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Angiotensin converting enzymes ACE and ACE2 in thyroid cancer progression

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Angiotensin-converting enzymes, ACE and ACE2, play not only a pivotal role in the regulation of blood pressure, but are involved in the processes of pathophysiology, including thyroid dysfunction or progression of several neoplasia such as cancers of skin, lungs, pancreas and leukemia. However, their role in the thyroid carcinogenesis remains unknown. We examined in this study the expression of ACE and ACE2 in thyroid tissues and their possible employment as biomarkers for thyroid cancer progression. Thyroid tissues, including 14 goiters (G), 12 follicular adenomas (FA), 10 follicular thyroid carcinomas (FTC), 14 papillary thyroid carcinomas (PTC) and 11 undifferentiated thyroid carcinomas (UTC), were subjected to RT-PCR and protein analyses with primers or antibodies specific for ACE and ACE2, respectively. FA revealed significantly increased ACE compared to other groups and FTC was significantly higher than UTC. ACE2 was significantly increased in PTC in comparison to G, FA and UTC, and in FTC as compared to G. The ratio ACE/ACE2 decreased, while ACE2/ACE increased with the differentiation grade of thyroid carcinoma. ACE was significantly diminished in individuals older than 50. Both ACEs were significantly diminished in M1 patients, ACE2 additionally in higher tumor masses. ACE and ACE2 are regulated within thyroid benign and malignant tissues. As the transcript ratio between both enzymes correlate proportional with the differentiation status of thyroid cancer, ACE and ACE2 may serve as new markers for thyroid carcinoma.

Key words: ACE, ACE2, thyroid cancer, follicular adenoma, renin, angiotensin

Thyroid carcinoma belongs to the most frequently observed endocrine malignancies and together with non-malignant, thyroid hormone (TH)-mediated disorders may affect the other systems, including renin angiotensin system (RAS). The components of RAS may act on individual organs systematically or locally and it is well known that abnormal levels of TH may affect their proper functioning [1–3]. The actions of the classical RAS are mediated by ACE, which generates Angiotensin II (AngII), a potent vasoconstrictor and main RAS effector. The pro-inflammatory and pro-proliferative actions of AngII may be counteracted by the recently described ACE2, which degrades AngII to Angiotensin 1–7 (Ang 1–7), a potent vasodilator possessing also anti-proliferative properties [4, 5]. Both ACEs, abundantly expressed in human cardiac, pulmonary and renal tissues [6–8], have also been detected on monocytes [9–11] and in human atherosclerotic lesions, where they were associated with a subset of lesion macrophages [12–15]. Furthermore, ACE and ACE2 may act as important components of tumor microenvironment and by interaction with different adhesion-related factors, such as E-cadherin [16] or cytokines, such as TGFb [17], are able to modulate the epithelial-to-mesenchymal transformation [18], crucial for processes of neoplasia [19].

Hypo- or hyperthyroidism which is potentially related to the insufficient or excess production of TH, respectively, may affect the levels of ACEs, AngII and Ang 1–7. It has been demonstrated that over-activation of thyroid gland is associated with increased ACE or AngII in plasma [20], heart [21, 22] and kidney [23], whereas hypothyroid state exerted contrary effects [24, 22]. With regards to the ACE2, recent data revealed that hyperthyroidism-induced cardiac hypertrophy was accompanied with cardiac activation of ACE2 and Ang 1–7 [25]. In patients with renal failure who frequently tend to present hypothyroidism and enlargement of the thyroid gland [26, 27], the levels of ACE on monocytes are increased, whereas ACE2 is noticeably decreased [28, 29]. Additionally, these patients obviously suffer from chronic inflammation related with increased levels of TNF and IL-1. As these two cytokines inhibit the proper conversion of TH in the target tissues, it seems explainable how uremia may impair the proper functioning of the thyroid gland [30–32].

Since there is a clear correlation between TH, RAS and different pathological conditions, and the increased number of tumor-associated monocyte-derived macrophages infiltrating thyroid carcinoma tissues could be detected [33–35], we investigated in this study the expression of both ACEs in thyroid gland and their possible employment as diagnostic markers for thyroid carcinoma.

