EXPERIMENTAL STUDY

Evaluation of insulin-like growth factor II, cyclooxygenase-2, ets-1 and thyroid-specific thyroglobulin mRNA expression in benign and malignant thyroid tumours

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Abstract

We evaluated three markers insulin-like growth factor II (IGF-II), cyclooxygenase-2 (COX-2) and ets-1 of thyroid growth stimulation and cell transformation together with a thyroid-specific marker thyroglobulin (Tg) for their potential to differentiate benign and malignant follicular thyroid neoplasia (FTN).

Design and methods: mRNA expression levels were determined by real-time PCR in 103 snap-frozen thyroid samples in benign thyroid nodules with different histology and function (19 cold (CTN) and 17 toxic thyroid nodules (TTN)), in 34 normal thyroid tissue of the same patients, eight Graves' disease (GD) thyroid nodules, 10 follicular thyroid carcinomas (FTC) and 10 papillary thyroid carcinomas (PTC).

Results: Mean IGF-II and COX-2 levels were not significantly altered between benign and malignant thyroid nodules (IGF-II=modular FTC TTN, CTN) and normal thyroid tissue (CTN-2). In contrast, the ets-1 expression in the FTC was significantly lower than in the TTN, CTN and GD samples compared with benign nodules and normal thyroid tissue. In addition, thyroglobulin mRNA expression was markedly deregulated (50-103-fold) in FTC, PTC and GD compared with benign nodules and normal thyroid tissue. We then studied IGF-II and Tg mRNA expression in fine needle aspiration cytology (FNAC) samples. However, a retrospective series of 40 FNAC samples only equivocal results were obtained in 38 benign and two malignant FTC thyroid tumour samples.

Conclusion: Upregulation of ets-1 and downregulation of Tg mRNA expression occur in differentiated thyroid cancer and can facilitate pre-operative identification of thyroid malignancy depending on further evaluation of these potentially promising markers in a larger series of benign and malignant thyroid tumours and their FNAC samples.

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Introduction

Fine needle aspiration cytology (FNAC) is the most sensitive and specific tool for pre-operative diagnosis of thyroid malignancy (1–4). However, there are several shortcomings to FNAC, such as sample quality and analysis as well as diagnostic limitations, in particular follicular thyroid neoplasia (FTN) (4, 5). As a consequence, patients with nodular thyroid disease will usually undergo thyroid surgery too frequently, because of the lack of tools to confidently assure the patient about the 'benignity' of the disease. This is most apparent in areas with iodide deficiency, where the clinician is faced with the dilemma to identify very rare thyroid cancer amongst very frequent thyroid nodular disease (1–3). The search for mutations in 'candidate genes', e.g. ras, has not been fruitful and PAX-8/PPARγ1 rearrangements seem to be too infrequent in follicular thyroid cancer (FTC) (<5%) to allow for a routine application as a diagnostic marker of FTN (4–8). Other markers, in particular galectin-3, have been reported to delineate benign and malignant FN with high sensitivity and specificity; however, more recent studies suggest that galectin-3 is useful for the diagnosis of papillary thyroid cancer and less so for FTC (9, 10). In view of the considerable heterogeneity of benign thyroid nodules (3, 5), it is conceivable that a combination of parameters rather than one single diagnostic parameter needs to be applied.

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In this study we evaluate a set of three novel markers of thyroid growth and cell transformation for their potential to distinguish benign and malignant follicular thyroid lesions: (i) insulin-like growth factor-II (IGF-II) has been suggested as a growth-promoting component of an autocrine loop involving IGF-II and an IGF-II sensitive insulin receptor isoform in thyroid cancer cells (11); (ii) cyclooxygenase-2 (COX-2) is the inducible form of cyclooxygenase, and represents an early-response gene, which can be upregulated by growth factors and oncogenes alike (12, 13). Increased COX-2 mRNA and protein expression levels have recently been described in malignant thyroid tumors, predominantly papillary carcinoma, but not in benign thyroid nodules (12); (iii) et-1 is a transcription factor involved in the regulation of cell proliferation and differentiation (14). Increased et-1 transcriptional activity was described in association with thyroid cell transformation (14) in several human thyroid carcinoma cell lines. Using real-time PCR technology we have performed quantitative mRNA analysis of IGF-II, COX-2 and et-1 in a series of benign and malignant thyroid pathologies. With the perspective to apply these markers for FNAC we also determined the mRNA expression of a thyroid-specific gene (thyroglobulin, Tg) as an internal thyroid-specific control and studied et-1 and Tg mRNA expression in a series of 40 consecutive FNAC samples.

Materials and methods

Thyroid samples

Nineteen cold nodules (CTN) (nine colloid nodules and 10 follicular adenomas). 17 toxic thyroid nodules (TTN) (nine colloid nodules and eight follicular adenomas), 36 corresponding normal thyroid tissues of the same patient, eight Graves' disease (GD) thyroids, 10 follicular thyroid carcinomas (FTC) and 10 classic papillary thyroid carcinomas (PTC) were studied. In addition, consecutive FNAC samples were obtained ex vivo in a separate series on 40 surgically removed solitary cold nodules (14 follicular adenoma, 24 adenomatous nodules, two FTC). Thyroid samples were obtained from the Department of Surgery, Martin-Luther University Hospital, Germany and local Leipzig hospitals. Analysis of the clinical and work-up results e.g. thyroid function tests, ultrasound, scintiscan and histology was performed by two independent investigators (D.F and R.P.). Informed consent was obtained from all patients and the local ethics committee approved the study.

