Expression of transcription and growth factors and the AKT/m-TOR signaling pathway components in papillary thyroid cancer

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Background: The molecular mechanism of thyroid cancer development is associated with changes in expression of transcription factors and growth factors accompanied by modified level of the AKT/m-TOR components.

Aims. The aim of study was to determine NF-κB p65, NF-κB p50, HIF-1α, HIF-2α, VEGF, CAIX, VEGFR2 expression and mRNA level of the AKT/m-TOR signaling pathway components in papillary thyroid cancer compared to those in benign lesions.

Material and methods: Forty patients aged 33—66 years with T1-4N0-2M0 papillary thyroid cancer (7 males and 33 females) were enrolled in the study. The mean age was 52.0±2.6 years. The comparison group included patients with benign lesions of thyroid tissue (4 males and 18 females) aged 38—66 years (mean age, 53.0±4.4 years). Expression levels of NF-κB p65, NF-κB p50, HIF-1α, HIF-2α, VEGF, CAIX, VEGFR2, and the AKT/m-TOR signaling pathway components were determined by RT-PCR using specific primers.

Results: Increased expression of transcription factors NF-κB and HIF-2α was found in papillary thyroid cancer. The levels of AKT and PTEN mRNA were elevated in transformed tissues. c-Raf expression was reduced 2.1-fold in cancer compared to that in thyroid tissues with benign lesions. Multiple positive correlations were revealed between transcription and growth factors and the AKT/m-TOR signaling pathway components in cancer. An association between PTEN expression and the NF-κB mRNA level was revealed, being a sign of deregulation in the signaling cascade in cancer tissues.

Conclusions: Overexpression of NF-κB, HIF-2α, AKT, PTEN and reduction of c-Raf expression is typical of thyroid papillary cancer.

Keywords: papillary thyroid cancer, transcription factors, growth factors, AKT/m-TOR signaling pathway components.
VEGF growth factor and carbonic anhydrase IX, which define neoangiogenesis. These events activate the AKT/m-TOR signaling cascade and underlie the growth and spread of the tumor [5].

Hyperactivation of the AKT/m-TOR signaling pathway is a characteristic feature of most cancer cells and, apparently, plays the key role in the mechanisms of tumor cell transformation and tumor progression [6]. Important components of this pathway include AKT, c-Raf, GSK-3, PDK1, as well as m-TOR, and its substrates p70-S64 and E-BP1. The activity of this signaling cascade is regulated by PTEN tumor suppressor protein.

The AKT/m-TOR signaling pathway has been studied less in tumors of endocrine organs than in tumors of other localization [7]. There are multiple relationships between levels of molecular markers, which reflects the intensity of pathological processes and can affect the prognosis of the disease. However, the contribution of the molecular markers associated with the activation of transcription and growth factors and the AKT/m-TOR signaling pathway components that determine the characteristics of papillary thyroid cancer (PTC) has not been studied.

Aim — the aim of the study was to compare the expression of transcription factors NF-κB p65 and p50, HIF-1α, HIF-2α, growth factors VEGF, CAIX and VEGFR2, as well as components of the AKT/m-TOR signaling pathway in PTC and in benign tumors of the thyroid gland.

Methods

Study Design
Observational one-stage continuous controlled study.

Inclusion Criteria
The study included patients at T1-4N0-2M0 stage of verified PTC aged 30 to 70 years, who voluntarily signed the informed consent. The control group included patients with benign thyroid gland lesions of comparable age under the same condition. Exclusion criteria were age over 70 years, disseminated thyroid cancer, presence of severe concomitant pathology, presence of primary-multiple tumors of other localizations, the patient’s refusal to participate in the protocol.

Conditions of the study
The study was conducted in the Scientific Research Institute of Oncology of the Tomsk National Research Medical Center of the Russian Academy of Sciences.

Description of medical intervention
The scope of diagnostics and treatment of the patients followed the recommended algorithms for the diagnosis and treatment of malignant neoplasms, approved by the Ministry of Health of the Russian Federation (2007), and clinical guidelines for the diagnosis and treatment of thyroid cancer (2014) [8, 9].

