Differential expression of IgG Fc binding protein (FcγBP) in human normal thyroid tissue, thyroid adenomas and thyroid carcinomas

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Abstract

The genetic events involved in thyroid carcinogenesis are still incompletely understood. Several rearrangements and mutations of oncogenes have been implicated in the development of thyroid papillary carcinomas, follicular adenomas and carcinomas. However, none of these molecular alterations is suitable either as a general marker for the diagnosis of thyroid carcinomas or to differentiate between thyroid follicular adenomas and carcinomas. In order to identify new genes with altered expression which could serve as such markers, we analyzed RNA from thyroid tumor and normal tissue using a novel technique called restriction-mediated differential display. Several differentially expressed genes were identified, including the gene for IgG Fc binding protein (FcγBP). Differential

expression of FcγBP was confirmed by quantitative real-time RT-PCR. Our experiments showed that IgG Fc binding protein (FcγBP) is differentially expressed in normal thyroid tissue, thyroid adenomas and thyroid carcinomas. While the FcγBP gene is constitutively expressed in normal thyroid tissue, its expression is significantly increased in follicular thyroid adenomas and significantly decreased in papillary and follicular thyroid carcinomas. Thus, measurement of the expression levels of FcγBP in thyroid biopsies might help to make the otherwise difficult distinction between a thyroid follicular adenoma and a follicular carcinoma.

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Introduction

Thyroid tumors are the most frequently occurring endocrine tumors. Thyroid nodules are found in about 4–7% of the Northern American population on physical examination (Ridgway 1998). Ultrasonographic and autopsy studies reveal an even higher (30–50%) prevalence of thyroid nodules in adults (Ezzat *et al.* 1994). Thyroid nodules are particularly frequent in older people. However, less than 10% of these nodules are malignant (Schlumberger 1998). To decide on the most appropriate therapy, it is therefore important to have means to distinguish between benign and malignant thyroid nodules.

Fine-needle aspiration biopsy cytology is currently the most useful single method for preoperatively distinguishing between benign and malignant thyroid tumors. However, a significant percentage (10–25%) of fine-needle aspiration biopsies are non-diagnostic, for example because of inadequate samples or the presence of an indeterminate cytological pattern, referred to as follicular neoplasia

(Russo *et al.* 1999). Specific molecular markers for thyroid carcinomas would be very useful to improve the proportion of definite diagnoses which can be made from fine-needle aspiration biopsies. To date, several potential markers have been found but none of them is completely satisfactory. The RET oncogene translocation (RET/PTC), for example, has been identified as a papillary thyroid carcinoma marker. However, it is only expressed in a minority of papillary carcinomas (11–25%) (Jhiang & Mazzaferri 1994). Another potential thyroid tumor marker is oncofetal fibronectin. An RT-PCR assay has been developed for detection of oncofetal fibronectin mRNA in fine-needle aspiration biopsies of the thyroid gland with a diagnostic sensitivity and specificity of 96.9% (Takano *et al.* 1998), but this is limited by the fact that oncofetal fibronectin is only expressed in papillary thyroid carcinomas. Mutations in the ras family of oncogenes are found in about 50% of follicular thyroid carcinomas (Lemoine *et al.* 1989). The most recently identified potential genetic marker for follicular carcinomas is a translocation of the thyroid transcription factor PAX8 (Kroll *et al.* 2000).

However, a genetic marker to distinguish accurately all thyroid carcinomas from benign tumors has not yet been identified.

The goal of the present study was to identify new genes which could serve as genetic markers for thyroid cancers. For this purpose we compared mRNA expression in thyroid tissue with nodular hyperplasia, in thyroid adenomas and in carcinomas with mRNA expression in normal tissue from the thyroids containing those pathological alterations. This comparison was carried out using restriction-mediated differential display (RMDD) (Fischer 1995). RMDD combines the power of restriction-based fragment generation for expression analysis with the unsurpassed convenience and resolution of direct-blotting electrophoresis, non-radioactive detection and direct sequencing of candidate bands without cloning steps. During the past few years, several investigators have successfully used cDNA-derived restriction fragments for comparative display of expressed genes (Fischer *et al.* 1995, Kato 1996, Prashar & Weissman 1996, Matz *et al.* 1997). Compared with differential display (DD), such restriction-based technologies provide significantly higher reproducibility and reliability than protocols based on arbitrary binding of PCR primers (Fischer *et al.* 1995), as is the case with DD and with the related RNA arbitrary fingerprinting (Welsh *et al.* 1992). The RMDD protocol, as employed in this study, allows systematic and unbiased coverage of all transcripts present in a sample, above a certain threshold. Spiking experiments with RNA showed a signal up to a dilution of 1:50 000 (authors' unpublished observation).

