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Changes in expression of human serine protease *HtrA1*, *HtrA2* and *HtrA3* genes in benign and malignant thyroid tumors

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Abstract. Human HtrA proteins are serine proteases involved in essential physiological processes. HtrA1 and HtrA3 function as tumor suppressors and inhibitors of the TGF-β signaling pathway. HtrA2 regulates mitochondrial homeostasis and plays a pivotal role in the induction of apoptosis. The aim of the study was to determine whether the HtrA proteins are involved in thyroid carcinogenesis. We used the immunoblotting technique to estimate protein levels of HtrA1, HtrA2, long and short variants of HtrA3 (HtrA3-L and HtrA3-S) and TGF-β1 in tissues of benign and malignant thyroid lesions, and control groups. We found that the levels of HtrA2 and HtrA3-S were higher in thyroid malignant tumors compared to normal tissues and benign tumors. The HtrA3-L level was increased in malignant tumor tissues compared to benign tumor tissues and control tissues from patients with benign lesions, and elevated in normal tissues from patients with thyroid carcinoma compared to normal tissues from patients with benign lesions. We also compared levels of HtrA proteins in follicular thyroid carcinoma (FTC) and papillary thyroid carcinoma (PTC) and found that these types of carcinoma differed in the expression of *HtrA3-S* and *HtrA1*. These results indicate the implication of HtrA proteins in thyroid carcinogenesis suggest that HtrA3 variants may play different roles in cancer development, and that the increased HtrA3-L levels in thyroid tissue could be correlated with the development of malignant lesions. The TGF-β1 levels in tumor tissues were not significantly altered compared to control tissues.

Introduction

Thyroid carcinoma is the most common endocrine malignancy in developed countries, accounting for ~1% of total human cancers, and is more frequent in women than in men. Thyroid cancer was recognized as the sixth most frequently diagnosed cancer in women (1).

The majority (95%) of thyroid carcinomas originate from follicular epithelial cells. They are classified as well differentiated, poorly differentiated or undifferentiated (anaplastic) neoplasms. The well differentiated thyroid tumors comprise follicular thyroid cancer (FTC) and papillary thyroid cancer (PTC). These cancers are highly curable, however cause death in over 10% of cases (2).

PTC is the most common thyroid malignancy, accounting for >80 % of cases and it does not have a benign counterpart. In contrast, follicular neoplasms comprise both benign and malignant (FTC) lesions. FTC, accounting for ~10% of thyroid carcinomas, morphologically overlaps benign follicular neoplasm. These two types of thyroid tumors do not differ appreciably. Currently, diagnostic criteria for malignancy are based on histological assessment of invasive features including full penetration of the tumor capsule and/or invasion of blood vessels in or beyond the capsule (3). Follicular lesions can not be accurately diagnosed by fine needle aspiration cytology as the crucial diagnostic features are missing in this method (3). Screening for molecular markers characteristic for thyroid tumors seems to be a promising way to improve traditional morphological cytopathology (reviewed in ref. 4).

The HtrA family of proteins consists of evolutionarily conserved ATP-independent serine proteases, homologues of the HtrA protein from the bacterium *Escherichia coli*. The common feature of HtrA proteins is the presence of trypsin-like proteolytic domain and at least one PDZ domain at the C-terminus. Members of the family have been identified in most organisms, from bacteria to humans. The defense against cellular stresses inducing aberrations in protein structure is believed to be a general function of the HtrAs (reviewed in refs. 5 and 6).

At least four human HtrA proteases have been identified. They are involved in maintenance of mitochondrial homeostasis, apoptosis and cell signaling, and disturbances in their functions may contribute to the development of several diseases including cancer, arthritis and neurodegenerative disorders (reviewed in refs. 6 and 7).

HtrA1 was originally identified as a gene downregulated in SV40-transformed fibroblasts (8). Several lines of evidence

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indicate that HtrA1 functions as a tumor suppressor, promoting cell death. Downregulation of *HtrA1* expression contributes to cancer cell survival and is correlated with tumor progression and metastasis (9-11). *HtrA1* expression was found to be downregulated in many types of cancer cell lines (10), primary tumors such as ovarian (10-12) and endometrial cancers (13,14), and also in metastatic foci of melanoma, lung and prostate cancers compared to primary tumors (9,15). Additionally, in primary ovarian cancer a progressive decrease of *HtrA1* expression correlated with an increasing degree of malignancy has been observed (12). In endometrial tumors *HtrA1* expression was decreased with increasing grades of endometrial cancer (13). In glioma and mesothelioma loss of *HtrA1* expression was correlated with poor prognosis (16,17). Moreover, it has been demonstrated that diminished level of HtrA1 protein in ovarian and gastric tumors is associated with poor clinical response to platinum-based chemotherapy (11).

