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RESEARCH

Amplification of Thymosin Beta 10 and AKAP13 Genes in Metastatic and Aggressive Papillary Thyroid Carcinomas

Liliána Z. Fehér · Gábor Pocsay · László Krenács · Ágnes Zvara · Enikő Bagdi · Réka Pocsay · Géza Lukács · Ferenc Győry · Andrea Gazdag · Erzsébet Tarkó · László G. Puskás

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Abstract Papillary thyroid carcinoma (PTC) is the most common well-differentiated thyroid cancer. Although the great majority of the cases exhibit an indolent clinical course, some of them develop local invasion with distant metastasis, and a few cases transform into undifferentiated/anaplastic thyroid carcinoma with a rapidly lethal course. To identify gene copy number alterations predictive of metastatic potential or aggressive transformation, array-based comparative genomic hybridization (CGH-array) was performed in 43 PTC cases. Formalin-fixed and paraffin-embedded samples from primary tumours of 16 cases without metastasis, 14 cases with only regional lymph node metastasis, and 13 cases with distant metastasis, recurrence or extrathyroid extension were analysed. The CGH-array and confirmatory quantitative real-time PCR results identified the deletion of the EIF4EBP3 and TRAK2 gene loci, while amplification of thymosin beta 10 (TB10) and Tre-2 oncogene regions were observed as general markers for PTC. Although there have been several studies implicating TB10 as a specific marker based on gene expression data, our study is the first to report on genomic amplification. Although no significant difference could be detected between the good and bad prognosis cases in the A-kinase anchor protein 13 (AKAP13) gene region, it was discriminative markers for metastasis. Amplification in the AKAP13 region was demonstrated in 42.9% and 15.4% of the cases with local or with distant metastasis, respectively, while no amplification was detected in non-metastatic cases. AKAP13 and TB10 regions may represent potential new genomic markers for PTC and cancer progression.

Keywords Thymosin beta 10 (TB10) · Tre-2 oncogene · AKAP13 · Genomic amplification · Metastasis · Papillary thyroid carcinoma (PTC)

Abbreviations Used

AKAP13	A-kinase anchor protein 13
BPTC	Papillary thyroid carcinoma with a bad disease outcome
CGH-array	Array-based comparative genomic hybridization
DOP-PCR	Degenerate oligonucleotide-primed PCR
EIF4EBP3	Eukaryotic initiation factor 4E-binding protein 3
FGF7	Fibroblast growth factor 7
GPTC	Papillary thyroid carcinoma with a good disease outcome

L. Z. Fehér · Á. Zvara · L. G. Puskás (✉)
Laboratory of Functional Genomics, Biological Research Centre,
Hungarian Academy of Sciences,
P.O. Box 521, Szeged 6701, Hungary
e-mail: pusi@brc.hu

G. Pocsay · R. Pocsay · A. Gazdag
1st Department of Internal Medicine,
Pándy Kálmán County Hospital,
Gyula, Hungary

L. Krenács · E. Bagdi
Laboratory of Tumour Pathology and Molecular Diagnostics,
Szeged, Hungary

G. Lukács · F. Győry
1st Department of Surgery, Medical and Health Science Centre,
University of Debrecen,
Debrecen, Hungary

E. Tarkó
2nd Department of Internal Medicine,
Borsod-Abaúj-Zemplén County Hospital,
Miskolc, Hungary

GPTC1	Papillary thyroid carcinoma with a good disease outcome without metastasis
GPTC2	Papillary thyroid carcinoma with a good disease outcome with regional lymph node metastasis
LOH	Loss of heterozygosity
PTC	Papillary thyroid carcinoma
QRT-PCR	Quantitative real-time polymerase chain reaction
TB10	Thymosin beta 10
TRAK2	Trafficking protein, kinesin binding 2
UTC	Undifferentiated thyroid carcinoma

Introduction

Papillary thyroid carcinoma (PTC) is the most common well-differentiated thyroid cancer. Although the great majority of the cases exhibit an indolent clinical course with a very high cure rate, approximately 10–15% of the cases develop distant metastasis, local invasion, recurrence, and a few cases transform into undifferentiated/anaplastic thyroid carcinoma (UTC) with a rapidly lethal course [1–3]. Although many parameters have been described that may allow stratification of the clinical risk of recurrence and death, including age, tumour size, the presence of distant metastasis, histological subtype and clinical staging (TNM, AGES, MACIS, IGR, EORTC and OSU), none of them has been found to be sufficient for a correct prognostication of the individual disease course [4–6].