In preliminary RMDD experiments, several genes that are differentially expressed in normal thyroid tissue and thyroid carcinomas were identified. One of them, the IgG Fc binding protein (FcγBP) gene was chosen for further investigation because its expression has not previously been described in thyroid tissue.

To obtain quantitative information on the expression of FcγBP we used quantitative real-time RT-PCR analysis on a larger number of thyroid tissue samples. We found that, in comparison with normal thyroid tissue and benign thyroid alterations such as nodular hyperplasia and adenomas, FcγBP expression was significantly reduced in thyroid (papillary and follicular) carcinomas. Assessment of FcγBP gene expression might thus be helpful in distinguishing benign from malignant thyroid alterations, particularly if it could be utilized on material obtained from fine-needle aspiration biopsies.

Materials and Methods

Tissue samples and cell lines

Thyroid tissue samples (from 6 papillary carcinomas, 3 follicular carcinomas, 6 adenomas, 3 oxyphilic (Hurthle cell) adenomas, 4 nodular hyperplasias, and normal tissues

from the corresponding thyroids) were obtained from patients undergoing thyroid surgery. The age of the patients at the time of surgery was between 16 and 80 years. Tissue specimens were immediately frozen in liquid nitrogen. Histological diagnoses were made by experienced thyroid pathologists according to the WHO classification of thyroid tumors (Hedinger 1988). The use of patient material and data was approved by the Ethics Committee of the Medical Faculty of the University of Bern.

Four thyroid carcinoma cell lines and one thyroid primary culture were also studied. Three follicular carcinoma cell lines established from a primary tumor (FTC133) and two metastases (FTC236, lymph node; FTC238, lung metastasis) from a single patient, were obtained from P E Goretzki, Dept of Surgery, University of Dusseldorf, Germany. A papillary carcinoma cell line, K1, was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). A thyroid cell culture (24HuSD) was derived from thyroid tissue from a Graves' disease patient.

RNA extraction

Total RNA was extracted from tissue samples by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski & Sacchi 1987). Total RNA was prepared from cell lines using Trizol reagent (Gibco BRL, Bethesda, MD, USA). The quality of each preparation was checked by agarose gel electrophoresis of the RNA samples and of test RT-PCR products.

Restriction-mediated differential display

From each tissue sample, 50 µg total RNA were reverse transcribed using cDNA primer CP28 V (5'-ACC TAC GTG CAG ATT TTT TTT TTT TTT TV-3' with V being a mixture of A, C and G, manufactured by Eurogentec, Seraing, Belgium). Double-stranded cDNAs were digested with MboI for 1 h at 37 °C and ligated to linker ML2025 (prepared by hybridization of oligonucleotides ML20, 5'-TCA CAT GCT AAG TCT CGC GA-3', and LM25, 5'-GAT CTC GCG AGA CTT AGC ATG TGA C-3') overnight at 16 °C. First round amplification was performed, using 1/20th of the ligation mix, in 1 × PCR buffer containing 1.5 mM MgCl₂, 2 µM dNTPs, 0.4 µM each of anchor primer CP28X (5'-ACC TAC GTG CAG ATT TTT TTT TTT TTT TX-3' (X=A, C or G)) and linker primer ML19Y (5'-TGC TAA GTC TCG CGA GAT CZ-3' (Z=A, C, G, or T)), and 1 U AmpliTaq polymerase (Perkin-Elmer, Foster City, CA, USA). After 15 cycles (20 s at 94 °C, 30 s at 60 °C, and 4 min at 72 °C), reactions were supplemented with dNTPs to a final concentration of 200 µM and amplified for another 10 cycles. This protocol will amplify only fragments at the 3' end of the cDNA. Second round