Molecular mechanism of HtrA1 function in cancer development is unclear. It was demonstrated that HtrA1 triggers death of cancer cells by induction of apoptosis (10,11). It was also shown that HtrA1 functions as an inhibitor of TGF-β proteins (18,19) which are regulators of cell growth and differentiation and whose involvement in cancer development has been proven. While at the very early stages of oncogenesis TGF-β proteins function as tumor suppressors, at the advanced stages they stimulate tumor progression and metastasis (20).

The HtrA2/Omi protein is a mitochondrial serine protease which under physiological conditions functions as a quality control protease and facilitates cell survival. However, in stressful conditions HtrA2 switches from a protector to a proapoptotic protein. Upon apoptotic stimuli HtrA2 is released into cytosol where it promotes apoptosis in a caspase-dependent manner by degradation of the Inhibitor of Apoptosis Proteins (IAPs) as well as in a caspase-independent manner via its proteolytic activity (21-26). HtrA2 degrades proteins other than IAPs, exhibiting antiapoptotic properties (e.g., Hax-1 and ped/pea15) (27,28). Moreover, Liu *et al* (29) showed that the protease is implicated in anoikis, cell death induced by disruption of cell attachment or contact with extracellular matrix. Resistance to anoikis is a common feature of cancer cells, contributing to metastasis (30). To date, several reports have been published arguing the involvement of HtrA2 in cancer development, with its expression variable, depending on cancer type (reviewed in ref. 7).

The HtrA3 protein was initially discovered as a pregnancy-related serine protease (PRSP) implicated in the development of embryo and placentation (31-33). So far, two isoforms of HtrA3, a long, 50 kDa variant (HtrA3-L) and a short, 40 kDa variant (HtrA3-S), produced by alternative mRNA splicing, have been identified (34). Both HtrA3 isoforms are serine proteases, but HtrA3-S lacks the PDZ domain at its C-terminal end. Since the PDZ domains are involved in substrate binding the HtrA3 isoforms may recognize different substrates and thus have slightly different functions. High homology of HtrA3 with HtrA1 (58%), an identical domain organization and similar expression pattern in human tissues suggest similar activity and physiological functions (34). Indeed, HtrA3 acts as an inhibitor of TGF-β signaling pathway (35). Moreover, evidence exists showing involvement of HtrA3 in modulation of chemotherapy-induced cytotoxicity (36,37). Recently, it has been proposed that HtrA proteins could be novel targets in cancer therapy (reviewed in refs. 6 and 7).

Table I. Characteristics of specimens.

Tissue type	No. of cases
Benign/Normal	20/20
Malignant	
Follicular carcinoma/Normal	12/12
Papillary carcinoma/Normal	8/8

HtrA proteins are involved in oncogenesis, are prospective therapeutic targets and finding new molecular markers in thyroid cancer is necessary. To date, there is no information available concerning involvement of HtrA proteins in thyroid cancer. These facts prompted us to evaluate levels of human HtrA1, HtrA2 and HtrA3 (long and short variants) proteins in thyroid normal and tumor (benign and malignant) tissues. Since HtrA1 and HtrA3 are believed to function in TGF-β signaling, we included evaluation of the TGF-β1 protein level. The relative protein levels were estimated by immunoblotting technique and correlations between the protein levels and thyroid tumor type, histopatological type of cancer and patient gender were analyzed.

Materials and methods

Patients and specimens. Specimens were collected from 40 patients undergoing surgical treatment for thyroid pathology at the Medical University of Gdansk. Both tumor and unchanged thyroid tissues (excised from thyroid in areas free of macroscopically visible abnormalities) were obtained from each patient. The collecting of tissues was supervised by a pathologist. Tissues were immediately frozen in liquid nitrogen and stored at -70˚C. Tissue characteristics are presented in Table I.

Chemicals. Immobilon PSQ Transfer Membrane (0.2 μm pore size) for immunoblotting was purchased from Millipore (Millipore Corp., Bedford, USA). Other chemicals were purchased from Sigma or Fluka (Poznan, Poland) and were of the highest purity.