Various molecular markers have been studied in PTC. Mutations leading to aberrant activation in the RAS-BRAF-MAPK pathway have been detected in up to 80% of PTC cases [7–15]. All three RAS genes (NRAS, KRAS and HRAS) can be mutated in 10% of adult sporadic PTCs [15], and the RAS mutations may be associated with aggressive tumour phenotypes [16]. The RAS-BRAF-MAPK pathway can also be activated by A-kinase anchor protein 9 (AKAP9)-BRAF fusion [17]. Rearrangements of the RET/PTC and NTRK1 proto-oncogenes in PTC have been widely studied [8, 11, 12, 14, 18–22]. The data on the roles of RET and BRAF gene alterations in the development of UTC are controversial [7, 8, 10, 12, 19]. Anomalies of the TP53 [23, 24], RAS [25], and β-catenin [26], genes have been suggested in the evolution into UTC.

DNA-microarray technology is a method widely accepted for the assessment of genomic and transcriptomic changes associated with thyroid cancers with different disease outcomes and in the search for specific genomic fingerprints as prediction markers [27–30]. A number of groups have used an array-based comparative genomic hybridization (CGH-array) approach alone [31–35] or in combination with mutation and gene-expresssion analysis in order to investigate genomic alterations associated with different types of thyroid tumours [33, 36]. Unger et al. recently could identify specific DNA aberration signatures that discriminated between RET/PTC-positive and RET/PTC-negative cases [37]. Kitamura et al. found significantly higher frequencies of loss of heterozygosity (LOH) on the 1q, 4p, 7q, 9p, 9q, and 16q loci among the PTC cases with a poor outcome, though tumour-suppressor genes or proto-oncogenes possibly involved were not identified [38]. Kjellman et al. demonstrated a gain of 1q and a loss of 9q21.3-q32 in PTC cases with a less favourable prognosis [32].

The early recognition of high-risk PTC cases is potentially of great importance. In an effort to identify gene copy number alterations that can predict the clinical outcome in PTC, we have performed a CGH-array study on 43 PTC cases.

Materials and Methods

Papillary Thyroid Carcinoma Samples

Fourty-three patients diagnosed with PTC were enrolled in the study. Formalin-fixed and paraffin-embedded thyroid tissue specimens were obtained from the files of the University of Debrecen (16 cases), University of Szeged (5 cases), the Jósa András County Hospital in Nyíregyháza (10 cases), the Erzsébet Hospital in Hódmezővásárhely (10 cases), the County Hospital in Kecskemét (1 case) and the Pándy Kálmán County Hospital in Gyula (1 case). All these cases were revised histopathologically by LK and EB during the selection procedure. There were 2 microcarcinomas. The study was approved by the local ethical committees.

All patients but one underwent total or near-total thyroidectomy. All were treated and followedup according to accepted protocols [39, 40]. Thirty-eight patients received post-surgical ^{131}I ablation. After 6 months of ^{131}I ablation"off thyroxine" Tg, Tg antibody measurement and an ^{131}I scan were performed, according to the consensus protocol [39]. These examinations were repeated yearly as long as they were needed. Repeated ^{131}I therapy, further surgery and oncological treatment were carried out in specific cases. In certain cases, CT, MR or FDG-PET was additional by performed.

Of the 43 PTC cases studied, 16 revealed no metastasis or recurrence (patients with a good disease outcome = GPTC1), while 14 presented with regional lymph node metastasis only (GPTC2), and 13 with an aggressive clinical course (a bad disease outcome = BPTC) (12 with distant metastases, 10 with recurrence, and 4 with extrathyroidal extension). The relevant data are given in Table 1.