Figure 1 Screen shoot of a LightCycler run. Arbitrary relative fluorescence versus cycle number is shown. Normal and pathologically altered samples have been tested in duplicate, for FcγBP and β-actin expression respectively. Cycle efficiency E (which is the slope of the linear part of the graph) was determined using the formula $\log E = \log F_2 - \log F_1/C_{12} - C_{11}$, where F is the arbitrary relative fluorescence and C_i is the cycle number at the crossing points of the slope with F_1 and F_2 . Factors of up- or down-regulation, R , were calculated using the formula $R = N/P$ where $N = E^{-(C_{12_Fc\gamma BP\ norm} - C_{12_\beta actin\ norm})}$ and $P = E^{-(C_{12_Fc\gamma BP\ path} - C_{12_\beta actin\ path})}$.

amplification was run for 20 cycles using 2 µl of a 1:100 dilution of the first round product and second round primers CP28XX1 (5'-ACC TAC GTG CAG ATT TTT TTT TTT TTT TXX1-3' (X1=A, C, G, or T)) and Bio-ML18ZZ1 (5'-Biotin-GCT AAG TCT CGC GAG ATC ZZ1-3' (Z1=A, C, G, or T)). This step increases the resolution and sensitivity of the detection of the 3'-cDNA fragments. Each primer set allows the amplification and analysis of a defined subset of fragments. Reactions were analyzed on a 6% denaturing polyacrylamide gel on a GATC 1500 Direct Blotting Electrophoresis apparatus (Beck & Pohl 1984) (GATC GmbH, Konstanz, Germany).

Electrophoresis took place for 3–4 hours with a constant power of 30 W applied. After a pre-run of 45 min, the conveyor belt with a 40-cm piece of Direct Blotting Membrane (GATC GmbH) attached was started. Transport speed was 12 cm/h. After air-drying, the membrane was UV fixed (10 000 µJ/cm²) in a Stratilinker (Stratagene, La Jolla, CA, USA) and color detection was

performed using a streptavidin-alkaline phosphatase conjugate (Sigma) and NBT/BCIP (Roche Molecular Biochemicals, Mannheim, Germany). Bands of interest were excised and stored in 50 µl 10 mM Tris-HCl/1 mM EDTA, pH 8.0, at 4 °C. Reamplification was performed on the excised bands in a PCR mixture containing 0.8 µM each of the previously used second round primers, 1.5 mM MgCl₂, 200 µM dNTPs, and 1 U AmpliTaq polymerase under the above cycling conditions, for 30 cycles. Reamplification products were gel-purified by a second round of direct blotting electrophoresis and band recovery. Purified fragments were sequenced using the cycle sequencing protocol on a 373 DNA sequencer (Perkin Elmer) using the primer CP28XX₁.

FcγBP RT-PCR

Two micrograms total RNA were reverse transcribed in a 20 µl RT reaction containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 1 mM

Table 1 List of differentially expressed genes in papillary thyroid carcinomas. Some of these genes are part of other expression studies (data not shown)

Gene	Accession number
IgG Fc binding protein	(FcγBP)
Alpha-B-crystallin	S45630
Cadherin-16	AF016272
EST	AA962212
Chromosome 7 sequence	AC006350
Alpha 1-antitrypsin	X01683
Lactadherin (BA46)	U58516
Fc-epsilon-receptor gamma chain	M33195
Fibronectin	X02761
Glycoprotein-39	M80927
Laminin-5 beta 3 chain	D37766
EST	AI738984

