

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

Objective: 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 (FN).

Design and methods: mRNA expression levels were determined by real-time PCR in 100 snap-frozen thyroid samples: 36 benign thyroid nodules with different histology and function (19 cold (CTN) and 17 toxic thyroid nodules (TTN)), 36 corresponding normal thyroid tissues of the same patients, eight Graves' disease (GD) thyroids, 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) or nodular (FTC, TTN, CTN) and normal thyroid tissues (COX-2). In contrast, eight- to tenfold upregulation of ets-1 was observed in PTC and three- to fourfold upregulation of ets-1 was observed in FTC (and GD) compared with benign thyroid nodules and normal thyroid tissues. In addition, thyroglobulin mRNA expression was markedly downregulated (50- to 100-fold) in FTC, PTC and GD samples compared with benign nodular and normal thyroid tissues. Hence an ets-1/Tg ratio > 20 distinguished differentiated thyroid cancer from benign nodular or normal thyroid tissue. We then studied ets1- and Tg mRNA expression levels in fine needle aspiration cytology (FNAC) samples. However, in a consecutive series of 40 FNAC samples only equivocal results were obtained on 38 benign and two malignant (FTC) thyroid tumour samples.

Conclusions: Upregulation of ets-1 and downregulation of Tg mRNA expression occur in differentiated thyroid cancer and may 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 (FN) (4, 5). As a consequence, patients with nodular thyroid disease will possibly 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/PPAR γ rearrangements seem to be too infrequent in follicular thyroid cancer (FTC) (<35%) to allow for a routine application as a diagnostic marker of FN (6–8). 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.

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 tumours, predominantly papillary carcinoma, but not in benign thyroid nodules (12); (iii) *ets-1* is a transcription factor involved in the regulation of cell proliferation and differentiation (14). Increased *ets-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 *ets-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 *ets-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 data and work-up results e.g. thyroid function tests, ultrasound, scintiscan and histology was performed by two independent investigators (DF and RP). 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 (250 mmol/l Tris-HCl pH 8.3, 375 mmol/l KCl, 15 mmol/l MgCl₂) (GibcoBRL, Karlsruhe, Germany), 0.5 mmol/l dNTPs, 5 mmol/l dithiothreitol (GibcoBRL), 15 U Prime RNase Inhibitor (PqLab, Erlangen, Germany), 0.5 µg oligo dt (Promega, Madison, WI, USA) and 200 U Moloney murine leukaemia 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). Intron spanning primer pairs were designed for IGF-II: forward 5'-CGG CGG GGA GCT GGT GGA C-3' and reverse 5'-CTC GGA CTT GGC GGG GGT AGC-3'; COX-2: forward 5'-CAA TCT GGC TGA GGG AAC ACA ACA-3' and reverse 5'-ATC TGC CTG CTC TGG TCA ATG GA-3'; *ets-1*: forward 5'-CCC CGT CCC CTT CCC CCT GTT ACT-3' and reverse 5'-TTC TGT CCA CTG CCG GGG GTC TTT-3' and 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 s, 66 °C for 7 s, and 72 °C for 8 s, 3 mmol/l MgCl₂; COX-2: 95 °C for 0 s, 64 °C for 7 s, and 72 °C for 13 s, 5 mmol/l MgCl₂; *ets-1*: 95 °C for 0 s, 52 °C for 7 s, and 72 °C for 11 s, 4 mmol/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 1:10 000) 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 *ets-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 ($\times 10^2$) of IGF-II, COX-2, *ets-1*, Tg (ng)/β-actin (ng) or *ets-1*, COX-2, IGF-II (ng)/thyroglobulin (ng) per sample (in this case correction for β-actin expression is nullified). 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 *ets-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 1 Messenger RNA expression of IGF-II, COX-2, ets-1 and Tg in snap-frozen thyroid tissue samples. Results are expressed as means \pm S.E.M.