Patients and methods

Patients and tissue preparation. A total of 61 thyroid tissues, including 14 goiter tissues (G), 12 follicular adenoma (FA), 10 follicular thyroid carcinomas (FTC), 14 papillary thyroid carcinomas (PTC) and 11 undifferentiated thyroid carcinomas (UTC), were collected from patients treated in the time interval from 1994 to 2011 at the Department of Surgery of the Martin-Luther-University Halle, by surgical resection. Pathological diagnosis of tissue sections was confirmed with hematoxylin and eosin staining (Table S1). All tissues were snap frozen in liquid nitrogen and stored at –80 °C for further proceedings. Out of 61 patients 21.3% suffered from cardiovascular disorders (CVD), 16.4% were taking calcium channel blockers (CBB) and 9.8% angiotensin II modifying medication (ACEi/ARB, Table S1). Total RNA was extracted from all samples by employment of Trizol reagent according to the manufacturer's instructions (Gibco).

Total RNA obtained from SW-480 cells representing colorectal adenocarcinoma, served as positive control, and was prepared and handled the same way as tissue extracts. Additionally, selected tissues from all groups were investigated in immunohistochemical studies with antibodies specific for ACE and ACE2, as described below. This study was approved by the ethics committee of the Martin Luther University, Faculty of Medicine, and all patients gave written consent.

RT-PCR. One μg of total RNA was reversely transcribed with Superscript II kit (Gibco, Munich, Germany) at 42 °C for 30 min followed by enzyme inactivation at 95 °C for 5 min.

Amplifications of target and housekeeping gene transcripts were performed with primer pairs mentioned in Table S2 and Rotor-Gene SYBR Green PCR Master Mix (Qiagen, Hilden, Germany). Thermal cycling conditions were as follows: hold 5 min at 95 °C, followed by 40 cycles of 5 s at 95 °C and 10 s at 60 °C. Data evaluation was performed with Rotor-Gene Q™ real-time PCR software. Dotted line within the qPCR graphs represents the expression of the target transcript in the reference sample (SW-480) and for the evaluation purposes was set as 100%. For the whole study, and for all qPCR reactions, the same internal calibrator, the same negative controls and the same normalizing marker were employed.

Immunohistochemistry. Freshly cut cryo-embedded serial 6 μm sections of selected thyroid tissues were washed with PBS and fixed in a 1:4 mixture of 3% H_2O_2 in ice cold 90% methanol for 20 min. After washing twice with PBS, cells were incubated overnight at 4 °C with the rabbit polyclonal antibodies against ACE and ACE2 (ab85955 or ab15348, respectively, both diluted 1:1000 with PBS; both from Abcam, Cambridge, MA, USA). Negative control sections were exposed to the secondary antibody only and processed as described below. After 3×10 min washing in PBS, cells were incubated for 30 min with a 1:1000 dilution of biotinylated goat anti-rabbit secondary antibody followed by incubation with an avidin-biotin-peroxidase complex. After 3×10 min washing in PBS, specific immunostaining was visualized with diaminobenzidine chromogenic solution (1:50). Finally, cells were lightly counterstained with Mayer's hematoxylin and photographed under light microscope (Biozero BZ-9000, Keyence Deutschland GmbH, Germany).

Western blot. Total protein extraction from liquid-nitrogen homogenized tissues was performed with a protein lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT) supplemented with protease inhibitors. Forty μg of total protein was separated on 12% SDS-PAGE and blotted onto PVDF membranes. Membranes were blocked 1h in 5% non-fat milk dissolved in TBST buffer. Thereafter primary ACE, ACE2 (both 1:1000, Abcam, ab85955 and ab15348, respectively) and β-actin (1:10000, Sigma Aldrich, A5441, clone AC-15) antibodies in blocking buffer were applied overnight at 4 °C and for 1 h at room temperature respectively. Finally, secondary anti-rabbit and anti-mouse- HRP-conjugated antibodies (both 1:10000, SCBT, sc-2004 and sc-2005, respectively) were employed (1 h at room temperature). Protein bands were visualized with ECL kit (Advansta, WesternBright ECL HRP substrate) and ChemiDoc™ MP Imaging System (Bio-Rad).

Statistics. Each experiment was performed in quadruplicates. Distribution of the quantitative variables was tested with D'Agostino & Pearson, Shapiro-Wilk or Kolmogorow-Smirnow normality tests. Depending on data distribution, parametric (differences between paired values are consistent) or non-parametric (Wilcoxon matched-pairs signed rank test) two-sided t tests were used. Data are presented as medians with inter quartile ranges. For comparisons within thyroid tissues, Bonferroni correction was applied and p-values *<0.01, **<0.001 and ***<0.0001 indicated statistical significance. All analyses were performed with GraphPad Prism 6 software.