dNTPs, 1 μM oligo dT₁₅ primer (Roche Molecular Biochemicals), 20 U RNasin (Promega, Madison, WI, USA) and 25 U M-MLV reverse transcriptase (Promega). PCR was performed with primers specific for IgG Fc binding protein (FcγBP): FcγBP forward (5'-CTG AGT ACT TCC GCC AAT GC-3') and FcγBP reverse (5'-GAG TTT ACC GGC AAG TAT CG-3'). As an internal control, PCR was also performed with β-actin primers: β-actin forward (5'-CCT CGC CTT TGC CGA TCC-3') and β-actin reverse (5'-GGA TCT TCA TGA GGT AGT CAG TC-3') (Raff *et al.* 1997). The RT reaction was diluted to 50 μl, and 2 μl were used as template in a 25 μl PCR containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1 × Triton X-100, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.4 μM of each primer and 1.25 U Taq polymerase (Promega). Cycling conditions were 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C for 30 cycles. PCR products were visualized by ethidium bromide staining of agarose gels.

Real-time quantitative PCR

Two micrograms total RNA were reverse transcribed in a 20 μl reaction as described above. Primers designed for real-time PCR were: β-actin forward, 5'-CTG CGT GTG GCT CCT GAG GAG CA-3'; β-actin reverse, 5'-CCA GTG TGA CGG CCA GAG GCG T-3'; FcγBP forward, 5'-TAC CGC CAA TGA TCG GCC AT-3'; and FcγBP reverse, 5'-GCC CTG GAG AGA CAG CTT-3'. Quantitative PCR was performed using the LightCycler DNA Master SYBR Green I mix (Roche Molecular Biochemicals), 4 mM MgCl₂ and 10 pM each of forward and reverse primers. Reactions were performed in triplicate. Amplification was performed in a Light-Cycler Thermocycler (Roche Molecular Biochemicals) and cycle parameters were 95 °C no hold, 59 °C for 6 s, and 72 °C for 30 s. For each sample, expression levels of

Figure 2 Restriction-mediated differential display membrane after electrophoresis on the Direct Blotting Electrophoresis system (GATC GmbH) and colorimetric detection of biotinylated PCR products. P, a papillary thyroid carcinoma sample and N, a normal thyroid sample. The differentially expressed band indicated by the arrow is the FcγBP fragment.

FcγBP mRNA were calculated, relative to β-actin, from the semi-logarithmic graphs of relative fluorescence versus cycle number (Fig. 1).

Cycle efficiency E (which is the slope of the linear part of the graph) was determined using the formula $\log E = \log F_2 - \log F_1 / C_{t2} - C_{t1}$, where F is the arbitrary relative fluorescence and C_t is the cycle number at the crossing points of the slope with F₁ and F₂. Factors of up- or down-regulation, R, were calculated using the formula $R = N/P$ where

$$N = E^{-(C_{t2_{Fc\gamma BP \text{ norm}}} - C_{t2_{\beta actin \text{ norm}}})}$$

and

$$P = E^{-(C_{t2_{Fc\gamma BP \text{ path}}} - C_{t2_{\beta actin \text{ path}}})}$$

Statistics

Statistical analysis was carried out using the StatView package (Abacus concepts, Berkeley, CA, USA). A three-sample comparison was performed for the ratio of FcγBP mRNA expression levels using the Kruskal-Wallis test for non-parametric data. The ratios compared were those found in malignant thyroid tissue relative to normal

Figure 3 (A) FcγBP and β-actin RT-PCR analysis of tissue samples from papillary carcinomas (P), follicular carcinomas (F), follicular adenomas (A), and normal thyroid tissues (N). The RT was performed using 2 μg total RNA with an oligo dT₁₅ primer and PCRs were performed for 25 cycles with an annealing temperature of 60 °C using primers specific for FcγBP and β-actin. The molecular weight marker in lane 1 is a 100 bp ladder. (B) RT-PCR analysis of RNA from cell cultures. RT-PCR conditions were as described for (A). FTC133 was derived from a primary follicular carcinoma and FTC236 and FTC238 were derived from a lymph node and a lung metastasis of the same follicular carcinoma. 24HuSD is a primary culture of thyroid tissue from a Graves' disease patient and K1 is a papillary thyroid carcinoma cell line.

thyroid tissue, in Hurtle cell adenomas relative to normal thyroid tissue and in thyroid adenomas and hyperplasias relative to normal thyroid tissue. A *P* value of less than 0.05 was considered significant.