Table 1 Summary of PTC cases studied. *GPTC1* papillary thyroid carcinoma cases without metastasis; *GPTC2* papillary thyroid carcinoma cases with only regional lymph node metastasis; *BPTC* papillary thyroid carcinoma cases with bad prognosis;
*2 microcarcinomas

		GPTC1	GPTC2	BPTC
Number of patients		16	14	13
Gender, female/male		15/1	10/4	10/3
Age at diagnosis (y), median (range)		39.9 (21–59)	37.7 (13–54)	59.4 (23–76)
Tumour size (cm), median (range)		1.25 (0.07–4)*	2.7 (0.6–6)	5.6 (1.5–15)
Extrathyroidal extension		0	0	4
Metastases	regional lymph node	0	14	6
	lung	0	0	7
	bone	0	0	5
Recurrence		0	1	11
TNM	Stage I	14	9	0
	Stage II	2	0	1
	Stage III	0	4	3
	Stage IV	0	1	9
Follow-up (y), median (range)		8.4 (3–11)	11.1 (4–26)	6.0 (1–22)
Death		0	0	11

Tumour fragments containing a minimum of 70% tumour cells were removed from the paraffin blocks for DNA extraction. The appropriate tumour field was selected in the relevant H&E-stained sections. Paraffin-embedded tissues (approximately 500 mg from each case) were deparaffinized in xylene, washed in ethanol, and purified with the DNA purification kit from Macherey-Nagel (Düren, Germany), according to the manufacturer's instructions.

Determination of Differences in Relative DNA Copy Numbers

Preparation of Genomic DNA Probes for Array Hybridization of PTC Samples

One hundred nanogram genomic DNA was amplified with a modified degenerate oligonucleotide-primed PCR (DOP-PCR) protocol by using a RotorGene 3000 quantitative real-time PCR (QRT-PCR) instrument, (Corbett Research, Mortlake, Australia) [41, 42]. The DNA concentration was assessed spectrophotometrically by means of NanoDrop (Rockland, DE, USA). Reactions were performed in a total volume of 100 µl. The cycling parameters were as follows: heat start at 95°C (15 min); 8 cycles denaturation at 94°C (50 s); annealing for 2 min from 45°C to 72°C with a 0.2°C/s ramp; and, extension at 72°C (90 s). After 8 cycles, the reaction mix was divided into two 50 µl aliquots and SybrGreen was added to both (1x final concentration, Molecular Probes, Eugene, USA). The following cycling protocol was performed in a QRT-PCR instrument: denaturation at 95°C (40 s); annealing at 58°C (60 s); and, extension at 72°C for 80 s. To avoid over-amplification of the products, cycling was terminated just before the reaction reached the plateau (i.e., 13–17 cycles). In the reactions, UN primer (5′-CCGACTCGAGNNNNNNATGTGG-3′) was used at 1 µM [42]. The reactions were performed with 1x ABsolute QPCR Mix (ABgene, Surrey, UK).

The PCR products were purified on PCR-purification columns (Bioneer, Daejeon, Korea). Three micrograms of amplified genomic DNA was fragmented with AluI restriction endonuclease, and was then tailed with dTTP, resulting in an oligo dT sequence at the 3′ ends of the products. A special capture oligo was subsequently ligated to the tailed DNA according to the Genisphere Array 900DNA labelling system (Genisphere, Hatfield, PA) as described previously [43].

Array Hybridization, Scanning, Data Processing

The tagged DNA was hybridized onto human cDNA microarrays comprising 3200 gene-specific samples in duplicate in a Ventana hybridization station (Ventana Discovery, Tucson, AZ, USA) by using the "antibody" protocol [43]. The CGH array was printed in house and was used as previously described [43]. Hybridization was first performed at 42°C for 6 h in "Chiphybe" hybridization buffer (Ventana) and 2.5 µl of Cy5 capture reagents (Genisphere) was then added to the slides in 200 µl of Chiphybe hybridization buffer (Ventana), followed by incubation at 42°C for 2 h. After hybridization, the slides were washed twice in 0.2x SSC at RT for 10 min and then dried and scanned. The probe and control samples were hybridized onto separate slides.

The presented data were calculated from the results of the four data points obtained from two separate labelling and hybridization protocols. The slides were scanned with a confocal laser scanner (ScanArray Lite, GSI Lumonics, Billerica, MA, USA). Image files were analysed with the GenePix Pro 3.0.5. program (Axon, Union City, CA, USA).