RNA extraction and RT-PCR

Snap-frozen tissue samples were pulverized and transferred into Trizol reagent (Invitrogen, Carlsbad, CA, USA) for RNA extraction. RNA clean up was performed using the RNeasy Mini Kit 50 (Qiagen Sciences, MD, USA). Total RNA (1 µg) was reverse transcribed in a 20 µl reaction. The reaction mixture consisted of 5× First Strand Buffer (250µmol/l Tris·HCl pH 8.3, 375 µmol/l KCl, 15 µmol/l MgCl₂) (GibcoBRL, Karlsruhe, Germany), 0.5 µmol/l dNTPs, 5 µmol/l dithiothreitol (GibcoBRL), 15 U Prime RNase Inhibitor (PepLab, Erlangen, Germany), 0.5 µg oligo dT (Promega, Madison, WI, USA) and 10 U Moloney murine leukemia virus reverse transcriptase (GibcoBRL). Reverse transcription was performed at 37°C for 60 min and at 94°C for 5 min.

Real-time PCR

Real-time PCR was performed using the LightCycler (Roche, Mannheim, Germany). Interon spanning primer pairs were designed for IGF-II: forward 5'-GGG GGA GGT GCT GGA C-3' and reverse 5'-CTC GGA CTT GCC GGG GGT AGC-3'; COX-2: forward 5'-CAA TCT GCC TTA GAC AAA ACA-3' and reverse 5'-ATC TCT CTC TGC TTC TCA ATG CAA-3'; et-1: forward 5'-CCC CCT CCT CTC CCT GCT ACT-3' and reverse 5'-TCC TCT GCA CTC CCG GGG TTT-3' were purchased from MWG Biotech AG (Ebersberg, Germany). An optimal PCR reaction for all investigated genes was established using the LightCycler-DNA Master SYBR Green I Kit (Roche). After initial denaturation (30 s) at 95°C PCR was carried out for 40 cycles (IGF-II: 95°C for 0.5 s, 66°C for 7 s, and 72°C for 8 s, 3 µmol/l MgCl₂; COX-2: 95°C for 0.5 s, 64°C for 7 s, and 72°C for 13 s, 5 µmol/l MgCl₂; et-1: 95°C for 0.5 s, 52°C for 7 s, and 72°C for 11 s, 4 µmol/l MgCl₂). PCR fragments were cloned into the pGEM-T vector (Promega) and calibration curves were obtained using the pGEM-T-cloned PCR products. Thyroid tissue samples were measured in duplicate and real-time PCR was repeated at least once. In all runs two dilutions (1:100 and 1:1000) of the plasmids were included to check for interassay variations. For all probes LightCycler software calculated the threshold cycles, which were used to ascertain the concentration of et-1, COX-2 and IGF-II. β-Actin and thyroglobulin (Tg) mRNA expression levels were determined as previously described (15, 16). Results are shown as ratios (× 10³) of IGF-II, COX-2, et-1, Tg (ng/β-actin (ng) or et-1, COX-2, IGF-II (ng)/thyroglobulin (ng) per sample (in this case correction for β-actin expression is nonilluditived). The Mann–Whitney U test was used to compare mRNA expression in benign thyroid nodules, Graves' disease tissues, normal thyroid tissues, follicular and papillary thyroid cancers.

Results

Messenger RNA expression for IGF-II, COX-2 and et-1 was demonstrated in all 100 snap-frozen thyroid tissue samples (Table 1): (i) β-actin-normalized IGF-II mRNA expression levels were lowest in toxic thyroid nodules
<table>
<thead>
<tr>
<th>Thyroid Tissue</th>
<th>IGf-1</th>
<th>COX-2</th>
<th>et-1</th>
<th>Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicular carcinoma</td>
<td>62.2±17.3</td>
<td>2.6±0.9</td>
<td>12.6±2.7</td>
<td>217±80</td>
</tr>
<tr>
<td>Papillary carcinoma</td>
<td>n.a.</td>
<td>n.a.</td>
<td>33.3±6.3</td>
<td>315±70</td>
</tr>
<tr>
<td>Graves' disease</td>
<td>34.8±10.5</td>
<td>2.4±0.11</td>
<td>12.3±1.9</td>
<td>448±150</td>
</tr>
<tr>
<td>Tc-induced thyroid nodule</td>
<td>9.1±5.4</td>
<td>1.5±0.3</td>
<td>3.0±0.7</td>
<td>43.4±14.2</td>
</tr>
<tr>
<td>Starving tissue of Tc-induced thyroid nodule</td>
<td>31.4±5.8</td>
<td>3.0±0.6</td>
<td>3.7±0.5</td>
<td>21.0±0.5</td>
</tr>
<tr>
<td>Cold thyroid nodule</td>
<td>13±5.6</td>
<td>1.2±0.6</td>
<td>2.0±1.2</td>
<td>45.0±12.9</td>
</tr>
<tr>
<td>Surrounding tissue of cold thyroid nodule</td>
<td>52±7.9</td>
<td>2.1±0.1</td>
<td>4.0±1.0</td>
<td>27.0±12.2</td>
</tr>
</tbody>
</table>

\*P < 0.05; \*P < 0.001 (Mann–Whitney U test).