Results

Differential display was performed using a modified technique, developed by A Fischer, named restriction-mediated differential display (RMDD) (Fischer 1995).

Initially, RMDD was performed with RNA from two thyroid papillary carcinomas and corresponding normal thyroid tissue from the same patients. Twelve cDNA fragments showing differential expression were purified and sequenced (Table 1). One of the cDNA fragments, approximately 175 bp in size (indicated by an arrow in Fig. 2), the expression of which was reduced in the papillary thyroid carcinoma samples, showed 99% homology to FcγBP (Harada *et al.* 1997). This gene was selected for further analysis because its expression has not previously

been described in thyroid tissue. Reduced expression of FcγBP mRNA in the papillary carcinoma samples was confirmed by RT-PCR (Fig. 3A).

To obtain quantitative data on the expression of FcγBP mRNA in thyroid carcinomas, thyroid adenomas, thyroid nodular hyperplasia and normal thyroid tissue, we performed real-time quantitative RT-PCR, which measures the incorporation of fluorescent SYBR green dye into newly synthesized double stranded DNA, after each round of elongation. Levels of FcγBP mRNA were normalized relative to β-actin mRNA, which was used as a control and internal standard. Table 2 shows the ratio of the relative expression of FcγBP mRNA between pathologically altered and normal thyroid tissues. In all carcinoma samples tested from six papillary and three follicular cancers, levels of FcγBP mRNA expression were lower than in the corresponding normal thyroid samples (ratio <1) (Table 2). In contrast, all six of the thyroid adenoma samples tested showed higher levels of FcγBP mRNA than the corresponding normal tissue (ratio >1). In three of the four thyroid nodular hyperplasias

Table 2 Expression of FcγBP mRNA in normal and pathological tissue, normalized to the respective expression of β-actin mRNA and ratio of FcγBP mRNA expression in benign and malignant thyroid nodules versus normal thyroid tissue, determined by quantitative real-time quantitative PCR

	Age	Sex	Diagnosis	Normal	Pathological	Ratio of FcγBP
Patient						
1	32	f	PTC	0.071	0.035	0.5
2	21	f	PTC	0.4	0.03	0.1
3	32	f	PTC	0.19	0.03	0.2
4	64	f	PTC	0.4	0.033	0.1
5	34	f	PTC	0.152	0.079	0.5
6	16	f	PTC	0.32	0.082	0.3
7	47	f	FTC	0.245	0.032	0.1
8	36	f	FTC	0.315	0.181	0.6
9	76	m	FTC	0.0257	0.0098	0.4
10	19	f	OA	0.257	0.09	0.4
11	61	f	OA	0.215	0.07	0.3
12	26	f	OA	0.274	0.211	0.8
13	84	m	FA	0.062	0.204	3.3
14	58	f	FA	0.0489	0.271	5.5
15	69	m	FA	0.0025	0.026	10.4
16	60	m	FA	0.234	0.52	2.2
17	66	m	FA	0.0082	0.03	3.7
18	62	f	FA	0.425	0.622	1.5
19	31	f	NH	0.263	0.257	1.0
20	50	f	NH	0.244	0.583	2.4
21	71	f	NH	0.0191	0.236	12.4
22	73	f	NH	0.316	0.712	2.3

PTC, papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; OA, oxyphilic adenoma; FA, follicular adenoma; NH, nodular hyperplasia.

tested, FcγBP mRNA expression was also higher in the hyperplastic nodule than in the corresponding normal thyroid tissue (ratio >1). The fourth hyperplasia sample showed similar levels of FcγBP mRNA in the hyperplastic nodule and the normal tissue (ratio=1). However, three Hurthle cell adenomas (oxyphilic adenomas) showed lower levels of FcγBP mRNA expression than normal tissue (ratio <1).

FcγBP expression was also assessed quantitatively (data not shown) and semi-quantitatively in four thyroid carcinoma cell lines (a papillary carcinoma cell line (K1), 3 follicular carcinoma cell lines (FTC133, FTC236, and FTC238)), and a primary cell culture of thyroid tissue from a Graves' disease patient (24HuSD). FcγBP mRNA expression was only detected in the 24HuSD Graves' disease primary cell culture (semi-quantitative results Fig. 3B).