Results

Expression of ACE and ACE2 on total tissue extracts. Total RNA obtained from patients' tissues with known diagnosis was subjected to RT-PCR with primers specific for ACE and ACE2. The expression of ACE transcripts was detected noticeably stronger in FA as compared with G and carcinoma tissues. ACE expression was significantly stronger in FA as compared to FTC and significantly lower in UTC as compared to FTC (Figure 1A). Increased expression of ACE2 was observed in PTC and FTC as compared to the other tissues investigated. Significantly increased levels of ACE2

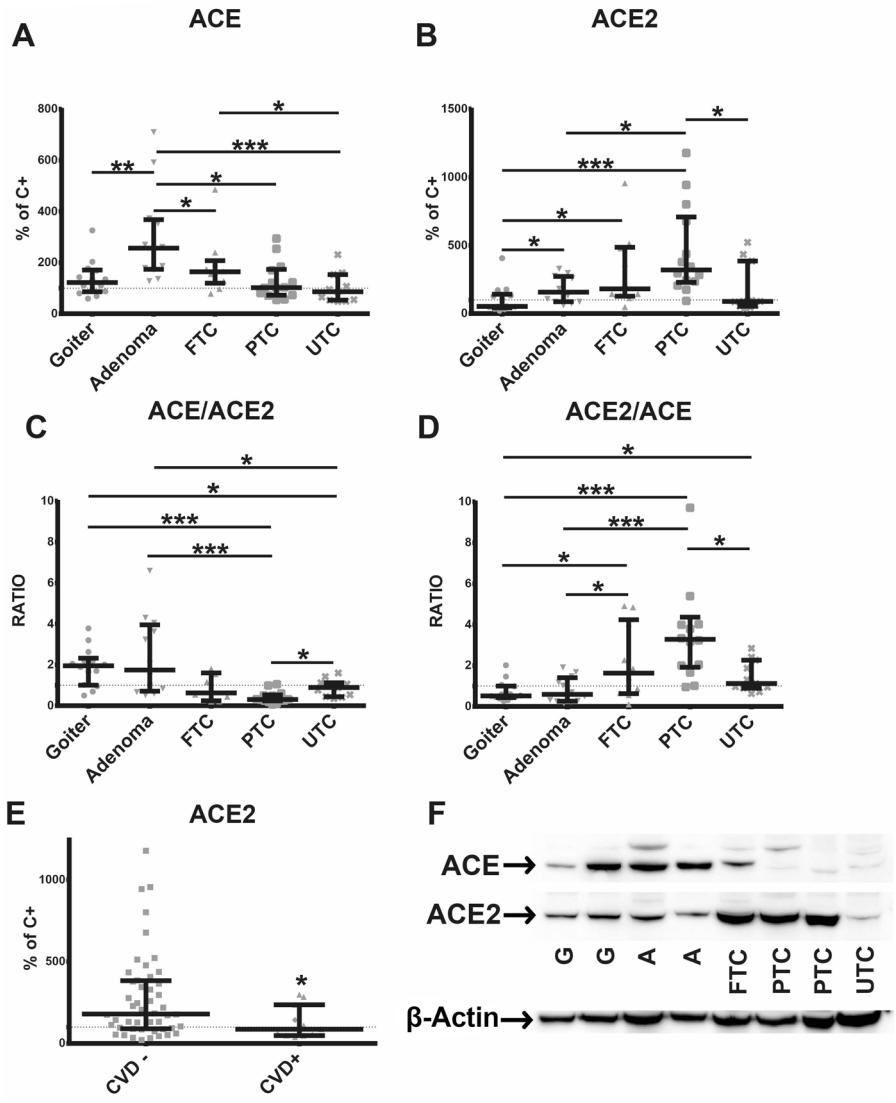

Figure 1. Quantitative RT-PCR analysis performed on human thyroid samples. Total RNA obtained from goiter (G), follicular adenoma (Adenoma, A), follicular thyroid carcinoma (FTC), papillary thyroid carcinoma (PTC) and undifferentiated thyroid carcinoma (UTC) were reversely transcribed and amplified with the primers specific for ACE and ACE2. Expression of ACE (A) and ACE2 (B) in thyroid samples was compared to the expression of both transcripts in SW-480 cells (positive control defined as 100% and C+) and evaluated as percentage difference (% of C+); dotted line in A and B represents the expression of the target transcripts (ACE and ACE2) in positive control. Expression ratio between (C) ACE and ACE2, and (D) ACE2 and ACE. ACE or ACE2 expression in each individual sample was divided by ACE2 or ACE expression in this same sample, respectively; dotted line in C and D represents the ratio between target transcripts in the reference sample and for evaluation purposes was set as 1. (E) Expression of ACE2 in patients with (CVD+) or without (CVD–) cardiovascular disorders. (F) Representative western blots demonstrating protein expression of ACE and ACE2 in thyroid tissues; note that β-actin served as loading control marker; medians with IRQs; p-values *<0.01, **<0.001 and *<0.0001 indicate statistical significance (Bonferroni correction was applied).**

were detected in PTC as compared to G or FA (Figure 1B). Expressions of ACE and ACE2 in UTC were relatively weak in comparison with differentiated thyroid carcinoma (Figure 1). The median percentage transcript expression for ACE in each histological subgroup was 122.0% (59.0–326.0, G), 256.50% (128.0–710.0, FA), 164.0% (78.0–485.0, FTC), 102.0% (54.0–294.0, PTC) and 86.0% (48.0–231.0, UTC). Analysis of ACE2 revealed following median percentages: 52.5% (21.0–406.0, G), 157.0% (61.0–329.0, FA), 181.0% (47.0–955.0, FTC), 320.0% (93.0–1177.0, PTC) and 89.0% (39.0–522.0, UTC).

Furthermore, investigations of the expression proportion between both transcripts revealed that ratio ACE/ACE2 decreased, while ACE2/ACE increased with the differentiation grade of thyroid carcinoma (Figures 1C, D). We found no correlations between ACEs expression and CBB or AngII modifying medication. ACE2 was significantly diminished in patients with CVD (Figure 1E).

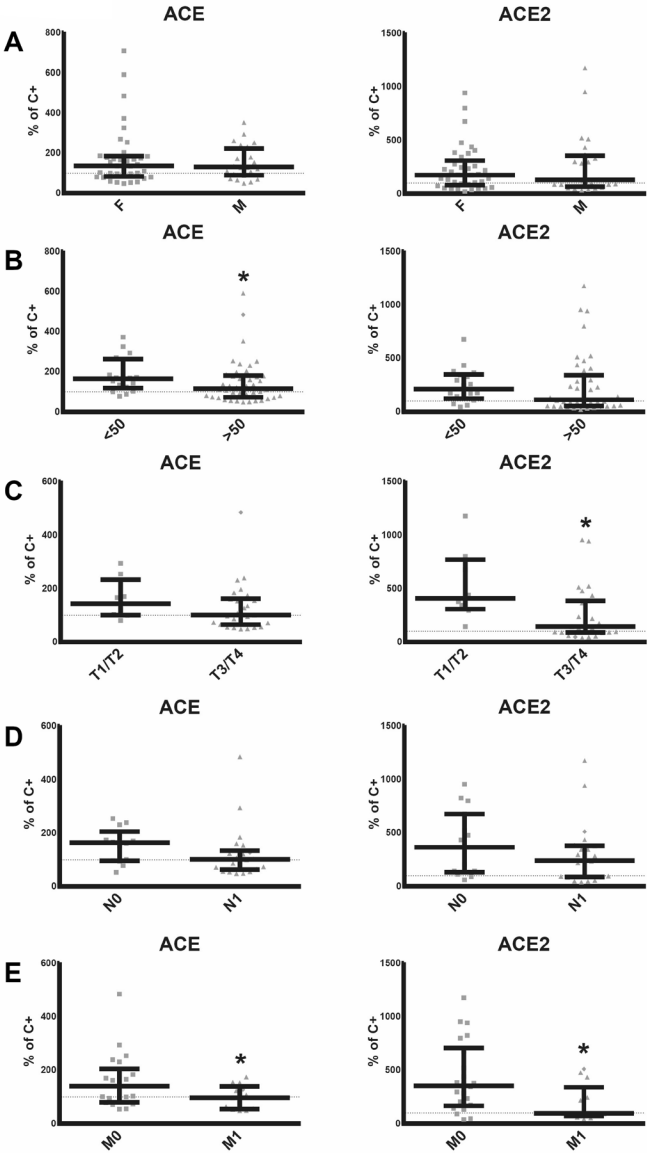

Figure 2. Evaluation of ACE and ACE2 expression regarding gender (A), age (B), tumor grade (C), lymph node (D) and metastatic status (E); dotted line represents the expression of the target transcripts (ACE and ACE2) in positive control and for evaluation purposes was set as 100%; medians with IRQs; p-values *<0.05 were considered to indicate statistical significance.

