reverse transcription-polymerase chain reaction

INTRODUCTION

Papillary thyroid carcinoma (PTC) is a well-differentiated malignant neoplasm derived from the thyroid follicular cells. It is the most common type of thyroid cancer and its incidence – in proportion to other thyroid tumors – has been steadily increasing nowadays (Leenhardt *et al.* 2004). The molecular background of PTC development is complex. The pathogenesis of PTC involves the perturbation of multiple signaling pathways, such as mitogen-activated protein kinase (MAPK) pathway and/or the phosphatidylinositol-3 kinase (PI3K)/PTEN/AKT pathway. Abnormal activation of MAPK pathway in thyroid cells results from mutation of the *BRAF* and *RAS* genes or chromosomal rearrangements involving *RET* and *NTRK1* genes (Frattini *et al.* 2004). PI3K has a fundamental role in regulation of cell growth, proliferation and survival. In human cancers oncogenic mutations or amplifications of gene coding the α-type catalytic subunits of PI3K kinase (*PIK3CA*) are common (Samuels *et al.* 2006, Karkas *et al.* 2006). Gene *PIK3CA* is located on chromosome 3q26.3 and consists of 20 exons, coding 1068 amino acids (Karkas *et al.* 2006). Recently, the presence of *PIK3CA* mutations and gene amplification have been observed in sporadic thyroid carcinomas (Garcia-Rostan *et al.* 2005, Wu *et al.* 2005, Hou *et al.* 2007, Wang *et al.* 2007, Abubaker *et al.* 2008). Mutations or amplification of *PIK3CA* gene have been found more frequently in follicular thyroid carcinoma (FTC) and anaplastic thyroid carcinoma (ATC), while being rather uncommon in PTC (Garcia-Rostan *et al.* 2005, Hou *et al.* 2007, Wang *et al.* 2007). However, as yet little is known about the precise role of *PIK3CA* in PTC and data are inconsistent.

New diagnostic approaches to thyroid diseases include genetic tests of cells from fine-needle aspiration biopsy (FNAB). Formerly, during FNAB procedure, at least one additional puncture was performed to provide material for molecular analysis. Nowadays, due to improvement of sensitivity of molecular biology diagnostic techniques, obtaining genetic material from a small number of cells remaining in the biopsy needle after routine cytological smear is feasible.

In the present research, levels of relative expression of *PIK3CA* gene in the material obtained from the FNAB washouts and in postoperative tissue collected from PTC patients were evaluated. Furthermore, the possible correlation between *PIK3CA* gene expression level in PTC postsurgical thyroid specimens and pTNM staging was analysed. As far as we are concerned, this is the first report worldwide which provides data on these subjects.

MATERIALS AND METHODS

Cytological specimens from 21 patients (17 women, 4 men) and postoperative tissues specimens from 20 patients (16 women, 4 men) with PTC were examined. All tissue samples were taken after patients' informed consents. Following the FNAB aspirates were smeared for routine cytology, the cell remnants were washed out of the needle immediately for further investigation. Contemporaneously, tissue samples from the postoperative specimens of PTC patients were obtained. Samples of macroscopically unchanged thyroid tissue, surgically removed from patients with nodular goitre, served as a control for *real-time* PCR test.

Total RNA was extracted by use of an RNeasy Micro Kit (Qiagen, Hilden, Germany) in FNAB material, and RNeasy Midi Kit (Qiagen, Hilden, Germany) in tissues material, both based on modified Chomczyński and Sacchi's method, according to manufacturers' recommendations. The purity of total RNA was assessed by NanoDrop® ND-100 spectrophotometr (data not presented). One hundred nanograms of total RNA were used in the first strand cDNA synthesis with TaqMan® Reverse Transcripton Reagents (Applied Biosystems, Branchburg, New Jersey, USA), according to manufacturers' instruction.

Real-time PCR was performed on the ABI PRISM® 7500 Sequence Detection System (Aplied Biosystem, Foster City, CA, USA) by using *Taq*Man® Universal PCR Master Mix (Applied Biosystem) and *Taq*Man® Gene Expression Assays probe and primer mix (Applied Biosystem), according to the manufacturer's specification. The Assay Identification number of *PIK3CA* was: Hs00180679_m1. Thermal cycler conditions were as follows: hold for 10 min. at 95 °C, follow by two-step PCR for 50 cycles of 95 °C for 15 s followed by 60 °C for 1 min. Amplification reactions, in triplicate for each sample, were performed and the results were normalized to the *ACTB* gene expression level.