Preparation of tissue extracts. Protein extracts were prepared as described previously by Narkiewicz *et al* (12,14). Briefly, ~30 mg of thyroid tissue was homogenized with 0.2 ml of cold 10 mM Tris pH 7.4 buffer containing 150 mM KCl and centrifuged (15000 x g, 30 min) at 4˚C. Supernatant was collected, stored at -70˚C and subjected to protein analysis.

Protein assay and electrophoresis. Proteins were quantified by Amido Black staining (38). SDS-PAGE electrophoresis was carried out according to Laemmli (39).

Immunoblotting (western blot analysis). The immunoblotting was performed essentially as described by Pound (40). Samples of tissue lysates containing equal amounts of total protein were resolved by SDS-PAGE and electrotransferred

onto Immobilon membrane. Samples of thyroid lesions and unchanged thyroid tissues were resolved on the same gel together with a reference sample. The same sample was used as the reference in all assays for a given protein (HtrA or TGF-β1). The chosen reference sample was the normal tissue in which the level of the tested protein was close to average. Proteins bound to the membrane surface were stained by a standard procedure with the Ponceau S dye. This step was introduced into the procedure in order to confirm proper sample loading, protein resolution by SDS-PAGE and electrotransfer, and also to check if protein amounts in the samples were comparable.

Polyclonal rabbit immunoglobulins against a given HtrA protein or monoclonal mouse anti-TGF-β1 immunoglobulins were used as primary antibodies. Polyclonal antibodies against the rat HtrA1 and human HtrA2 recombinant proteins were raised as described previously (41). Polyclonal rabbit anti-HtrA3 and monoclonal mouse anti-TGF-β1 antibodies were purchased from Abcam (USA). As the secondary antibodies, goat anti-rabbit or anti-mouse HRP-conjugated IgG were used (Sigma). To visualize HtrA and TGF-β1 proteins chemiluminescence detection was performed using Lumi-Light Western Blotting Substrate from Roche (Warsaw, Poland). The relative levels of the HtrA or TGF-β1 proteins were calculated as a ratio of the tested protein band intensity to the intensity of a corresponding protein (HtrA or TGF-β1) in the reference sample resolved on the same gel. The blots were also probed with mouse monoclonal antibody against GAPDH (Abcam) used as a loading control.

Statistical analysis. Protein band intensity was assessed by densitometric analysis using 1Dscan EX 3.0 software (Scanalytics, Inc., USA) and band intensity ratios were calculated. Each assay was repeated 3 times and the differences did not exceed 10%. Statistica 7.1 PL was used for statistical analysis. The Student's t-test was used to compare the groups. Analysis of variance was performed using ANOVA tests and post-hoc NIR Fisher's test. Statistical significance was assumed at $P<0.05$.

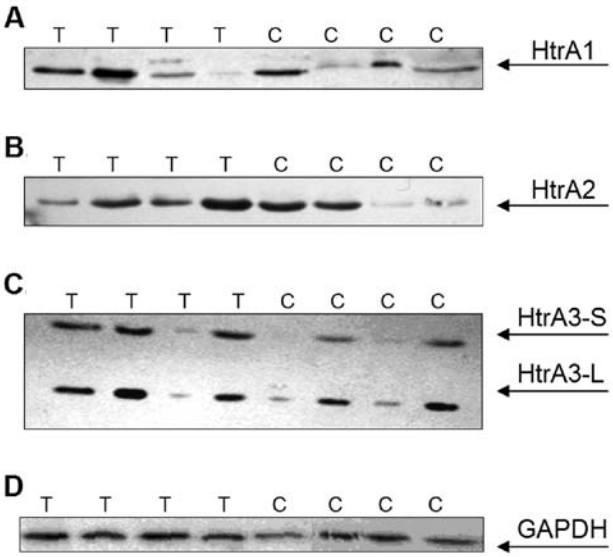

Figure 1. Detection of HtrA1 (A), HtrA2 (B), HtrA3 (C) and GAPDH (D) proteins in thyroid tumors (T) and normal tissues (C). Representative western blotting results.

Table II. Relative levels of HtrA proteins and TGF-β1 in different histological types of thyroid carcinoma.