The patients with the thyroid gland pathology underwent surgical treatment in the form of hemithyroidectomy or thyroidectomy. At the second stage of treatment, the patients received radioactive iodine therapy in case of metastases in lymph nodes. The study examined tumor and histologically unchanged tissue from the thyroid gland, obtained from the patients of both groups after the surgery. Tissues specimen were frozen and stored at -80 °C.

The primary endpoint of the study
The expression of NF-κB p65 and p50, HIF-1α, HIF-2α, growth factors VEGF, CAIX and VEGFR2, as well as components of AKT/m-TOR signaling pathway was determined in PTC tissue and benign thyroid gland tumors.

Subgroup analysis
Two groups were identified over the course of the study:
— Group A included patients with PTC (T1-4N0-2M0 stage).
— Group B included patients with benign neoplasms of the thyroid gland.

Methods of recording endpoints
RNA was isolated using RNeasy mini Kit, containing DNase I (Qiagen, Germany). NanoDrop-2000 (Thermo Scientific, USA) spectrophotometer was used to assess concentration and purity of RNA isolation. The concentration of RNA ranged from 80 to 250 ng/μl, A260/A280 = 1.95—2.05; A260/A230 = 1.90—2.31. RNA integrity was assessed using capillary electrophoresis with TapeStation (Agilent Technologies, USA) and R6K ScreenTape kit (Agilent Technologies, USA). RIN was 5.6—7.8.

The level of gene expression was assessed by quantitative reverse transcriptase PCR in real time (RT-qPCR) using SYBR Green dye on iCycler amplifier (Bio-Rad, USA). To obtain the cDNA on the RNA template, a reverse transcription reaction was performed using the m-MulV-RH kit (BioLabmix, Russia) with random hexanucleotide primers according to the instructions. PCR was performed in three replicates in a volume of 25 μl containing 12.5 μl of HS-qPCR SYBR Blue Biomaster (BioLabmix, Russia), 300 nM of forward and reverse primers and 50 ng of cDNA. The two-step amplification program included 1 cycle —94 °C, 10 min — preliminary denaturation; 40 cycles — 1 step at 94 °C, 10 sec and 2 step 20 sec — at 60 °C. The primers were selected using Vector NTI Advance 11.5 and NCBI database (http: //www.ncbi.nlm.nih.gov/nucleccore) (Table 1).

The housekeeping gene of GAPDH enzyme (glyceraldehyde-3-phosphate dehydrogenase) was used a reference and the level of expression of each target gene was normalized relative to GAPDH expression. Quantitative analysis of the expression was performed with 2ΔΔCt relative to the constitutively expressed GAPDH gene.
**Table 1. Sequence of primers for the investigated genes probes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Amplicon</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAIX</td>
<td>217 bp</td>
<td>F 5'-GTTGCTGTCTGGTCTGGAA-3', R 5'-CAGGAGTCTGCTAGAGG-3'</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>188 bp</td>
<td>F 5'-CAAGAACCTACTGCTAATGCA-3', R 5'-TTTGTTGAGCTCTGGAG-3'</td>
</tr>
<tr>
<td>EPAS1</td>
<td>265 bp</td>
<td>F 5'-TTTGTTGAGCCTGTCTGGAG-3', R 5'-GGGATTGTCAGAGAGGTGT-3'</td>
</tr>
<tr>
<td>RELA</td>
<td>217 bp</td>
<td>F 5'-GGGATTGTCAGAGAGGTGT-3', R 5'-GGGATTTGGTCAGAGAGGTGT-3'</td>
</tr>
<tr>
<td>PTEN</td>
<td>188 bp</td>
<td>F 5'-GGGATTTGGTCAGAGAGGTGT-3', R 5'-GGGATTTGGTCAGAGAGGTGT-3'</td>
</tr>
<tr>
<td>VEGFα</td>
<td>244 bp</td>
<td>F 5'-CCAAGACCTACTGCTAATGCA-3', R 5'-TTTGTTGAGCTCTGGAG-3'</td>
</tr>
<tr>
<td>KDR</td>
<td>217 bp</td>
<td>F 5'-TTTGTTGAGCTCTGGAG-3', R 5'-GGGATTGTCAGAGAGGTGT-3'</td>
</tr>
<tr>
<td>C-RAF</td>
<td>188 bp</td>
<td>F 5'-CCAAGACCTACTGCTAATGCA-3', R 5'-TTTGTTGAGCTCTGGAG-3'</td>
</tr>
<tr>
<td>GSK3β</td>
<td>267 bp</td>
<td>F 5'-CCAAGACCTACTGCTAATGCA-3', R 5'-TTTGTTGAGCTCTGGAG-3'</td>
</tr>
<tr>
<td>70S kinase alpha</td>
<td>244 bp</td>
<td>F 5'-CCAAGACCTACTGCTAATGCA-3', R 5'-TTTGTTGAGCTCTGGAG-3'</td>
</tr>
<tr>
<td>m-TOR</td>
<td>160 bp</td>
<td>F 5'-CCAAGACCTACTGCTAATGCA-3', R 5'-TTTGTTGAGCTCTGGAG-3'</td>
</tr>
<tr>
<td>PDK1</td>
<td>187 bp</td>
<td>F 5'-CCAAGACCTACTGCTAATGCA-3', R 5'-TTTGTTGAGCTCTGGAG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>138 bp</td>
<td>F 5'-CCAAGACCTACTGCTAATGCA-3', R 5'-TTTGTTGAGCTCTGGAG-3'</td>
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</tbody>
</table>