| Thyroid tissue | IGF-II | COX-2 | ets-1 | Tg |
|--|-----------------|----------------|------------------|---------------------|
| Follicular carcinoma | 62.2 \pm 17.3 | 2.6 \pm 0.9 | 12.6 \pm 2.3* | 217 \pm 80* |
| Papillary carcinoma | n.a. | n.a. | 33.0 \pm 6.3** | 315 \pm 70* |
| Graves' disease | 34.8 \pm 10.2 | 2.4 \pm 0.11 | 12.2 \pm 1.9* | 448 \pm 150* |
| Toxic thyroid nodule | 9.1 \pm 5.4 | 1.5 \pm 0.3 | 3.3 \pm 0.7 | 43 431 \pm 14 120 |
| Surrounding tissue of toxic thyroid nodule | 31.4 \pm 5.8 | 3.0 \pm 0.6 | 2.7 \pm 0.5 | 21 205 \pm 9570 |
| Cold thyroid nodule | 13.2 \pm 5.6 | 1.3 \pm 0.8 | 3.0 \pm 1.2 | 43 983 \pm 19 280 |
| Surrounding tissue of cold thyroid nodule | 52.0 \pm 7.9 | 2.1 \pm 0.11 | 4.0 \pm 0.8 | 27 900 \pm 12 220 |

* $P < 0.01$, ** $P < 0.001$ (Mann-Whitney U test).
n.a., not analyzed.

(TTN) (IGF-II/ β -actin ratio \times 100: 9.1 \pm 5.4). Comparison of IGF-II mRNA expression levels in benign and malignant thyroid nodules and their corresponding normal tissues showed a tendency for lower mRNA expression in nodular tissues but these did not reach statistical significance (Table 1, Fig. 1a). (ii) COX-2 mRNA expression levels were similar in benign and malignant follicular neoplasia (CTN, TTN, FTC), normal thyroid tissue and GD samples (Table 1, Fig. 1b). (iii) In contrast, differential mRNA expression was noted for ets-1 with the highest levels observed in PTC (ets-1/ β -actin ratio \times 100: 33.0 \pm 6.3), followed by FTC (ets-1/ β -actin ratio \times 100: 12.6 \pm 2.3) and GD (ets-1/ β -actin ratio \times 100: 12.2 \pm 1.9) (Table 1, Fig. 2a). Compared with the group of benign nodular thyroid tissues and normal thyroid tissues, ets-1 upregulation was three- to fourfold in FTC and eight- to tenfold in PTC respectively, reaching significance ($P < 0.01$ – 0.001 , Mann-Whitney U test) for all samples (Table 1).

When mRNA expression of the thyroid-specific gene thyroglobulin (Tg) was studied, we found a marked Tg downregulation (50- to 100-fold) in all FTC (Tg/ β -actin ratio \times 100: 217 \pm 80), PTC (Tg/ β -actin ratio \times 100: 315 \pm 70) and Graves' disease (Tg/ β -actin ratio \times 100: 448 \pm 150) samples compared with benign nodular (Tg/ β -actin ratio \times 100: 43 600 \pm 16 500) and normal thyroid (Tg/ β -actin ratio: 24 300 \pm 11 200)

tissues. Calculation of an ets-1, COX-2 or IGF-II/Tg expression ratio showed that an ets-1/Tg ratio > 20 clearly distinguished differentiated thyroid cancer (PTC, FTC) and GD from benign nodular and normal thyroid tissues (Fig. 3, $P < 0.01$, Mann-Whitney U test), while IGF-II/Tg and COX-2/Tg ratios were less strong candidates, because there was some overlap between the different thyroid sample subgroups (Fig. 4a,b, $P < 0.3$, Mann-Whitney U test).

Subsequently, ets-1 and Tg mRNA expression were determined in a consecutive series of 40 FNAC samples, obtained *ex vivo* on surgically removed solitary cold thyroid nodules ($n = 38$ benign nodules and two FTC). In contrast to the findings on snap-frozen tissue samples reported above, there was no significant difference between Tg (CTN: Tg/ β -actin ratio \times 100: 19 270 \pm 4520 and FTC: Tg/ β -actin ratio \times 100: 11 905 \pm 6340) and ets-1 (CTN: ets-1/ β -actin ratio \times 100: 5.34 \pm 0.52 and FTC: ets-1/ β -actin ratio \times 100: 6.33 \pm 1.80) gene expression levels in benign and malignant thyroid tumours.

