

The Expression and Activation of Extracellular Signal-Regulated Kinase-1/2 and Proliferating Cell Nuclear Antigen Content in Normal Tissue and Human Thyroid Tumors

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Key words Thyroid tumors; ERK1/2; PCNA

Abbreviations ERK1/2: Extracellular signal-Regulated Kinase-1/2; PCNA: Proliferating Cell Nuclear Antigen; RET: Rearranged during Transfection; PTC: Papillary Thyroid Cancer; MAPK: Mitogen-Activated Protein Kinase; EGFR: Epidermal Growth Factor Receptor; Ras: Rat Sarcoma viral oncogene homolog; MEK: Mitogen-Activated Protein Kinase; BRAF: Rapidly Accelerated Fibrosarcoma B-type (murine sarcoma viral oncogene homolog B1)

Abstract

Extracellular signal-regulated kinase-1/2 (ERK1/2) activation and expression and proliferating cell nuclear antigen (PCNA) content in normal tissues, benign and malignant (metastatic and not metastatic) human thyroid tumors were studied.

In malignant tumors and follicular adenoma tissue 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 nonencapsulated, 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.

Total ERK content was significantly lower in tumors compared to normal tissue, except goiter. Activation of the ERK was almost completely suppressed in tumors but not in normal tissue.

Thus, ERK activation is not associated with proliferative processes in thyroid tumor tissue.

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.

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 [4,5]. This pathway is often activated in certain tumors by chromosomal translocations *RET-PTC*, mutations in rapidly accelerated fibrosarcoma B-type (*BRAF^{V600E}*), rat sarcoma viral oncogene homolog (*RAS*), some cytokine receptors or overexpression of wild type and mutated receptors such as epidermal growth factor receptor (*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 mitogen-activated protein kinase (*MAPK*) cascade [7]. Suppressing of this pathway with specific inhibitors

enhanced cancer cells sensitivity (and thyroid cancer cells as well) to cancerostatic drugs [8-12]. On the other hand, dysregulated MAPK cascade can trigger innate tumor-suppressive mechanisms [13-17].

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 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 48 patients gave a written informed consent on further use of postoperative material for diagnosis and research.

The thyroid tissues of 28 patients with thyroid tumors without concomitant diseases were selected for investigation. There were young women, which were 1-8-year children at the moment of Chernobyl disaster or were born during next 10 years). Age of the patients ranged from 21 to 36 (average – 28.6 years).

3 cases of Follicular Carcinoma (FTC), 3 cases of Follicular Adenoma (FA), 6 cases of papillary carcinoma (encapsulated tumors - IPTC), 6 cases of papillary carcinoma (nonencapsulated, metastatic tumors - NPTC), 3 cases of Multinodular Goiter (MNG) have been selected for the analysis. The same tissue was used both for ERK1/2 and PCNA determination.

As a control, the unchanged areas of thyroid tissue (conditionally normal) for each case were used after the examination of tissue by pathologist.

After removal, thyroid tissue was placed on ice and then frozen at -80 °C. The tissue was homogenized in a homogenizer TissueLyser – perhaps homogenizer II from Retsch (Germany) in special buffers from Enzyme-Linked Immunosorbent Assay (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 Bicinchoninic Acid (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 pair *t*-test and presented as $M \pm SD$. Differences were considered statistically significant at $P < 0.05$.

Results and Discussion

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 practically absent (Figure 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

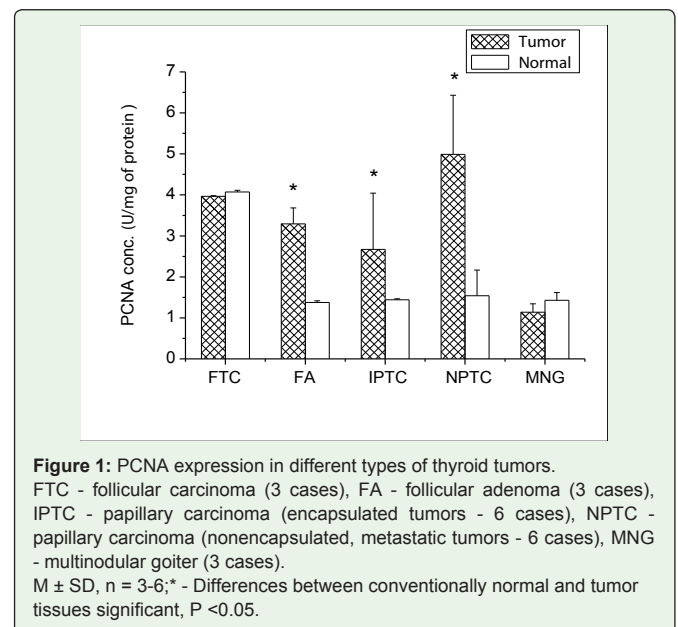
was higher than in normal tissue (Figure 1). It should be noted that in the encapsulated tumors that excess was only 85% whereas in non-encapsulated, metastatic tumors PCNA content was on average more than 3 times above the normal tissue (Figure 1), and in some tumors with metastases in the lungs - even 4 times (6.215 U/mg protein in tumors versus 1.539 U/mg protein in conventionally normal tissue). The PCNA content ratio: tumor/normal was significantly higher in NPTC compared to IPTC – 3.24 vs. 1.85 ($P < 0.05$).