**Note:** NM — number of RNA sequence in NCBI Nucleotide Database (http://www.ncbi.nlm.nih.gov/nuccore); F — forward primer; R — reverse primer.

**Ethical expertise**

This study was approved by the local ethics committee of the Tomsk Scientific Research Institute of Oncology (Minutes No. 5 of April 24, 2015).

**Statistical analysis**

The sample size was not calculated in advance. The statistical processing of the results was carried out using Statistica 8.0 software package. The results of determining the expression of genes are presented as mean ± mean error. The significance of differences was estimated using the Mann—Whitney test. Differences were considered significant at p < 0.05. The existence of correlation between the markers was determined using correlation analysis; the strength of the relationship between the variables was estimated by calculating the Spearman rank correlation coefficient (r).

**Results**

**Study participants**

Group A included 40 patients with PTC (7 men, 33 women) aged 33 to 66 years (mean age 52.0±2.6 years) at stage T1-4N0-2M0 of the tumor process. Group B included 22 patients with benign thyroid neoplasms (4 men, 18 women) aged 38 to 66 years (mean age 53.0±4.4 years). The diagnosis was morphologically verified in all patients.

**Main outcomes of the study**

Table 2 shows the mRNA levels of the studied markers in the tissues of benign neoplasms and PTC. The 8.7-fold (p=0.041) and 5.6-fold (p=0.036) increase in the expression of transcription factors NF-κB p65 and p50, respectively, as well as 5.3-fold (p=0.042) increase in the expression of nuclear factor HIF-2α was identified in PTC tissue in comparison with the tissue from benign neoplasms.

The expression of VEGF growth factor, its receptor VEGFR2 and CAIX in PTC tissue did not differ from that in the tissue of benign tumors of the gland.

At the next stage of the study we examined the expression of the components of AKT/m-TOR signaling pathway (AKT, c-Raf, GSK-3β, PDK1 and PTEN) in the tissue of thyroid gland neoplasms (Table 2). The 8.6-fold (p=0.041) increase in the level of mRNA of AKT protein kinase accompanied by the 8.1-fold (p=0.037) compen-
Table 2. Expression of transcription and growth factors in the tissue of follicular adenoma and papillary thyroid cancer (X±SD)

<table>
<thead>
<tr>
<th>Factor. rel. units</th>
<th>Benign neoplasm of thyroid gland (n=22)</th>
<th>PTC (n=40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB p65</td>
<td>0.17±0.09</td>
<td>1.48±0.38*</td>
</tr>
<tr>
<td>NF-κB p50</td>
<td>0.79±0.63</td>
<td>4.44±1.89*</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>0.40±0.16</td>
<td>2.94±1.30</td>
</tr>
<tr>
<td>HIF-2α</td>
<td>0.44±0.25</td>
<td>2.33±0.63*</td>
</tr>
<tr>
<td>VEGF</td>
<td>0.63±0.26</td>
<td>3.70±1.71</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>3.67±2.37</td>
<td>2.37±0.97</td>
</tr>
<tr>
<td>CAIX</td>
<td>5.15±4.49</td>
<td>2.30±0.95</td>
</tr>
</tbody>
</table>

Note: * — p <0.05 when compared with benign neoplasms.