The background-corrected intensity data were filtered for flagged spots and weak signals. After automatic flagging, manual flagging was performed to exclude spots displaying irregularities, such as scratches and dust particles. Technical replicates on the same array were averaged. Data were excluded in cases where the technical replicates differed significantly. Data obtained via the median of feature pixels were accepted only if at least 30% of the pixels were above 2x the standard deviation of the background intensity. Normalization was performed with the print-tip LOWESS method [44]. We next used the one-sample t-test in order to determine the genes to be regarded as changed in copy number. Logarithms of each ratio were taken to satisfy the *t*-test requirement for normal distribution. Genes for which the mean of the log ratios across the biological replicates was equal to zero at a significance level α=0.05 were considered to have an unchanged copy number. On the other hand, genes with a p value smaller than α and the average-fold change (over- or under-representation) of the four data points that were at least 1.5-fold were regarded as changes in DNA copy number.

Detection of DNA Copy Number Changes with QRT-PCR

QRT-PCR was carried out in a final volume of 20 μl with 100 ng of non-amplified genomic DNA in a RotorGene 3000 instrument (Corbett Research, Mortlake, Australia). The reactions were performed with 5 pmol/each of the forward and reverse gene-specific primers in 1x ABsolute QPCR Mix (ABgene, Surrey, UK) with SybrGreen (1 μM final concentration, Molecular Probes, Eugene, USA) with the following protocol: heat start for 15 min at 95°C, 45 cycles of 25 s denaturation at 95°C, 25 s annealing at 59°C and 25 s extension at 72°C. Fluorescent signals were collected after each extension step at 72°C. Curves were analysed with the RotorGene software, through the use of dynamic tube and slope correction methods, data from cycles close to the baseline being ignored. Primers were designed by using the PrimerExpress software (Applied Biosystems, Foster City, CA, USA). Relative ratios were normalized to the copy numbers of alpha1-fetoprotein transcript factor and H3 histone (family 3A) and calculated with the Pfaffl method [45]. The PCR primers used are listed in Table 2. All PCRs were performed three times in separate runs.

Results

The copy number changes were related to two normal non-neoplastic thyroid samples. Relative DNA losses and gains were determined for each tumour sample from the group of patients with an aggressive disease course by normalizing the intensities to the values obtained after hybridization with labelled probes from indolent cases. 20 chromosomal regions exhibiting changes (deletion or amplification) were selected in order to confirm our CGH results with QRT-PCR, which was performed on the original, non-amplified genomic DNA (Table 3). Relative copy number changes (relative ratios) were normalized to the copy numbers of the alpha1-fetoprotein transcription factor and the H3 histone (family 3A) as internal control gene loci.

These results indicated the amplification of TB10 (thymosin beta 10), Image EST-Acc. No. R78712: A-kinase anchor protein 13 (AKAP13), and Tre-2 oncogene, and the deletion of FGF7 (fibroblast growth factor 7), EIF4EBP3 (eukaryotic initiation factor 4E-binding protein 3) and TRAK2 (Trafficking protein, kinesin binding 2) in both indolent and aggressive cases (Table 4).

Table 2 Oligonucleotide sequences (5′–3′) used in QRT-PCR experiments

Gene (Accession No.)	Chromosome location	Forward primer	Reverse primer	Product size (bp)
Tre-2 (X63547)	17p13	TCCAGCGGCCCATTTG	AGGGCGTGGAAAAACGAGAT	57
Thymosin beta 10 (BC016731.1)	2p11.2	AATCGCCAGCTTCGATAAGG	GGTCGGCAGGGTGTTCTTC	67
Image EST (R78712) - AKAP13	15q25	GGTCATGTGTCGTGAAATATTATTGTT	CCCGGCTGAGATTTTACATTTT	76
Euk. initiation factor 4EBP3 (AF038869)	5q31.3	ACCTGGCATGTGGAGTTACAGA	TGGATGCCCCAGGAAGAG	70
TRAK2 (KIAA0549) (AB011121)	2q33	TTGCCTGGACAGTTACAGTTTCC	AATCGGACAATTTAACGTTGTACTACTC	87
Fibroblast growth factor 7 (M60828)	15q15-q21.1	GAACAAAATTTCTAATGCTGCTCAAG	CATCAATCACTGTTGCTATCTTATATACAAG	93
Alpha 1-fetopr. transcript. fact. (U93553)	1q32.1	GGTGTCCAGGAACAAGTCAATG	CTCTGTCTGCTGCGGGTAGTT	71
H3 histone, family 3A (BC081561)	1q41	TGCAGGAGGCAAGTGAGGC	CTGGATGTCTTTTGGCATAATTGTT	101