Correlation with age, gender, tumor grade, lymph node and metastatic status. We found no difference in ACE and ACE2 expression between females and males. However, ACE2 expression in male group revealed a falling tendency (Figure 2A). The transcripts of both enzymes demonstrated decreased expression in the group of patients older than 50 years (Figure 2B), as well as in individuals with diagnosed T3/T4 tumors (Figure 2C), lymph node (Figure 2D) and distant metastases (Figure 2E) as compared to corresponding controls. It is worth to note that expression of ACE was significantly decreased in patients older than 50 (Figure 2B) and with M1 status (Figure 2E). ACE2 was significantly diminished in patients with higher tumor masses (Figure 2C) and distant metastases (Figure 2E). The median percentage transcript expression for ACE in each group investigated was 136.0% (48.0–710.0, F) vs. 131.0% (49.0–353.0, M); 165.5% (78.0–373.0, <50 years) vs. 115.5% (48.0–592.0, >50 years); 143.5% (81.0–294.0, T1/T2) vs. 104.0% (48.0–485.0, T3/T4); 164.0% (53.0–254.0, N0) vs. 101.5% (48.0–485.0, N1); 140.5% (54.0–485.0, M0) vs. 97.0% (48.0–174.0, M1). Analysis of ACE2 revealed following median percentages: 175.0% (21.0–943.0, F) vs. 129.5% (32.0–1177.0, M); 211.5% (45.0–677.0, <50 years) vs. 113.0% (21.0–1177.0, >50 years); 407.5% (143.0–1177, T1/T2) vs. 159.0% (39.0–955.0, T3/T4); 365.0% (60.0–955.0, N0) vs. 241.0% (39.0–1177.0, N1); 354.5% (39.0–1177.0, M0) vs. 96.0% (46.0–512.0, M1).

Protein expression of ACE and ACE2 on thyroid tissues. To investigate the cellular localization and expression of both proteins within benign and malignant thyroid tissues, selected surgical resectates were subjected to immunohistochemistry and western blot with specific ACE and ACE2 antibodies. Both proteins were mostly localized in the cytoplasm of the thyroid cells, although sporadic positive ACE and ACE2 staining could be detected in nucleus. Weaker ACE immunoreactivity was detected in G and most thyroid carcinoma tissues representing FTC, PTC and UTC as compared with FA (Figure 3A). In contrast, ACE2 was noticeably higher in FTC and PTC as compared with other investigated tissues (Figure 3B). In addition to these findings, ACE2 protein expression in FTC and PTC was noticeably stronger than that of ACE, further supporting the results obtained on transcript level (Figure 1F, Figure 3).

Discussion

In this study, we identified ACE and ACE2 transcripts as novel RAS indicators of the malignant state of thyroid gland. We found that levels of both transcripts are regulated within benign and malignant tissues, and that expression ratios between ACEs correlate proportional with the differentiation status of thyroid cancer. Furthermore, and the most importantly, based on the expression of ACE or ACE2/ACE ratio we could distinguish between FA and FTC of the thyroid gland. To our knowledge, this is the first study demonstrating that both RAS components, ACE and ACE2 may be involved in the processes of thyroid carcinogenesis and possess the diagnostic properties.

Several studies demonstrated that RAS may be involved in tumor progression and angiogenesis. The results originating from a mouse melanoma model suggest that enhanced AngII signaling supports the infiltration of tumor-associated macrophages, resulting in enhanced tissue VEGF protein levels [36]. Furthermore, administration of AngII-modifying medication, especially employment of ACE-inhibitors