In multinodular goiter tissue PCNA expression level was low and no difference between tumor and conventionally normal tissues was detected (Figure 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 [18].

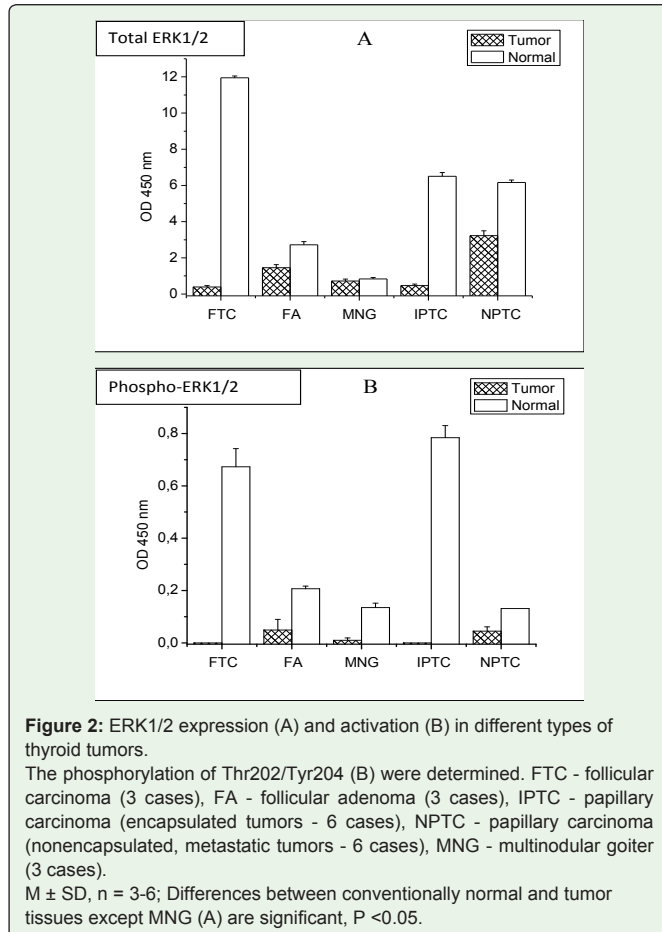
Now studies are in progress to develop inhibitors of PCNA, which could have a therapeutic effect. Thus, data on inhibition of PCNA with triiodothyronine (T3) became the basis of the synthesis of a small, non-protein and, importantly, non-hormonal inhibitor - T2-amino alcohol derivative of T3. Inhibitor binds to PIP-box (PCNA-interacting protein box) of antigen, preventing the latter to interact with DNA and DNA polymerase [19].

Hence, the tumor/normal PCNA content ratio for papillary carcinoma probably could serve as a diagnostic and prognostic marker for assessing the tumor aggressiveness. Perhaps there are certain limitations (likely even - mechanical) for the growth of the encapsulated tumor and division of its cells.



Ret/Ras/Raf/MEK/ERK cascade transmits mostly mitogenic signals, and is considered as the main pathway controlling cell division [20]. 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. Figure 2A shows that in all tumor types of thyroid except goiter, ERK1/2 content in tumor tissue was lower than in normal. The level of ERK expression in multinodular goiter was lower than in other tissues and no difference between normal and goiter tissue was observed. Even more surprising was ERK activation in these tissues. The kinase activity levels in tumor tissues was near zero and significantly lower than in normal tissue (Figure 2B).



Thus, PCNA expression obviously does not correlate with activity and expression of ERK1/2. 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 [13,14]. 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) [13]. 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 p21^{CIP1} up-regulation) and 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) [16].

It is possible that cancer cells induce special defensive mechanisms like upregulation of heat shock protein mortalin [17], which inhibit both the ERK expression and activation and thus protect the cell from senescence, growth arrest and apoptosis.

Conclusion

1. The PCNA content ratio: tumor/normal was significantly higher in nonencapsulated, metastatic tumors compared to encapsulated tumors and probably could serve as prognostic marker for assessing the PTC tumor aggressiveness.
2. The level of ERK1/2 content and activation in thyroid tumor tissue was significantly lower than in normal.
3. PCNA expression in thyroid tumors does not correlate with activity and expression of ERK1/2.

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