Table 3 DNA copy number alterations in prognostic group of PTC cases studied Dark-grey denotes DNA gains common amplification of TB10 and AKAP13, while light-grey denotes separately amplification. (*N* no change; *A* amplification; *D* deletion; *F* female, *M* male)

Gene name		TB10		AKAP13		TRE2		TRAK2		FGF7		EIF4EBP3	
		2p11.2		15q25		17p13		2q33		15q15-q21.1		5q31.3	
	Sex (age	Mean (SD)		Mean (SD)		Mean (SD)		Mean (SD)		Mean (SD)		Mean (SD)	
GPTC1	F (32)	1.31 (0.64)	N	1.26 (0.64)	N	2.62 (1.76)	A	0.59 (0.38)	N	0.86 (0.70)	N	0.53 (0.30)	D
	F (49)	1.29 (0.34)	N	0.46 (0.30)	D	1.80 (0.62)	A	0.54 (0.26)	D	0,74 (0.36)	N	0.31 (1.12)	D
	F (48)	1.12 (0.82)	N	0.49 (0.50)	D	2.63 (2.28)	A	0.46 (0.36)	D	0.52 (0.32)	D	0.35 (0.20)	D
	F (33)	0.95 (0.54)	N	0.67 (0.52)	N	2.74 (1.60)	A	0.58 (0.44)	N	0.45 (0.10)	D	0.89 (0.78)	N
	F (52)	1.88 (0.26)	A	1.27 (0.68)	N	3.14 (1.90)	A	0	D	1.11 (0.20)	N	0.52 (0.16)	D
	F (45)	1.14 (0.60)	N	0.98 (0.68)	N	1.64 (0.56)	A	1.10 (0.50)	N	1.13 (0.48)	N	0.69 (0.48)	N
	F (27)	2.96 (2.36)	A	0.81 (0.58)	N	2.19 (0.24)	A	0.64 (0.72)	N	0.97 (0.64)	N	1.20 (0.86)	N
	F (37)	1.18 (0.70)	N	0.64 (1.12)	N	2.24 (1.66)	A	0.48 (0.08)	D	0.91 (0.62)	N	0.40 (0.70)	D
	F (47)	0.70 (0.44)	N	0.89 (0.44)	N	0.69 (0.64)	N	0,09 (0.14)	D	1.65 (0.54)	A	0.18 (0.14)	D
	F (33)	4.07 (2.00)	A	2.88 (2.11)	A	3.87 (3.41)	A	1.85 (0.98)	A	1.20 (0.64)	N	1.86 (1.10)	A
	F (41)	0.72 (0.09)	N	1.36 (0.73)	N	4.06 (2.31)	A	1.51 (0.42)	A	1.68 (0.11)	A	0.76 (0.29)	N
	F (49)	10.21 (6.66)	A	2.99 (2.69)	A	0.94 (0.91)	N	0.94 (0.65)	N	0.70 (0.47)	N	4.37 (3.32)	A
	F (28)	1.78 (0.18)	A	1.23 (0.63)	N	4.14 (2.62)	A	0.81 (0.20)	N	0.81 (0.11)	N	1.15 (0.40)	N
	F (43)	1.33 (0.24)	N	1.02 (0.53)	N	5.67 (3.97)	A	1.04 (0.28)	N	1.25 (0.30)	N	1.03 (0.37)	N
	F (47)	2.01 (0.45)	A	1.34 (0.72)	N	3.66 (2.66)	A	0.78 (0.23)	N	0.95 (0.27)	N	1.15 (0.44)	N
	F (21)	1.89 (0.29)	A	1.04 (0.57)	N	8.63 (4.69)	A	0.86 (0.26)	N	1.00 (0.07)	N	1.10 (0.44)	N
GPTC2	F (27)	1.13 (0.58)	N	0.66 (0.36)	N	3.93 (3.94)	A	0.39 (0.18)	D	0.40 (0.28)	D	0.20 (1.12)	D
	F (19)	0.92 (0.82)	N	1.09 (0.82)	N	1.26 (0.42)	N	0.82 (0.50)	N	1.03 (0.42)	N	0.79 (0.52)	N
	M (13)	2.02 (1.20)	A	0.70 (0.68)	N	3.82 (0.60)	A	0.36 (0.18)	D	0,47 (0.46)	D	0.64 (0.46)	N
