ROLE OF MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) IN PROCESSES OF PROLIFERATION IN HUMAN THYROID TUMORS

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Effector kinase of the MAPK (mitogen-activated protein kinase)-pathway – extracellular signal-regulated kinase-1/2 (ERK1/2) activation and expression and proliferating cell nuclear antigen (PCNA) content in normal tissues, benign and well-differentiated malignant (metastatic and not metastatic) human thyroid tumors were studied.

In malignant tumors and tissue of follicular adenoma's increased PCNA expression was observed, and the amount of antigen in tumor tissue, except for follicular carcinoma, exceeded the amount in the conditionally normal tissue. Importantly, in encapsulated papillary carcinomas this excess was 85%, while in non–encapsulated, metastatic tumors PCNA content was on average more than 3 times above normal, and in the cases of most aggressive tumors with metastases in lungs – even 4 times.

PCNA amount in thyroid tissue could serve as one of diagnostic and prognostic markers, and development of effective antigen inhibitors may be a promising trend in thyroid cancer treatment.

Total ERK content was significantly lower in tumors compared to normal tissue, except goiter. Phosphorylation (activation) of the ERK was almost completely suppressed in tumors but not in normal tissue. Evidently, ERK activation is not associated with proliferative processes in thyroid tumor tissue.

The possible mechanisms of MAPK cascade inhibition in thyroid tumors are discussed.

Key words: thyroid tumors, extracellular signal – regulated kinase-1/2, proliferating cell nuclear antigen.

Introduction

The proliferation potential of tumor cells is one of the main factors of tumor progression. Its quantitative evaluation is extremely important for diagnostic and prognostic criteria of tumor development. For the diagnosis of thyroid cancer (TC) it is necessary to develop objective methods to obtain organ-specific indicators of proliferation status based on gene expression data.

Proliferating cell nuclear antigen (PCNA) is a highly conserved protein essential for the proper assembly of the components involved in the processes of DNA repair and replication. Proteins are combined within interdomain connecting loop of PCNA, and many of the regulatory impacts result from competing in this docking site. In case of modification of this site, for example, in cancer cells, DNA replication and repair processes can be changed. In this case, the possibility of target therapy arises for some types of cancer [1].

The prognosis for thyroid cancer varies considerably depending on cases with presence and absence of metastases. To determine biomarkers useful for the diagnosis of thyroid cancer and to compile of marker

panels for early detection of metastatic thyroid carcinoma, a series of studies were carried out which, in particular, have shown that PCNA level in metastatic tumors was almost 2 times higher than in tumors without metastases [2, 3].

The Ret/Ras/Raf/MEK/ERK cascade couples growth signals from cell surface receptors to transcription factors, which regulate expression of genes controlling important cellular processes, such as proliferation, angiogenesis, cell growth, survival and apoptosis [4]. This pathway is often activated in certain tumors by chromosomal translocations RET-PTC, mutations in BRAF (BRAFV600E), RAS, some cytokine receptors or overexpression of wild type and mutated receptors such as EGFR [5, 6]. At the core of the molecular pathogenesis of thyroid cancer also underlies the uncontrolled activity of various signaling pathways, and in the first place MAPK cascade [7]. Suppressing of this pathway with specific inhibitors enhanced cancer cells sensitivity (and thyroid cancer cells as well) to cancerostatic drugs [8, 9]. On the other hand, dysregulated MAPK cascade can trigger innate tumor–suppressive mechanisms [10-14].

Thus, a study of the MAPK expression and activation as well as PCNA expression and development of methods of blocking the antigen are of current interest.

The aim of this paper was to compare the expression and activation of ERK1/2 and PCNA expression in normal, benign and well-differentiated malignant (metastatic and non-metastatic) human thyroid tumors.

Materials and methods

Studies were performed on postoperative material of patients, obtained in the Department of Surgery of the Institute. The study protocol was approved by the Ethics Committee of the Institute of Endocrinology, and all patients gave a written informed consent on further use of postoperative material for diagnosis and research.

After removal, thyroid tissue and tumor were placed on ice and then frozen at -80 °C. The tissue was homogenized in a homogenizer TissueLyser II from Retsch (Germany) in special buffers from ELISA kit QIA59 for PCNA determination (Calbiochem USA) or ab176660 for determination of total ERK1/2 and phosphorylated ERK1/2 (Thr202/Tyr204) (Abcam, UK), containing a mixture of protease and phosphatase inhibitors, to save intactness and activity of proteins. The study was conducted in triplets. The protein concentration in the lysate was determined using BCA protein assay kit (Novagen, USA). Bio-tek Instruments (USA) microplate reader was used for measurements of PCNA and ERK1/2 content/activity at 450 nm.

The data obtained were processed statistically using Student t-test and presented as M±SD. Differences were considered statistically significant at $p<0.05$.

Results and discussion

Fig. 1 shows that the obtained calibration curve for PCNA, total ERK1/2 and phosphorylated ERK1/2 (Thr202/Tyr204) determination almost perfectly coincides with the theoretical exponent and lines (X), which indicates a lack of data scattering.