Table 3. Expression of AKT. c-Raf. GSK-3β. PDK1. PTEN. m-TOR and its substrates in follicular adenoma tissue and papillary thyroid cancer (X±SD)

<table>
<thead>
<tr>
<th>Factor. rel. units</th>
<th>Benign neoplasm of thyroid gland (n=22)</th>
<th>PTC (n=40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT</td>
<td>0.38±0.18</td>
<td>3.28±2.12*</td>
</tr>
<tr>
<td>c-Raf</td>
<td>3.13±2.16</td>
<td>1.53±0.93*</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>2.95±2.19</td>
<td>2.87±1.98</td>
</tr>
<tr>
<td>PDK1</td>
<td>0.88±0.28</td>
<td>1.85±1.29</td>
</tr>
<tr>
<td>PTEN</td>
<td>0.46±0.23</td>
<td>3.73±1.89*</td>
</tr>
<tr>
<td>m-TOR</td>
<td>5.23±4.47</td>
<td>2.02±1.29</td>
</tr>
<tr>
<td>p70-S6</td>
<td>5.02±4.50</td>
<td>0.88±0.35</td>
</tr>
<tr>
<td>4E-BP1</td>
<td>0.65±0.29</td>
<td>1.62±1.12</td>
</tr>
</tbody>
</table>

Note: * — p<0.05 when compared with benign neoplasms.

Saturatory increase in the level of PTEN mRNA was observed in PTC tissue. In contrast, the expression of c-Raf gene in PTC tissue was 2.1 times lower (p=0.048) than in benign tumors. The expression of m-TOR protein kinase, its substrates p70-S6 kinase and 4E-BP1 did not differ from that in benign tumors (Table 3).

Correlation analysis revealed numerous associations between the studied molecular markers in PTC tissue (Fig. 1). Positive relationships was identified between the expression of NF-κB p65, VEGFR2 (r=0.7; p<0.05), CAIX (r=0.8; p<0.05), HIF-1α (r=0.6; p<0.05) and HIF-2α (r=0.6; p<0.05); between VEGFR2 and VEGF (r=0.5; p<0.05), CAIX (r=0.6; p<0.05) and HIF-1α (r=0.7; p<0.05); there was also positive relationships between VEGF, CAIX (r=0.6; p<0.05) and HIF-2α (r=0.7; p<0.05) and direct relationships between the expression of HIF-1α, CAIX (r=0.7; p<0.05) and HIF-2α (r=0.6; p<0.05). These associations between transcription and growth factors are the basis of molecular mechanisms of tumor development.

There were direct relationships between the expression of PTEN, c-Raf (r=0.6; p<0.05) and p70-S6 (r=0.8; p<0.05), as well as between m-TOR and PDK1 (r=0.7; p<0.05) in PTC tissue, which indicates the existence of a regulatory system between kinases and their inhibitors.

Fig. 1. Relationships between transcription and growth factors in papillary thyroid cancer.

The lines denote direct relationships between molecular markers.

We also identified associations between expression of PTEN tumor suppressor and transcription factors NF-κB p65 (r=0.76; p<0.05) and NF-κB p50 (r=0.72; p<0.05) (Fig. 2, 3), which describes the features of the signaling cascade functioning. The expression of NF-κB p65 was also correlated with the expression of PDK1 (r=0.71; p<0.05), and that of NF-κB p50 with c-Raf (r=0.76; p<0.05).
Discussion

Summary of the main outcome of the study

Expression of the transcription factor HIF-1 in PTC tissue is linked to the disease prognosis and defines the outcome of the pathological process [10, 11]. In our study, we have demonstrated that molecular and biological features of this tumor also include an increase in expression of HIF-2, NF-κB p65 and NF-κB p50. These changes result in modified expression of the components of the AKT/m-TOR signaling cascade.