	M (48)	1.79 (1.26)	A	0.54 (0.56)	D	3.99 (2.32)	A	0.40 (0.42)	D	0.49 (0.46)	D	0.55 (0.52)	D
	F (35)	4.13 (3.7)	A	0.67 (0.40)	N	3.67 (1.00)	A	0.25 (0.24)	D	1.47 (0.36)	N	0.42 (0.28)	D
	F (48)	2.00 (1.12)	A	0.93 (0.58)	N	3.92 (2.28)	A	0	D	0.72 (0.14)	N	0.58 (0.40)	N
	M (43)	4.27 (2.38)	A	1.86 (1.94)	A	2.33 (1.48)	A	1.14 (0.70)	N	1.16 (0.28)	N	0.37 (0.20)	D
	F (54)	1.67 (0.68)	A	2.25 (2.54)	A	1.73 (0.86)	A	3.39 (2.08)	A	1.90 (0.78)	A	1.10 (0.58)	N
	F (51)	1.35 (0.40)	N	3.28 (1.93)	A	0.75 (0.14)	N	0.58 (0.52)	N	0.99 (0.20)	N	0.39 (0.32)	D
	F (17)	0.83 (0.26)	N	3.64 (3.28)	A	2.82 (0.34)	A	1.06 (0.52)	N	5.26 (1.08)	A	1.14 (0.66)	N
	F (50)	4.87 (2.16)	A	2.38 (2.14)	A	5.00 (3.02)	A	1.08 (0.38)	N	8.14 (1.68)	A	0.85 (0.62)	N
	M (40)	1.32 (0.58)	N	1.30 (0.73)	N	3.24 (1.68)	A	2.03 (1.08)	A	1.18 (0.56)	N	0.73 (0.34)	N
	F (44)	1.02 (0.26)	N	1.12 (0.92)	N	1.97 (1.04)	A	0.72 (0.34)	N	1.18 (0.54)	N	0.81 (0.32)	N
	F (39)	3.03 (1.98)	A	2,36 (1.88)	A	1.51 (0.74)	A	1.33 (0.52)	N	0.65 (0.32)	N	0.79 (0.46)	N
BPTC	F (73)	0.72 (0.26)	N	0.68 (0.44)	N	0.91 (0.36)	N	0.38 (0.30)	D	0.62 (0.38)	N	0.51 (0.38)	D
	F (76)	4.16 (2.38)	A	0.53 (0.56)	D	2.29 (1.38)	A	0.21 (0.22)	D	0.66 (0.72)	N	0.21 (0.12)	D
	F (52)	3.27 (1.58)	A	0.84 (0.80)	N	2.95 (1.20)	A	0.06 (0.08)	D	0,76 (0.60)	N	0.28 (0.20)	D
	F (76)	2.07 (1.32)	A	2.11 (1.32)	A	1.15 (0.82)	N	0.76 (0.30)	N	0.85 (0.64)	N	0.65 (0.40)	N
	M (51)	1.35 (0.68)	N	0.53 (0.40)	D	4.47 (3.30)	A	0.58 (0.46)	N	0.50 (0.36)	D	0.70 (0.54)	N
	F (71)	0.80 (0.30)	N	0.71 (0.62)	N	1.75 (0.50)	A	1.91 (1.04)	A	0.97 (0.34)	N	1.18 (0.70)	N
	F (67)	1.50 (0.68)	A	0.72 (0.38)	N	1.15 (0.64)	N	0.46 (0.40)	D	0.83 (0.84)	N	0.35 (0.22)	D
	M (50)	2.95 (2.20)	A	0.90 (0.64)	N	1.28 (0.92)	N	0.52 (0.52)	D	0.49 (0.30)	D	0.63 (0.30)	N
	M (63)	1.72 (1.32)	A	0.74 (0,76)	N	1.93 (2.10)	A	0.49 (0.64)	D	0.50 (0.44)	D	0.70 (0.52)	N
	F (66)	3.77 (2.50)	A	0.53 (0.42)	D	3.46 (3.18)	A	0.65 (0.42)	N	0.37 (0.44)	D	0.69 (0.52)	N
	F (41)	0.77 (0.36)	N	7.34 (6.65)	A	3.52 (1.80)	A	0.71 (0.48)	N	2.95 (1.84)	A	0.78 (0.44)	N
	F (33)	5.11 (3.23)	A	1.19 (1.19)	N	4.09 (3.91)	A	1.12 (0.75)	N	0.94 (0.62)	N	2.80 (2.06)	A
	F (63)	1.14 (0.11)	N	0.94 (0.49)	N	4.13 (2.45)	A	1.28 (0.33)	N	1.39 (0.12)	N	0.78 (0.28)	N