Determination of the PCNA content in various types of thyroid tumors revealed that high level of PCNA expression was observed in follicular carcinoma tissue but the difference between conventionally normal and tumor tissue was absent (Table 1). In follicular adenomas, in contrast to follicular carcinomas, the amount of antigen in tumor tissue exceeded its content in normal tissue almost 2.5-fold. The level of PCNA expression in tumor tissue of papillary carcinomas was higher than in normal tissue (Table 1). It should be noted that in the encapsulated tumors that excess was only 85% whereas in non-encapsulated, metastatic tumors PCNA content was on average more than 3 times above the normal tissue (Table 1), and in some tumors with metastases in the lungs – even 4 times (6.215 U/mg protein in tumors versus 1.539 U/mg protein in conventionally normal tissue).

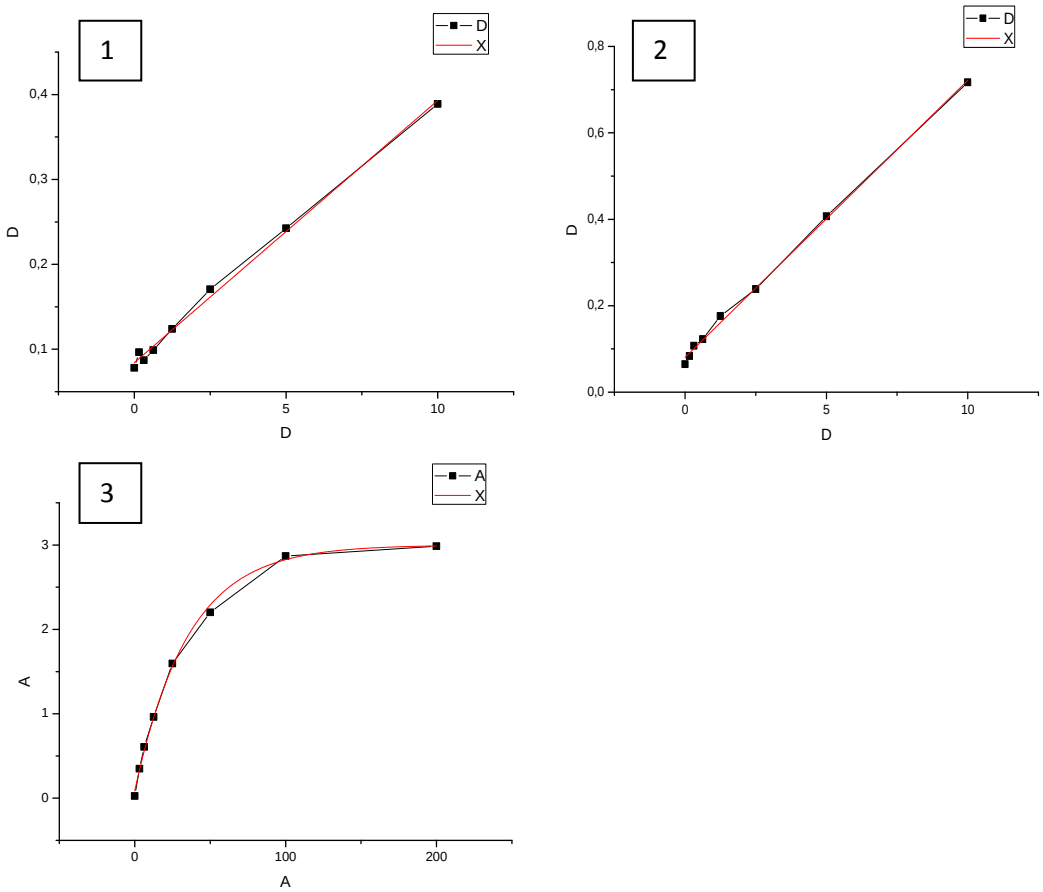

Fig. 1. Calibration curves for determining of the ERK1/2 expression (1) and activated ERK1/2 (2), PCNA amount (3) in thyroid homogenates using the ELISA kit QIA59 for PCNA determination and ab176660 for total ERK1/2 and phosphorylated ERK1/2 (Thr202/Tyr204) determination.

Abscissa – the PCNA, total ERK1/2 and phosphorylated ERK1/2 amount in U/ml, ordinate – optical density at 450 nm. A – the calibration curve, X – theoretical line (curve).

Table 1. PCNA expression in different types of thyroid tumors

Tumor type	Tumor tissue		Normal tissue	
	M	SD	M	SD
FTC	3,96479	0,01468	4,07276	0,03957
FA	3,29295	0,39046*	1,37763	0,04152
IPTC	2,67012	1,0725*	1,44275	0,02479
NPTC	4,98787	1,14335*+	1,5387	0,62638
MNG	1,13984	0,20179	1,42951	0,1903

Notes: FTC – follicular carcinoma, FA – follicular adenoma, IPTC – papillary carcinoma (encapsulated tumors), NPTC – papillary carcinoma (non-encapsulated, metastatic tumors), MNG – multinodular goiter; M±SD, n=3-6; * – differences between conventionally normal and tumor tissues significant, $p<0.05$. + – differences between encapsulated and non-encapsulated tumors significant, $p<0.05$.