Protein	FTC (n=12)	PTC (n=8)	P-value
HtrA1	2.10	1.01	0.045
HtrA2	2.56	2.47	0.882
HtrA3-S	0.88	3.17	<0.001
HtrA3-L	1.41	2.26	0.331
TGF-β1	0.76	0.67	0.544

FTC, follicular thyroid carcinoma; PTC, papillary thyroid carcinoma.

Results

The levels of HtrA2, HtrA3-S and HtrA3-L are increased in thyroid cancer. We assayed the relative levels of HtrA1, HtrA2 and of the two isoforms of HtrA3 proteins in thyroid malignant (n=20) and benign tumor tissues (n=20) as well as in control macroscopically unchanged thyroid tissues. The control tissues were grouped according to their source, e.g., the thyroids with benign or malignant lesions. To measure relative levels of HtrA proteins, lysates of the tissues containing equal amounts of total protein were resolved electrophoretically and subjected to western blot analysis.

As predicted, polyclonal antibodies against HtrA1 and HtrA2 detected bands of 50 and 40 kDa, respectively, corresponding to the mature forms of these HtrA proteins (Fig. 1A and B). Immunoblotting with anti-HtrA3 antibodies revealed two bands of around 50 and 40 kDa, representing a long (HtrA3-L) and a short (HtrA3-S) mature isoform of the protein (Fig. 1C).

The levels of HtrA2 and HtrA3-S were significantly higher in a group of thyroid malignant tumors compared to control tissues and benign thyroid lesions (Fig. 2B and C), with more dramatic increase found for HtrA3-S. The HtrA3-S protein level in malignant tumor tissues was 2.3-fold higher compared to benign tumor tissues and ~1.6-fold higher compared to each of the control groups (one from patients with benign lesions and one from patients with malignant tumors). The HtrA3-L protein level was significantly increased in malignant tumor tissues compared to benign tumor tissues, and also to control tissues from patients with benign tumors. Interestingly, the *HtrA3-L* expression was higher in control tissues from patients with thyroid cancer compared to control tissues from patients with benign lesions (Fig. 2D).

We did not find any statistically significant differences in the *HtrA1* expression between the thyroid tumors and the control tissues (Fig. 2A). No statistical correlation between the HtrA protein relative levels and patient gender was found (data not shown).

Follicular thyroid carcinoma and papillary thyroid carcinoma differ in expression of HtrA1 and HtrA3-S. To evaluate the relationship between the HtrA gene expression and histological