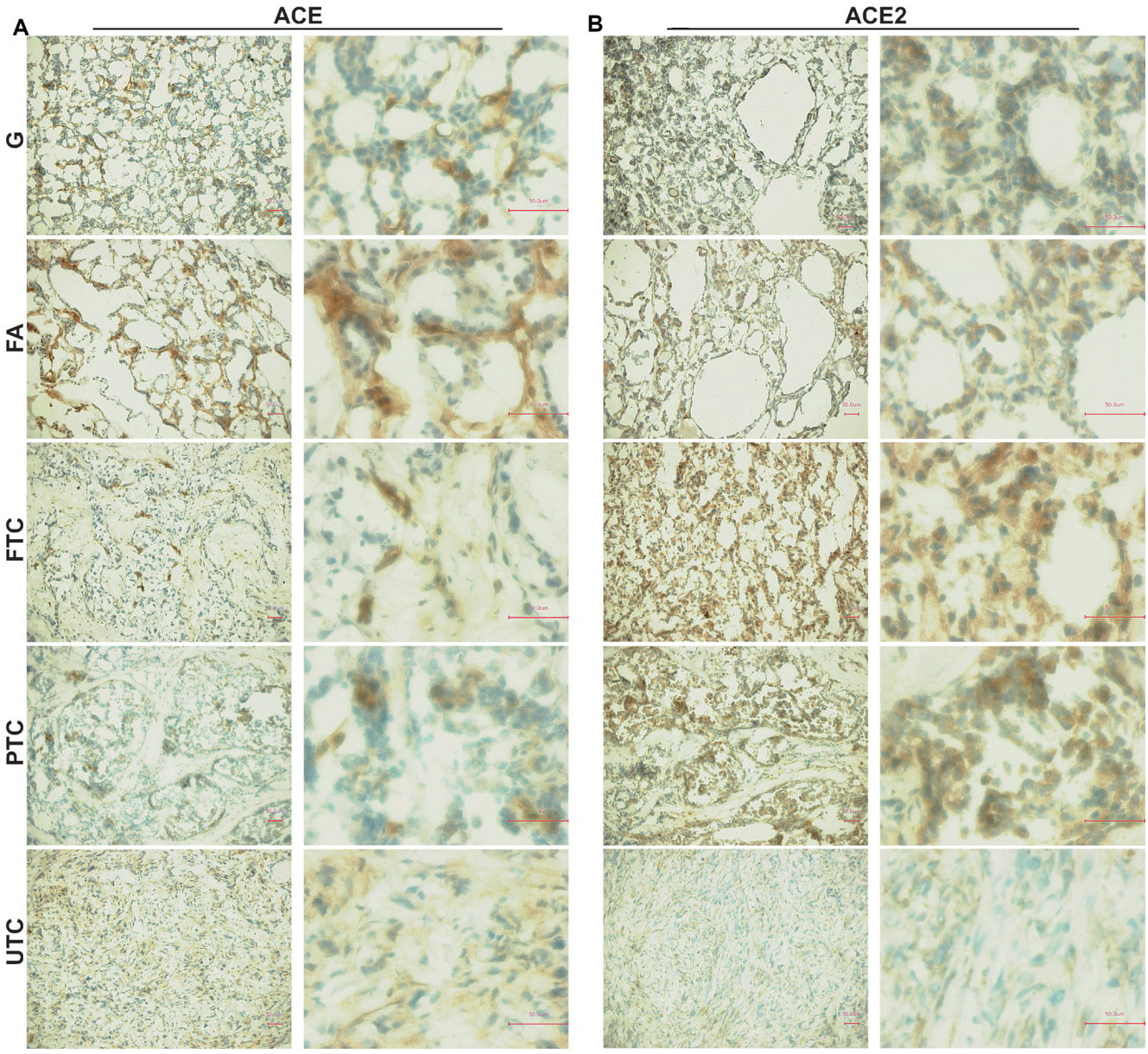

Figure 3. Representative immunohistochemical analysis of ACE (A) and ACE2 (B) expression on thyroid tissues. Frozen, freshly cut serial 6 μm cryosections were stained with specific ACE or ACE2 antibodies, visualized with diaminobenzidine chromogenic solution and counterstained with Mayer's hematoxylin. Note strong ACE2 staining in papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC) as compared to ACE immunoreactivity in those tissues; note stronger ACE staining in follicular adenoma (FA) than in other tissues investigated; note strong ACE2 production in FTC and PTC as compared to goiter (G), FA and undifferentiated thyroid carcinoma (UTC).

(ACEi), was linked with anti-cancer effects as reflected in decreased proliferation and invasion of tumor cells, as well as reduced angiogenesis or metastatic potential both *in vitro* and *in vivo* [37–39]. On the other hand, in ACE 10/10 mice model the high levels of ACE on macrophages provided a consistent and marked resistance to melanoma proliferation. It is worth to note that in the absence of any tumor environment, these macrophages reacted to LPS stimuli with increased levels of IL-12 and nitrite production, whereas IL-10 production was diminished [40].