Discussion

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

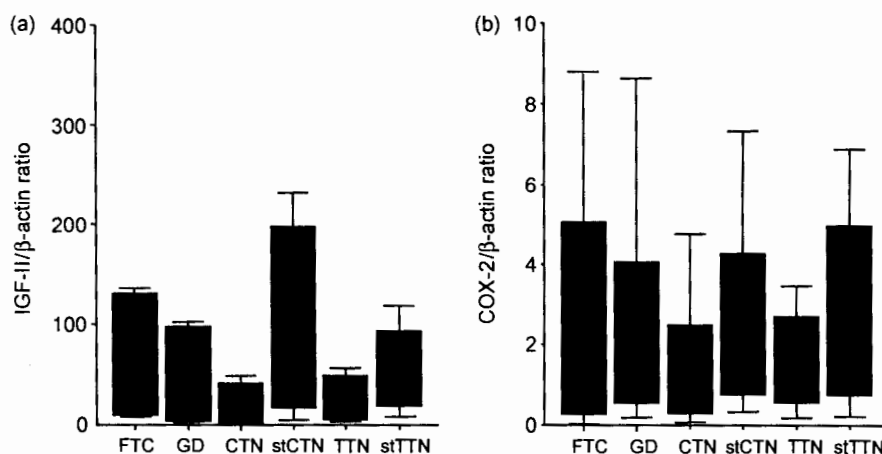


Figure 1 Box plots showing median and distribution (box area = 50% of samples) of (a) IGF-II mRNA expression and (b) COX-2 mRNA expression in different thyroid pathologies and normal thyroid tissues, normalized for β -actin expression $\times 10^2$. FTC, follicular thyroid cancer; GD, Graves' disease; CTN, cold thyroid nodule; stCTN, corresponding normal tissue of same patients; TTN, toxic thyroid nodule; stTTN, corresponding normal thyroid tissue of same patients.

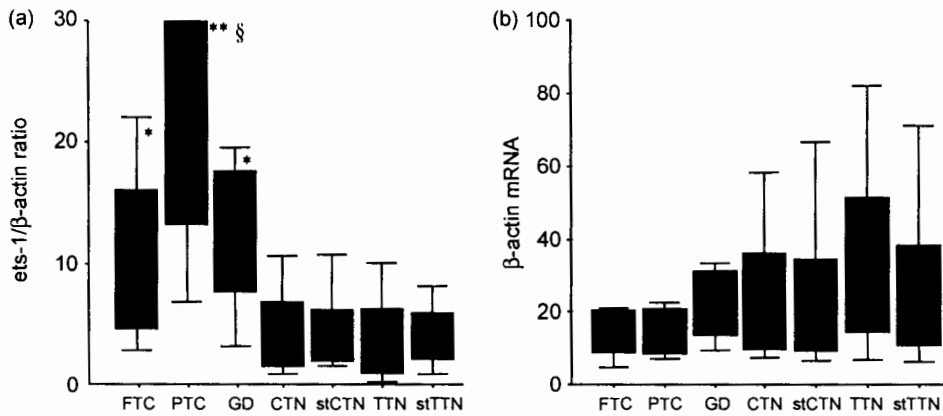


Figure 2 Box plots showing median and distribution (box area = 50% of samples) of (a) ets-1 mRNA expression in different thyroid pathologies and normal thyroid tissue, normalized for β -actin expression $\times 10^2$; § ets-1/ β -actin ratio in PTC ranges from 7.5 to 52. * $P < 0.01$, ** $P < 0.001$ (Mann–Whitney U test). (b) Similar β -actin mRNA expression levels in different thyroid tissues. FTC, follicular thyroid cancer; PTC, papillary thyroid cancer; GD, Graves' disease; CTN, cold thyroid nodule; stCTN, corresponding normal tissue of same patients; TTN, toxic thyroid nodule; stTTN, corresponding normal thyroid tissue of same patients.

(thyroglobulin) for their diagnostic potential to differentiate benign and malignant follicular thyroid neoplasia (FN). Using quantitative mRNA expression analysis neither IGF-II nor COX-2 were found to be useful molecular markers for follicular thyroid neoplasia, 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). With the perspective to apply diagnostic markers for FNAC of thyroid nodules, we included a thyroid-specific internal 'quality' control in our study, since determination of gene/ β -actin expression levels in mRNA analysis of fine needle aspirates may be biased by 'contamination', for example with non-thyroid cells. When we quantitatively assessed thyroglobulin mRNA expression in our thyroid tissue samples, we found a striking variation in thyroglobulin expression, which was markedly downregulated (50- to 100-fold, $P < 0.01$, Mann–Whitney U test) in all FTC, PTC and GD samples compared with benign nodular tumours and normal thyroid tissue (Table 1, Fig. 2b). Messenger RNA downregulation of thyroid-specific genes has