An analysis of relative gene expression data was performed, using the $2^{-\Delta\Delta CT}$ method on an ABI PRISM® 7500 Sequence Detection System Software (Applied Biosystems, Foster City, CA, USA). The fold change

in studied gene expression, normalised to endogenous control, was calculated using: RQ = $2^{-\Delta\Delta CT}$.

Statistical analysis

All statistical analyses were performed using software package Statistica 7.0. Basic measures of location (i.e. mean, median), measures of dispersion (SD, SEM), and minimum, maximum, lower quartile and upper quartile values were calculated to provide detailed descriptions of gene expressions in selected groups. Subsequentially, the data were statistically analyzed, using non-parametric Mann-Whitney's *U* test, in order to compare the level of expression values (RQ) among the two studied independent groups (FNAB, post-surgical tissues). Mann-Whitney's *U* test and Spearman's rank correlation coefficient were used to describe the correlation between *PIK3CA* gene expression level in postsurgical specimens and tumor size, according to pTNM scale. However, as pTNM group – which was being assessed – was numerically very small (14 patients), thus, solely two subgroups were distinguished: pT1 (n=8) and pT2–T4 (n=6).

RESULTS

The elevated levels of *PIK3CA* gene expression in both studied groups (material from FNAB washouts and from postoperative tissue), in comparison with macroscopically unchanged thyroid tissues were demonstrated. In turn, no statistically significant (*p*>0.05) difference in *PIK3CA* gene expression levels between two studied groups was observed. The box-and-whis-

ker plot diagrams, representing the expression levels of *PIK3CA* (median and mean values) in FNAB washouts and postsurgical tissue in PTC, are presented in Figure 1 and Figure 2, respectively. Moreover, no correlation of *PIK3CA* gene expression level with tumor size, according pTNM scale, was found (*p*>0.05). Respective expression levels of *PIK3CA* (median and mean values) in two studied groups: pT1 and pT2–T4, are presented in Figure 3 and Figure 4, respectively.

DISCUSSION

An important question is whether FNAB washout samples can substitute for a surgically resected tissue samples in the routine evaluation of tumors, using gene expression profiling. All classifiers, reported to date, have relied on databases derived from surgically resected tumor specimens. Consequently, these may not be suitable for FNAB washouts specimens, which often are composed of relatively pure population of tumor cells. However, new methods of molecular biology are of such sensitivity that they allow for a precise assessment of gene expression, even when small amount of genetic material is accessible. In our study, we have clearly presented no significant difference (*p*>0.05) in *PIK3CA* gene overexpression between FNAB-derived samples of PTC, in comparison with tissue material collected postoperatively. Our results confirmed the significance of genetic analysis in material from FNAB. Thus, in our opinion, increased expression of *PIK3CA* gene may be the genetic preoperative marker in the diagnosis of thyroid nodules suspected for PTC.
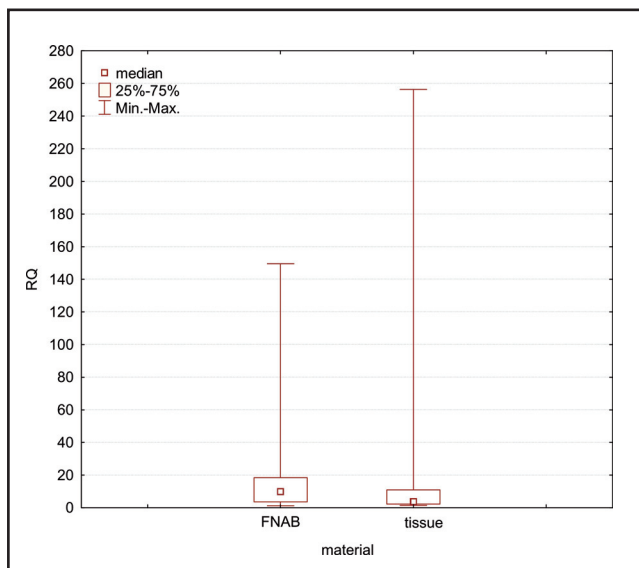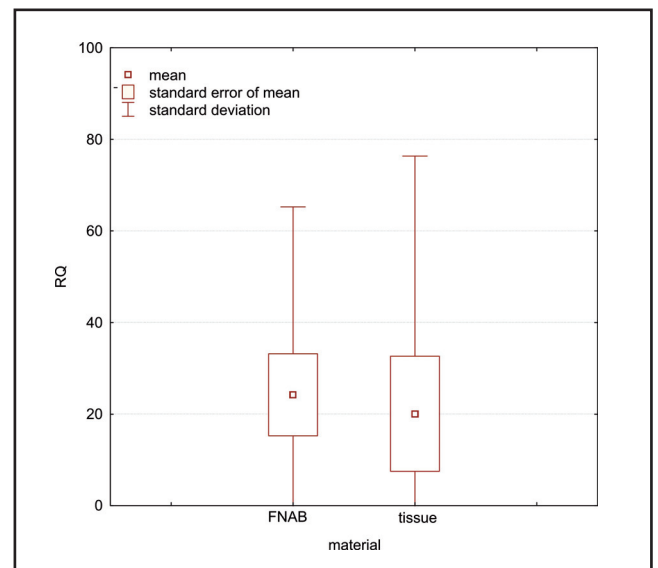