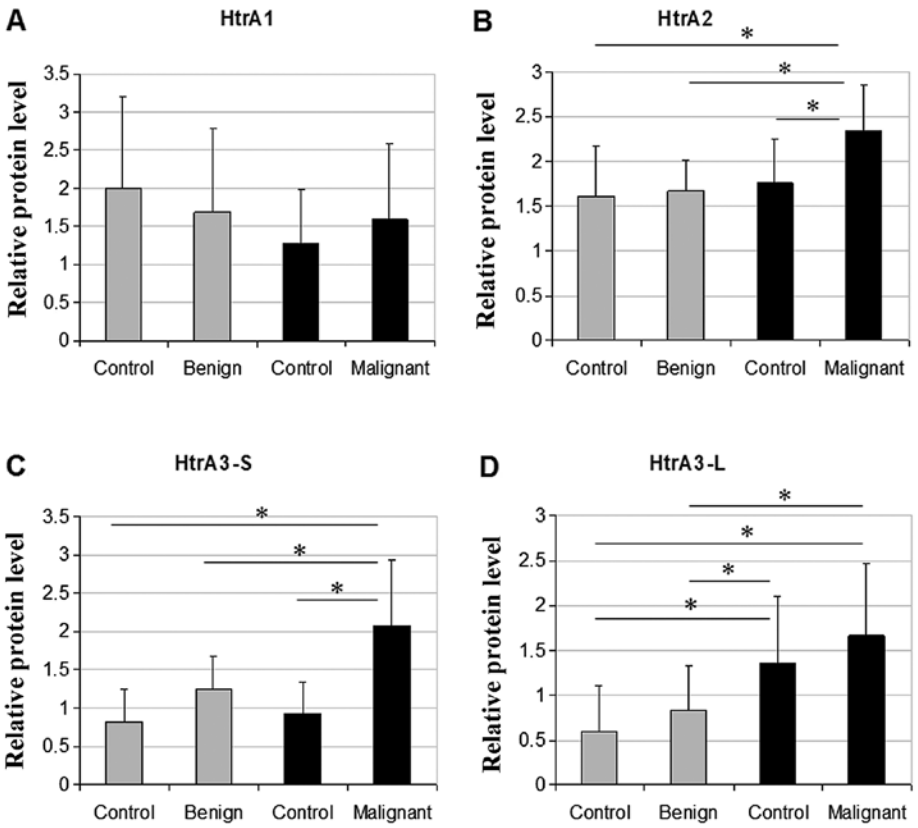

Figure 2. Relative levels of HtrA proteins in benign and malignant thyroid tumors. The HtrA protein levels were estimated in tissue lysates by western blot method. Grey boxes represent groups of thyroid control (n=20) and tumor (n=20) tissues from patients with benign lesions and black boxes represent groups of thyroid control (n=20) and tumor (n=20) tissues from patients with malignant neoplasms. Data were analyzed using ANOVA and Fisher's post-hoc test with a P-value of <0.05 considered significant. The asterisk refers to statistical significance at P<0.05.

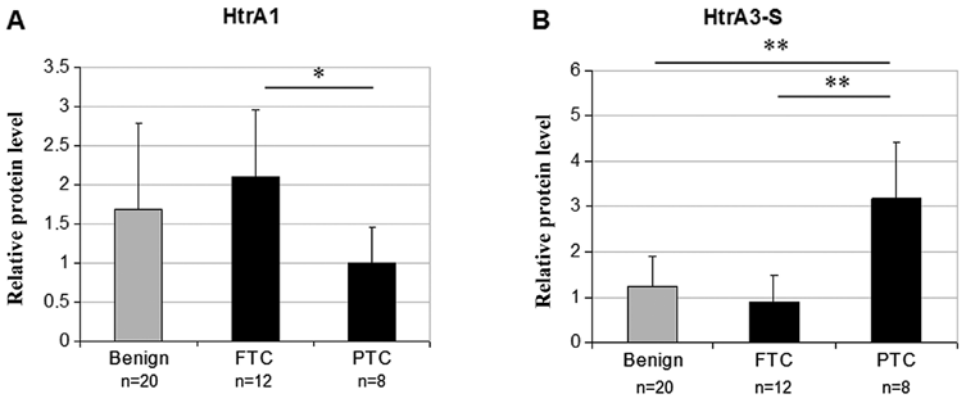

Figure 3. Comparison of the relative protein levels of HtrA1 (A) and HtrA3-S (B) in follicular and papillary thyroid carcinomas and benign tumors. Statistical significance between tested groups was determined by Student's t-test, data significantly different at *P<0.05 and **P<0.001.

type of thyroid cancer, we divided patients with thyroid carcinoma (n=20) according to histopathological analysis of the tumor tissues into two groups: patients with follicular thyroid carcinoma (FTC) (n=12) and patients with papillary thyroid carcinoma (PTC) (n=8), and then compared the relative HtrA protein levels in these groups. As shown in Table II, the HtrA3-S protein level was 3.6-fold higher in PTC compared to the FTC (P<0.001) while the relative HtrA1 protein level was ~2-fold higher in FTC compared to PTC (P=0.045). We also found that the relative protein level of HtrA3-S in PTC but not in FTC was significantly higher compared to benign tumor tissues (Fig. 3). There were no significant differences in HtrA2 and HtrA3-L levels between FTC and PTC (Table II). Thus, these two types of thyroid carcinoma differ in expression of HtrA3-S and HtrA1.

The TGF-β1 expression in thyroid benign and malignant tumor tissues. As shown in Fig. 4 no significant differences between the TGF-β1 relative protein levels in healthy controls and thyroid benign or malignant tumor tissues were found. We did not find any statistically significant correlation between the *TGF-β1*

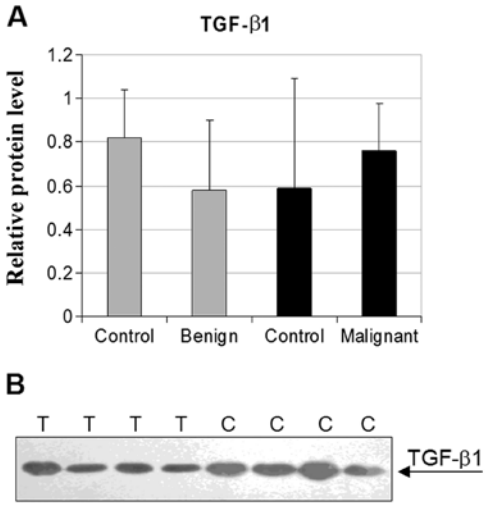

Figure 4. Relative levels of TGF-β1 in thyroid tumors. The TGF-β1 protein level was determined by western blot method. Grey boxes represent groups of thyroid control (n=20) and tumor (n=20) tissues from patients with benign lesions and black boxes represent groups of thyroid control (n=20) and tumor (n=20) tissues from patients with malignant neoplasms (A). Representative western blot results (B). Data were analyzed using ANOVA and Fisher's post-hoc tests with a P-value of <0.05 considered significant.

expression and histological type of thyroid cancer (Table II) or patient gender (data not shown).