Statistical analysis confirmed that the ratio of FcγBP mRNA expression in malignant relative to normal thyroid tissue (mean=0.311) and Hurthle cell adenomas (relative to normal tissue) (mean=0.5) is significantly lower than in benign follicular adenomas and nodular hyperplasia (relative to normal tissue) (mean=4.47) ($P=0.0003$, Kruskal-Wallis test).

Discussion

The purpose of this study was to identify genes that are differentially expressed in malignant and benign thyroid tumors. Such genes could be useful in the important distinction between the majority of thyroid nodules, which are benign, and the minority of thyroid nodules, which are malignant.

The FcγBP gene investigated in this study has potential as a genetic marker in thyroid cancer. In each of the malignant thyroid tumors tested the ratio of FcγBP mRNA expression (relative to normal tissue) was less than one, whereas in all the thyroid adenomas and in three out of four of the hyperplastic nodules the ratio of FcγBP expression was greater than one. Measurement of FcγBP mRNA expression in thyroid tumors and surrounding normal tissues would thus have enabled us to predict the benign or malignant nature of these thyroid nodules.

Oxyphilic (Hurthle cell) adenomas seem to be an exception to the above rule since – contrary to the findings in thyroid follicular adenomas – FcγBP mRNA expression in the oxyphilic adenoma samples tested was lower than in the surrounding normal thyroid tissue. This

suggests a difference in the regulation of FcγBP mRNA expression between thyroid oxyphilic and follicular adenomas.

Diagnosis of thyroid cancer usually involves ultrasound-guided fine-needle aspiration biopsy. Papillary carcinomas have distinctive cytological features, which are usually easily recognized in a fine-needle aspiration biopsy specimen. However, follicular carcinomas are often difficult to distinguish from follicular adenomas because they have a similar microscopic appearance (Fagin 1994). RT-PCR for a carcinoma-specific marker, from fine-needle aspiration biopsies, would therefore be an attractive alternative to cytological examination. Fine-needle aspiration biopsy RT-PCR for oncofetal fibronectin has already shown promising results for the identification of papillary carcinomas (Takano *et al.* 1997). If down-regulation of FcγBP expression is characteristic of all thyroid carcinomas, measurement of FcγBP mRNA expression (using RT-PCR) could have great potential as a diagnostic marker to distinguish between benign and malignant thyroid tumors, in particular between follicular adenomas and follicular carcinomas. The fact that FcγBP mRNA expression seems to be down-regulated in Hurthle cell adenomas should not pose too great a problem, since Hurthle cell adenomas have quite distinct cytological characteristics (Hedinger 1988). It will be interesting to measure FcγBP mRNA expression in Hurthle cell carcinomas to determine if regulation of FcγBP expression differs between Hurthle cell adenomas and carcinomas.

The precise role of the product of the FcγBP gene, the expression of which was analyzed in this study, is as yet unclear. FcγBP was first identified as an IgG Fc binding site in intestinal and colonic epithelia (Kobayashi *et al.* 1989). It binds monomeric and aggregated IgG but is immunologically distinct from already known IgG Fc receptors (Kobayashi *et al.* 1989). It is produced by goblet cells in the colon and seems to be secreted into the bowel lumen with mucus, which suggests that it might be involved in immune protection and inflammation in the intestine. The role of FcγBP in the thyroid is equally unclear. It might possibly also contribute to immune protection in the thyroid. Down-regulation of FcγBP expression in papillary and follicular carcinomas could potentially facilitate immune evasion and therefore play a role in thyroid carcinogenesis and the progression of thyroid cancer.

In summary, we report the identification of FcγBP as a differentially expressed gene in thyroid papillary and follicular cancer. We found that FcγBP is expressed in normal thyroid and, more significantly, it is down-regulated in papillary and follicular thyroid carcinomas. Further studies are required to determine the function of FcγBP in the thyroid and the feasibility of using FcγBP expression as a diagnostic marker or prognostic indicator for thyroid cancer.

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