Fig. 1. Box-and-whisker plots, representing the expression of *PIK3CA* gene in the studied groups (FNAB, post-surgical tissues). The results are calculated as RQ values. Whiskers represent median and minimum and maximum values for particular groups. Boxes represent lower quartile and upper quartile. The results were statistically analyzed, using Man-Whitney's *U* test, *p*<0.05.

Fig. 2. Box-and-whisker plots, representing the expression of *PIK3CA* gene in the studied groups (FNAB, post-surgical tissues). The results are calculated as RQ values. Whiskers represent means±SD (standard deviation) for particular groups. Boxes represent means±SEM (standard error of mean). The results were statistically analyzed, using Man-Whitney's *U* test, *p*<0.05.
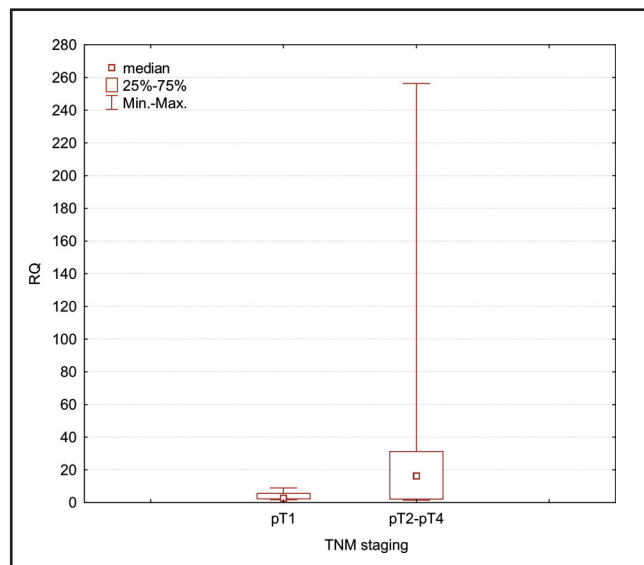
Fig. 3. Box-and-whisker plots, representing the expression of *PIK3CA* gene in the studied groups (pT1 and pT2–T4). The results are calculated as RQ values. Whiskers represent median and minimum and maximum values for particular groups. Boxes represent lower quartile and upper quartile. The results were statistically analyzed, using Man-Whitney's *U* test and Spearman's rank correlation coefficient, *p*<0.05.
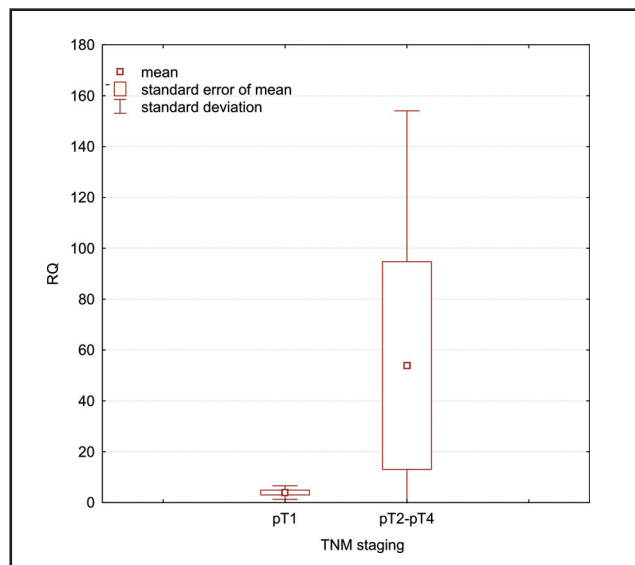
Fig. 4. Box-and-whisker plots, representing the expression of *PIK3CA* gene in the studied groups (pT1 and pT2–T4). The results are calculated as RQ values. Whiskers represent means±SD (standard deviation) for particular groups. Boxes represent means±SEM (standard error of mean). The results were statistically analyzed, using Man-Whitney's *U* test and Spearman's rank correlation coefficient, *p*<0.05.