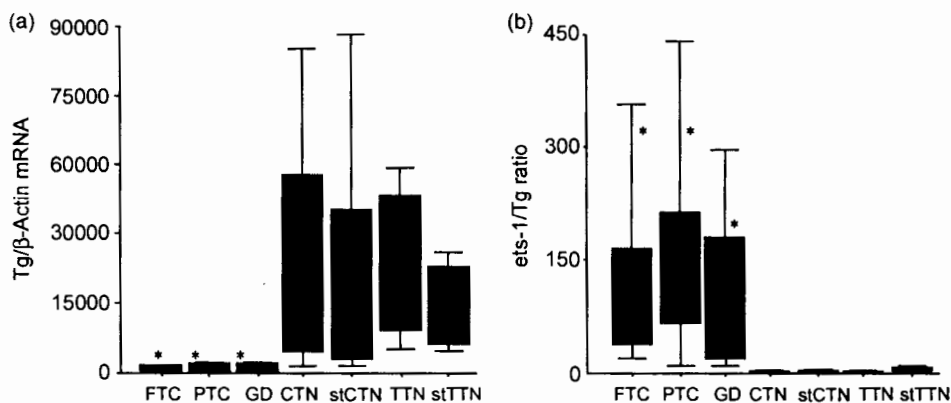


Figure 3 Box plots showing median and distribution (box area = 50% of samples) of (a) Tg mRNA expression in different thyroid pathologies and normal thyroid tissue, normalized for β -actin expression $\times 10^2$ and (b) ets-1/Tg ratio $\times 10^3$ in different thyroid pathologies and normal thyroid tissues. * $P < 0.01$ (Mann–Whitney U test). FTC, follicular thyroid cancer; PTC, papillary thyroid cancer; GD, Graves' disease; CTN, cold thyroid nodule; stCTN, corresponding normal tissue of same patients; TTN, toxic thyroid nodule; stTTN, corresponding normal thyroid tissue of same patients.

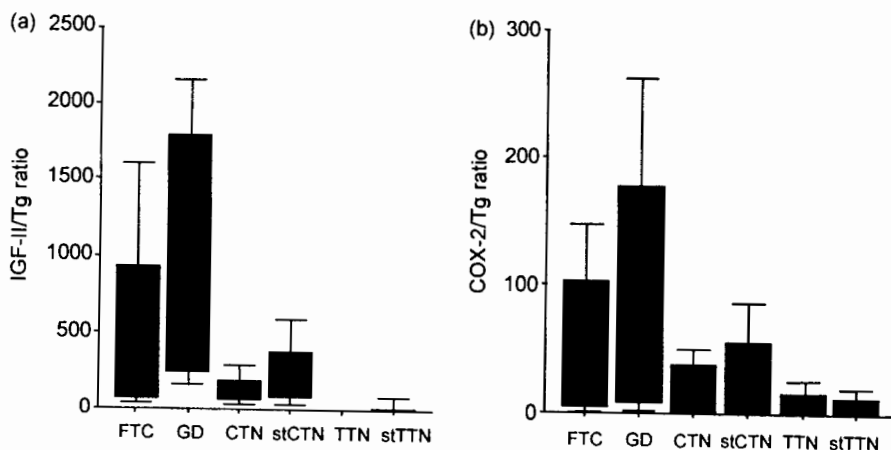


Figure 4 Box plots showing median and distribution (box area = 50% of samples) of (a) IGF-II/Tg ratio $\times 10^3$ and (b) COX-2/Tg ratio $\times 10^3$ in different thyroid pathologies and normal thyroid tissues. All *P* values were < 0.3 (Mann–Whitney U test). FTC, follicular thyroid cancer; GD, Graves' disease; CTN, cold thyroid nodule; stCTN, corresponding normal tissue of same patients; TTN, toxic thyroid nodule; stTTN, corresponding normal thyroid tissue of same patients.

previously been reported also by Lazar *et al.* (18) 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 (> 20) allowed a clear and highly specific separation of FTC (and GD) from TTN, CTN and normal thyroid tissues (*P* = 0.01), Mann–Whitney U test, Fig. 3b). Using this approach, 0/36 normal thyroids, 0/17 TTN, 1/19 CTN (one follicular adenoma) but all 10 FTC would have been diagnosed as 'suspicious of malignancy', in addition to the 10 PTC. 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 tumours. The reasons for the discrepancy between FNAC results and our previous findings on snap-frozen tumour 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 tumour 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 pre-operative identification of thyroid malignancy. In view of the morphological and genetic heterogeneity of thyroid tumour samples (3–5) further evaluation of these potentially promising markers in a larger series of benign and malignant thyroid tumours and their FNAC samples is required.

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