In multinodular goiter tissue PCNA expression level was low and no difference between tumor and conventionally normal tissues was detected (Table 1).

Other authors also observed PCNA overexpression in thyroid carcinomas compared with adenomas [3]. The highest level of PCNA expression was observed in the most aggressive types of thyroid cancer – anaplastic and medullary carcinomas. In differentiated tumors antigen content was somewhat lower, but significantly increased in invasive variants of these tumors [15].

Now studies are in progress to develop inhibitors of PCNA, which could have a therapeutic effect. Thus, data on inhibition of PCNA with triiodothyronine (T3) became the basis of the synthesis of a small, non-protein and, importantly, non-hormonal inhibitor – T2-amino alcohol derivative of T3. Inhibitor binds to PIP-box (PCNA-interacting protein box) of antigen, preventing the latter to interact with DNA and DNA polymerase [16].

Hence, the content of PCNA in tumor tissues could serve as a diagnostic and prognostic marker for assessing the tumor aggressiveness and the development of effective inhibitors of antigen may be a promising direction in the treatment of thyroid cancer.

Ret/Ras/Raf/MEK/ERK cascade transmits mostly mitogenic signals, and is considered as the main pathway controlling cell division [17]. Therefore, it was of interest to find out how PCNA content correlates with the expression and activity of protein kinases of this signaling pathway.

For the study of ERK we used a kit ab176660, which allows determining both the activation and the total content of protein kinase in the same tissue sample. Table 2 shows that in all tumor types of thyroid other than goiter, ERK1/2 content in tumor tissue was lower than in normal. The most significant difference

between normal and tumor tissues was observed in IPTC and, especially, FTC. The level of ERK expression in normal tissue of multinodular goiter was lower than in other tissues and no difference between normal and modular goiter tissue was observed. Even more surprising was ERK activation in these tissues. The levels of activated kinase in tumor tissues was near zero and significantly lower than in normal tissue (Table 3). As in the case of the expression, the most significant difference between normal and tumor tissues was observed in FTC and IPTC.

Table 2. Total ERK1/2 content in different types of thyroid tumors

Tumor type	Tumor		Normal	
	M	SD	M	SD
FTC	0,390	0,08*	11,952	0,098
FA	1,456	0,167*	2,723	0,177
MNG	0,720	0,109	0,834	0,077
IPTC	0,466	0,084*	6,506	0,206
NPTC	3,226	0,272*	6,161	0,136

Notes: FTC – follicuar carcinoma, FA – follicular adenoma, IPTC – papillary carcinoma (encapsulated tumors), NPTC – papillary carcinoma (nonencapsulated tumors), MNG – multinodular goiter; M±SD, n=3-6; Differences between conventionally normal and tumor tissues except MNG are significant, $p<0.05$.

Table 3. Phosphorylated (activated) ERK1/2 content in different types of thyroid tumors

Tumor type	Tumor		Normal	
	M	SD	M	SD
FTC	0,000	0	0,673	0,069
FA	0,049	0,041	0,207	0,010
MNG	0,010	0,009	0,135	0,017
IPTC	0,000	0	0,784	0,046
NPTC	0,045	0,016	0,131	0

Notes: the phosphorylation of Thr202/Tyr204 were determined. FTC – follicuar carcinoma, FA – follicular adenoma, IPTC – papillary carcinoma (encapsulated tumors), NPTC – papillary carcinoma (nonencapsulated tumors), MNG – multinodular goiter: M±SD, n=3-6; differences between conventionally normal and tumor tissues except MNG are significant, $p<0.05$.

Thus, PCNA expression obviously does not correlate with ERK1/2 phosphorylation (activation) and expression. Moreover, a contradiction arises between the proliferative functions of ERK and its low activation level in thyroid tumors. Perhaps the most plausible explanation for this discrepancy was obtained by Park and coauthors. It was found that, although the Ras and Raf oncogenes are often involved in cell transformation, in many cases constitutive activation of this cascade in tumor tissues leads to growth arrest or senescence [10, 11]. Thus, in human medullary thyroid cancer cells, activated Ras or c-Raf-1 can induce growth arrest by producing and secreting an autocrine–paracrine factor (leukemia inhibitory factor) [10]. Sustained activation of the Raf/MEK/ERK pathway induces growth arrest, accompanied by appropriate changes in cell cycle regulators (decreased pRb phosphorylation, E2F1 down-regulation, and p21CIP1 up-regulation), cell type-specific changes in morphology and expression of c-Myc or RET in the human tumor lines LNCaP, U251, and TT (thyroid medullary carcinoma) [13].

It is possible that cancer cells induce special defensive mechanisms like upregulation of heat shock protein mortalin [14], which inhibit both the ERK expression and activation and thus protect the cell from senescence, growth arrest and apoptosis.

Aside from the fact of decreased expression and almost complete inactivation of the ERK in the tumor tissue, these data suggest to researchers a very important question: If the main mitogenic cascade is not working in the cell – what is the mechanism that provides an intensive division of cancer cells? The answer to this question could explain why even specific inhibitors of MAPK-cascade are often not effective in the treatment of cancer.

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