Thyroid nodules are a common clinical problem, and FNAB is widely used for its elucidation. Less than 5% are malignant, with PTC being the most frequent cancer. Approximately 20% are classified as indeterminate or suspicious for malignancy. Patients whose thyroid nodules show indeterminate or suspicious cytologic features in FNAB samples, however, would require thyroidectomy because of a 20% risk of thyroid carcinoma. The gene-expression pattern may be useful for final diagnosis of PTC in difficult or ambiguous cases. Heretofore, only few studies have proved molecular methods to be suitable for routine FNAB examination, showing a similar pattern of genes overexpressions in FNAB and tissue samples (da Silveira Mitteldorf *et al.* 2010). Kebebew *et al.* (2005) presented the results of gene expression profiling for FNAB samples to aid in distinguishing benign from malignant lesions in the thyroid. The same authors used a real-time quantitative RT-PCR assay and provided a sensitivity of 91.0% and specificity 95.0% for distinction of benign from malignant disease (Kebebew *et al.* 2006).

Activation of PI3K pathway (particularly AKT-1 kinase) is associated with tumor advancement (regional invasion and metastasis) into FTC and/or PTC. The localization of activated AKT differs between the two forms of thyroid carcinoma, although nuclear localization is related with tumor invasion in both subtypes (Vasko *et al.* 2004). Recent studies have showed that *AKT-1* mutation may also occur in thyroid cancer, especially in recurrent or metastatic lesions, suggesting that this is relatively late genetic event in thyroid carcinogenesis (Ricarte-Filho *et al.* 2009). Additionally,

further study has disclosed that also *PIK3CA* mutation can occur in some cases of metastases derived from aggressive cancer such as poorly differentiated thyroid carcinoma (PDTC) or ATC (Ricarte-Filho *et al.* 2009). It has been demonstrated that simultaneous activation of PI3K and MAPK pathways could drive to aggressiveness and progression of well-differentiated thyroid carcinoma into ATC (Xing 2010). In our study, despite the increased level of *PIK3CA* gene expression (mean and median of RQ values) in pT2–T4 group of PTC, statistically significant correlation between *PIK3CA* gene expression and pTNM staging was not confirmed. However, the study group might be numerically too small (14 patients) to demonstrate statistical significance, and, thus, clear view on the subject is hardly possible. Furthermore, the correlation regarding only the tumor size (T) was analyzed; relationship of PIK3 expression and lymph node involvement (N) and presence of distant metastasis (M) remained unknown, but its existence could not be excluded.

Thyroid carcinoma is the most common malignant tumor of the endocrine system. In well-differentiated types of thyroid carcinomas (PTC and FTC) the prognosis is satisfactory, due to optimisation of combined therapy. Unfortunately, some of the well-differentiated thyroid carcinomas, due to molecular disturbances accumulation, may transform into more aggressive types, such as PDTC or ATC, which may become radio-iodine-resistant. Thus, it is so important to understand the multistep molecular background of thyroid carcinogenesis process to make – in these cases – targeted selective therapy feasible. The current knowledge of

alterations in PI3K pathway opens the possibility for development of the specific targeted therapy.

In future, strong scientific evidence for the role of PI3K pathway in progression of well-differentiated thyroid carcinomas to PDTC or ATC, could be crucial for proper medical management.

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