Discussion

In the present study we evaluated the expression of the genes encoding members of HtrA serine proteases family, human *HtrA1*, *HtrA2* and *HtrA3*, and of the transforming growth factor β1 (*TGF-β1*) in thyroid normal and tumor tissues. The relative HtrA and TGF-β1 protein levels were evaluated by western blot technique and correlated with histological type of cancer and patient gender. Moreover, for the first time, the levels of both isoforms of HtrA3, HtrA3-S (short) and HtrA3-L (long) in tumor tissues were analyzed.

We found an increase in the HtrA2 level in thyroid malignant tumors compared to normal thyroid tissues and to benign thyroid lesions (Fig. 2). This indicates involvement of HtrA2 in the development of thyroid cancer and suggests that the elevated HtrA2 level may be correlated with thyroid cancer malignancy. These results are in agreement with several other reports showing HtrA2 involvement in oncogenesis. *HtrA2* was found to be overexpressed in prostate carcinoma while normal prostate tissue and benign prostatic hyperplasia showed none or weak expression (42); it was highly expressed in advanced gastric adenocarcinomas (43), in lung cancers (44,45) and in advanced tumors induced in Syrian hamster kidneys by prolonged estrogenization (41). On the other hand, the HtrA2 protein levels were reduced in endometrial (13,14), ovarian (12) and breast cancers (46,47). The mechanism of HtrA2 function in oncogenesis is not known, however, its involvement in promoting apoptosis and anoikis makes an obvious link with cancer cells which evade both types of cell death. The increased HtrA2 levels observed in this study might reflect a cellular defense response to oncogenesis aimed at triggering cell death.

However, the question arises why overexpressed HtrA2 with its proapoptotic function does not protect from tumor development. One possibility is that HtrA2 level might not be sufficient to trigger cell death. Trencia *et al* (27) demonstrated that overproduction of antiapoptotic ped/pea15 prevents apoptosis triggered by HtrA2. Thus induction of apoptosis mediated by HtrA2 might be dependent on the relative levels of the active protease and antiapoptotic proteins, such as ped/pea15 or IAPs. On the other hand, HtrA2 might be sequestered in mitochondria and not released into the cytoplasm.

It was demonstrated that activating point mutations of *ras* are frequent in differentiated thyroid cancers and regarded as crucial oncogenic events contributing to the transition from follicular adenoma to follicular carcinoma (reviewed in ref. 48). Activated oncogenic Ras is a strong inhibitor of *anoikis*. Recent studies by Liu *et al* (29) showed that Ras inhibits *anoikis* by preventing the release of mitochondrial HtrA2 protease into the cytoplasm. Thus, it is possible that sequestration of HtrA2 in mitochondria may repress cell death and facilitate metastasis contributing to cancer promotion.

In western blotting experiments using anti-HtrA3 antibody we detected two bands corresponding to both, the 50 (HtrA3-L) and 40 kDa (HtrA3-S) isoforms of HtrA3 in thyroid tumor and control tissues, and the short isoform was predominant in both tissue types. Both isoforms of *HtrA3* transcripts were found in a range of human tissues with long (heart, skeletal muscle) or short form (placenta, kidney) being predominant in some of them. In some of tissues only long (lung, small intestine) or short (brain) form was detected (34).