Recent population based cohort study by Hicks et al. suggested that the use of ACEi was associated with an increased risk of lung cancer, particularly in individuals taking this medication for more than five years [41]. A meta-analysis study by Song et al. revealed that administration of angiotensin modifying medication exerted beneficial effects

on urinary tract cancer, colorectal cancer, pancreatic cancer and prostate cancer, but not on the breast and hepatocellular cancer [38]. Various studies reported the regulation of ACE and ACE2 in several cancer types. Increased ACE serum levels, reported in patients with laryngeal cancer, were associated with poor prognosis [42], whereas in patients suffering from primary lung cancer, higher amounts of circulating ACE predicted better clinical outcome [43]. Prochazka et al. demonstrated a decreased ACE activity in primary human lung tumors as compared to corresponding normal lung tissue [44]. Furthermore, in patients with bronchial carcinoma elevated plasma ACE activity reflected the effective response to chemotherapy or radiotherapy [45, 46]. In patients with acute myeloid leukemia, ACE was noticeably overexpressed in leukemic myeloid blast cells as compared with control group, representing individuals with non-malignant hematological disorders [47]. Also the multiple myeloma patients demonstrated significantly elevated ACE serum levels as compared with control groups [48].

With regards to ACE2, it has been demonstrated that breast cancer cells overexpressing ACE2, possess decreased metastatic ability in immunodeficient mouse model. The same study reported highly expressed ACE2 in adjacent and ductal cancer tissues, whereas in samples with distant or lymph node metastasis, low levels of ACE2 were observed [49]. In other animal models, injection of Ang 1–7, a product of ACE2 actions, led to decreased growth and metastatic ability of lung and prostate cancers [50–52]. Similar results were also observed for pancreatic cancer, where cellular overexpression of ACE2 led to diminished tumorigenicity and tumor growth, both *in vitro* and *in vivo* [53].

We found in our studies significantly low levels of ACE and ACE2 in the most malignant samples representing UTC, whereas in differentiated thyroid carcinoma tissues, such as PTC, ACE expression was low, but ACE2 was noticeably upregulated as compared to non-malignant samples. Furthermore, both enzymes demonstrated a falling tendency in samples originating from higher tumor sizes and metastatic lesions. With regard to ACE2, it worth to note, that its expression reached significantly low levels in bigger tumors and in individuals producing distant metastases.

What is the possible mechanism or role of both enzymes in thyroid cancer progression? It seems that enzymatic activity of at least one of the ACEs may play an important role in tumor growth and metastases. Yu et al. demonstrated that anti-proliferative effects of ACE2 might be abolished upon inhibition of Ang 1–7 receptor and the silencing of ACE2/Ang-(1–7)/Mas axis was related with increased calcium influx and activation of PAK1/NF-κB/Snail1 signaling [49]. Based on our data and especially the ratio between both enzymes, we speculate that pro-inflammatory and pro-proliferative actions of AngII generation cannot be effectively counterbalanced by ACE2 activity or Ang 1–7 actions. This fact may be further supported by the observations that although thyroid tumor samples possess relatively high ACE2/ACE ratios, the tissues producing lymph node or distant metastases reveal significantly diminished ACE2 levels that *per se* may correlate with decreased ACE2 activity.

Although the exact functional or biological rationale of ACE or ACE2 remains not entirely understood for thyroid carcinogenesis, both enzymes proved to have a valuable diagnostic potential. Furthermore, as the expression of ACE allows discriminating between FA and FTC lessons, it would be of great interest to verify whether the circulating blood levels of both enzymes may support the development of serum/plasma non-invasive assay useful for diagnostic of thyroid cancer.

Limitations. Although this study was investigated on human tissues originating from patients with different thyroid disorders we must address several limitations. By design, the expression of both ACEs could be obtained on the transcript and protein levels, however the measurements of enzymatic activity of both enzymes in the circulation or thyroid tissues are missing. The lack of functional data, especially the influence of AngII-modifying medication on thyroid cancer progression, which is a noticeable limitation of this study, should be addressed in the future. We are aware that this study was conducted on relatively small patients' collective, but the promising results presented in this report justify future investigations on larger cohorts.

Supplementary information is available in the online version of the paper.

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