Discussion of the main outcomes of the study

Increased expression of AKT protein kinase, one of the key components of the AKT/m-TOR signaling cascade, has been observed in PTC tissue. This can be attributed to predominance of anabolic processes in tumor cells. There is evidence that the thyroid cancer tissue has increased content of AKT [12, 13]. In other words, the increase in expression of AKT gene is accompanied by the increase in the content of its protein product.

PTEN phosphatase downregulates the AKT/m-TOR signaling pathway. We have observed an increase in the level of PTEN mRNA in PTC. It is believed that this pathology develops and progresses in the presence of mutation-al changes in PTEN gene, which is one of the causes of development of cancer syndromes (Cowden Syndrome) and is associated with the production of a functionally defective protein. In a study by S. Beg et al. a reduction in PTEN content was observed in 24.5% of PTC samples, however, fluorescent hybridization in situ (FISH) detected PTEN gene defect only in 4.8% of the samples [14].

We observed a decrease in the level of c-Raf mRNA in PTC tissue, which may be due to the inhibitory effect of AKT kinase. In the literature, there are other reports on the expression of c-Raf protein kinase in PTC tissue [15]. It is worth noting this work did not examine the relationship between c-Raf expression and AKT/m-TOR signaling cascade activity.

The nuclear factor NF-κB is known to affect the expression of grown factor VEGF both directly and via regulation of the transcription of HIF-1α nuclear factor [16, 17]. The link between the expression of HIF-1α and HIF-2α, CAIX, VEGFR2 and between the expression of HIF-2α and HIF-1α, VEGF can also be attributed to HIF-1α and HIF-2α ability to enhance transcription of the genes. Similar information is available in recent studies [18, 19]. A joint regulation of the expression of VEGF, its receptor and CAIX has also been demonstrated, which is an evidence of effective regulation of angiogenesis.

The link between the expression of m-TOR and PDK1 has been identified in PTC tissue, which indicates the role of PDK1 in the activation of AKT protein kinase, which has m-TOR as a substrate [6]. The relationships between expression of PTEN phosphatase and p70-S6 kinase is probably due to the regulation of the AKT/m-TOR signaling pathway activity by PTEN tumor suppressor [20]. The direct relationship between c-Raf mRNA levels and PTEN expression shows that this phosphatase can also affect the activity of MAPK signaling cascade, which is in-
volved in the regulation of cell proliferation, differentiation and apoptosis [10, 11].

The positive relationship identified between the expression of nuclear factors NF-κB p65 and NF-κB p50 and PTEN is of particular importance. A study by K.M. Vasudevan showed that activation of the transcriptional activity of this nuclear factor occurs under the conditions of PTEN suppression [21] and pronounced activity of the kinases of the signaling cascade under study. They include c-Raf and PDK1. The loss of functional activity of PTEN tumor suppressor results in increased expression of NF-κB due to activation of the AKT/m-TOR signaling cascade [22]. High levels of PTEN phosphatase mRNA is an indirect evidence of the altered activity of this tumor marker, which leads to even higher AKT activity.

**Conclusion**

This study has established important molecular and biological characteristics of PTC. They include high level of expression of transcription factors NF-κB and HIF-2α, AKT protein kinase and PTEN phosphatase, as well as low level of c-Raf mRNA. The peculiarities of expression of transcription and growth factors, as well as the AKT/m-TOR signaling pathway components, can influence the course of the disease, defining the effectiveness of the treatment. The findings are important for both fundamental and clinical oncology.

**Supplementary Information**

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**Conflict of interests.** The authors declare the absence of obvious and potential conflicts of interest related to the publication of this article.

**Contribution of authors:** L.V. Spirina - elucidation of the expression of the studied markers, preparation of the article for publication; S.Yu. Chizhevskaya - formation of the study groups; I.V. Kondakova - coordination of research, general management.