Discussion

A set of three molecular markers (IGf-1, COX-2, et-1) of thyroid growth stimulation and cell transformation was evaluated together with a thyroid-specific marker.

Figure 1 Box plot showing median and distribution box area in 50% of samples of (a) IGf-1 mRNA expression and (b) COX-2 mRNA expression in different thyroid pathologies and normal thyroid tissues. Normalised for β-actin expression x 10^6. PTC: follicular thyroid cancer; GD: Graves' disease; CTN: cold thyroid nodule; sCTN, corresponding normal tissue of same patients; TTN: toxic thyroid nodules; sTTN, corresponding normal thyroid tissue of same patients.
(thyroglobulin) for their diagnostic potential to differentiate benign and malignant follicular thyroid neoplasia (FTN). Using quantitative miRNA expression analysis, neither KIF4A nor COX-2 were found to be useful molecular markers for follicular thyroid cancer, in contrast to the transcription factor ets-1. Ets-1 was upregulated in follicular thyroid cancer and ets-1 upregulation was even further pronounced in papillary thyroid cancer (PTC, Fig. 2, Table 1). Overexpression of ets proteins has been described in several human malignancies, including lung, gastric and prostate cancers (17). In addition, de Nigris et al. (14) have previously reported increased ets-1 transcriptional activity and protein expression in different human thyroid cancer cell lines and PTC. Based on elegant in vitro studies they have suggested that ets-1 is required for the maintenance of the neoplastic phenotype of thyroid carcinoma cell lines, possibly through changes in apoptosis, and that increased ets-1 activity in thyroid cancer may not depend on specific oncogene activation (14).

In our study, we introduced a new internal control (IIF) in the analysis of the data. We calculated the relative expression of the target miRNA in each sample by normalizing the relative amount of miRNA to the level of the internal control. The expression of the target miRNA was then calculated as the fold change compared to the normal sample.

Figure 2. Box plots showing median and distribution (box area = 50% of samples) of (a) ets-1 mRNA expression in different thyroid pathologies and normal thyroid tissue (mRNA levels were determined by qRT-PCR and normalized to 18S rRNA). The expression levels were significantly higher in FTN compared to normal thyroid tissue (p < 0.01, Student's t-test). (b) Similar results were obtained for the expression of ets-1 mRNA in different thyroid tissue samples. The expression levels were significantly higher in FTN compared to normal thyroid tissue (p < 0.01, Mann–Whitney U test).

Figure 3. Box plots showing median and distribution (box area = 50% of samples) of (a) Tg mRNA expression in different thyroid pathologies and normal thyroid tissue (mRNA levels were determined by qRT-PCR and normalized to 18S rRNA). The expression levels were significantly higher in FTN compared to normal thyroid tissue (p < 0.01, Student's t-test). (b) Similar results were obtained for the expression of Tg mRNA in different thyroid tissue samples. The expression levels were significantly higher in FTN compared to normal thyroid tissue (p < 0.01, Mann–Whitney U test).
previously been reported also by Lazar et al. [118] in thyroid malignancies using TaqMan PCR technology. These findings prompted us to assess whether correction of either of the three investigated markers for tissue-specific TG expression could help to define a better diagnostic cut-off point for benign and malignant FN (whereby correction of mRNA gene expression with β-actin or any other housekeeping gene is nullified). In fact, calculation of an -ets-1/TG ratio (C20) allowed a clearer and highly specific separation of FTC from TCTN, CTHN and normal thyroid tissue [25, 31], Mann–Whitney U test. Fig. 9b, using this approach, 0/36 normal thyroid, 0/17 TCTN, 0/19 CTHN (one follicular adenoma) but all 10 FTC would have been diagnosed as "suspicious of malignancy," in addition to the 10 FTC. We then studied ets-1 and TG mRNA expression levels in consecutive FNAC samples of a further 40 solitary cold thyroid nodules, but no significant differences between the ets-1 and/or TG mRNA levels were obtained for benign (n = 38) and malignant (n = 2) thyroid tumors. The reasons for the discrepancy between FNAC results and our previous findings on snap-frozen tumor samples are not obvious. Samples were obtained by the same investigators and there was no difference between the clinical data of the patients and their histopathological results. However, one important factor may be the limited number of malignant tumor samples in our FNAC series. In conclusion, upregulation of ets-1 together with downregulation of TG mRNA expression occur in differentiated thyroid cancer and may be useful for preoperative identification of thyroid malignancy. In view of the morphological and genetic heterogeneity of thyroid tumor samples (1–5) further evaluation of these potentially promising markers in a larger series of benign and malignant thyroid tumors and their FNAC samples is required.

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