Former studies concerning the *HtrA3* expression in diverse tumors were based on analysis of the HtrA3-S protein level only, since the HtrA3-L level was usually too low to be quantified (12,14). In the present study we found that both *HtrA3-S* and *HtrA3-L* expression levels were elevated in malignant tumors when compared to benign lesions. Furthermore, HtrA3-S but not HtrA3-L level was increased in cancers when compared to control tissues. The observed differences in expression of the *HtrA3* isoforms in thyroid tumors indicate the implication of both HtrA3 isoforms in the development of thyroid cancer and also suggest that HtrA3-S and HtrA3-L could play different roles in thyroid carcinogenesis. Contrary to our results, showing a significant increase in HtrA3 level, previous reports revealed a significant decrease of HtrA3 expression in ovarian (12), endometrial (13,14) and lung cancers (37). However, the molecular mechanisms of HtrA3 function in cancer development remains unclear. Recent studies demonstrated that HtrA3 is localized in mitochondria and upon cytotoxic stress induced by etoposide or cisplatin treatment is released into the cytosol where it promotes apoptosis via its proteolytic activity (36,49). Thus, overexpression of the *HtrA3* gene in thyroid tumors might be a response to stress created by oncogenesis, aimed at enhancing apoptosis but not sufficient to activate death of tumor cells due to a prevalence of antiapoptotic signals.

Interestingly, we have also found a significant increase in expression of *HtrA3-L* in control tissues from patients with thyroid cancer compared to control tissues from patients with benign lesions which suggests that the elevated level of the long form of HtrA3 might predispose to the development of malignant form of thyroid tumor.

In the present study we have observed a correlation between HtrA1 and HtrA3-S protein levels and histological type of thyroid cancer. We found that the HtrA1 level was increased in follicular thyroid carcinoma (FTC) compared to papillary thyroid carcinoma (PTC), while the HtrA3-S level was much higher in PTC compared to FTC (Table II). This observation suggests that the HtrA1 and HtrA3 proteins may play different roles in the development of PTC and FTC. It is worth to note that not only was the HtrA3-S level in PTC significantly higher compared to FTC but also compared to benign lesions (Fig. 4). Since there were no significant differences in HtrA3-L levels between PTC and FTC (data not shown), these findings taken together support the hypothesis of diverse functions of HtrA3 isoforms in thyroid cancer development. Both, the long and the short form of HtrA3 are serine proteases, but HtrA3-S lacks the PDZ domain at the C-terminal end. The PDZ domains are known as regulatory elements and specificity determinants of the HtrA proteins (reviewed in refs. 5 and 6). It was demonstrated that HtrA3 requires its proteolytic activity to trigger apoptosis and PDZ-deleted HtrA3 variant was slightly less prone to trigger platinum-induced apoptosis of lung cancer cells than its full-length counterpart (36). Taking into account these facts, the HtrA3 isoforms may recognize different substrates and play slightly distinct roles. However, to date no physiological substrates have been identified and further studies are required to clarify the precise roles of HtrA3 isoforms in carcinogenesis.

Currently, fine-needle aspiration (FNA) is the most widely used preoperative test for initial evaluation of thyroid nodule and diagnostic criteria for malignancy are based on the histological assessment of thyroid specimens (3). Several studies focused on molecular markers that could improve the diagnostic accuracy of cytological analysis after FNA (4). It is tempting to speculate that assaying levels of HtrA2, HtrA3-S and HtrA3-L might serve as an adjuvant facilitating the differentiation between the benign and malignant lesions. However, more thyroid cancer samples need to be analyzed. We evaluated expression level of *TGF-β1* in control and tumor thyroid tissues. However, no significant differences between the TGF-β1 relative protein levels in healthy controls and thyroid benign or malignant tumor tissues have been found. Also, we did not find any correlation between the *TGF-β1* expression and histological type of thyroid cancer or patient gender. It has been shown that the TGF-β1 protein level was elevated in endometrial cancer (14). Moreover, a significant negative correlation between the levels of TGF-β1 and HtrA1 and HtrA3 suggested the involvement of HtrA proteins in TGF-β1 protein regulation (14). However, such correlation has not been found in ovarian cancer (12). These findings together with our results suggest that the regulation of TGF-β1 pathway by HtrA proteins may be tissue-specific and could play different role in the development of distinct tumors.

To our knowledge this is the first study demonstrating upregulation of HtrA2 and HtrA3 proteins levels in thyroid tumors and presenting differences in expression of the long and the short isoforms of HtrA3 in cancer.

Our results indicate involvement of HtrA2 and HtrA3 proteins in thyroid cancerogenesis and suggest different roles of HtrA3-S and HtrA3-L in the cancer development. Our findings open the way for further investigation of the role of the HtrA proteins in thyroid pathogenesis and also suggest the possibility to consider HtrA proteins as molecular markers in diagnostics of thyroid cancer.

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