The Tre-2 oncogene locus was found to be over-represented in 86.7% and 69.2% of the GPTC and BPTC groups, respectively. Deletion of the EIF4EBP3 and TRAK2 gene loci could be detected in most of the cases, with no discriminative marker potential between the BPTC and GPTC groups. The FGF7 locus showed both DNA gains and losses in a few samples in both groups, but loss of the FGF7 locus was observed in more cases in the BPTC group (30.8% vs. 16.7%).

Image EST (Acc. No. R78712) is mapped to the middle of the AKAP13 gene. No significant differences could be detected between the aggressive and indolent cases in the

Table 4 DNA copy number alterations in six chromosomal regions in two groups of PTCs (GPTC: PTC with a good prognosis; BPTC: patients with a bad disease outcome)

	Thymosin beta 10			AKAP13			Tre-2 oncogene			TRAK2			Fibroblast growth factor 7			Eukaryotic initiation factor 4EBP3		
	Deletion	No change	Amplification	Deletion	No change	Amplification	Deletion	No change	Amplification	Deletion	No change	Amplification	Deletion	No change	Amplification	Deletion	No change	Amplification
GPTC	0.0%	50.0%	50.0%	10.0%	63.3%	26.7%	0.0%	13.3%	86.7%	33.3%	53.3%	13.3%	16.7%	66.7%	16.7%	36.7%	56.7%	6.7%
BPTC	0.0%	38.5%	61.5%	23.1%	61.5%	15.4%	0.0%	30.8%	69.2%	46.2%	46.2%	7.7%	30.8%	61.5%	7.7%	30.8%	61.5%	7.7%

AKAP13 and TB10 gene regions. In the AKAP13 region, DNA gains were shown in 2 cases (15.4%) in the aggressive group with distant metastases, and in 6 cases (26.1%) in the indolent group. In the TB10 region, 61.5% of the aggressive group with distant metastases and 50.0% of the indolent group exhibited amplification (Table 4).

However, when the GPTC cases were divided into two groups, with or without local metastasis, significant differences in gene amplification pattern were identified (Table 5). Amplification of the AKAP13 region was detected in 42.9% and 15.4% with local or distant metastasis, respectively, while 12.5% amplification was detected in the PTC cases without metastasis. Small difference between these subgroups could be recorded in case of TB10 gene. 43.8% of the cases with no metastasis displayed amplification, while approximately 60% of those with regional or distant metastasis demonstrated amplification in the TB10 region. As concerns all the other genes investigated in this study, no difference was observed between the metastatic and non-metastatic cases.

Discussion

We performed a CGH-array study in order to identify gene copy number alterations predictive of the clinical behaviour of PTC. Six regions were selected from the results of CGH-array and QRT-PCR confirmation. A relatively small number of regions were selected from the first CGH-array screen, because of the optimization protocol and our focus on specific gene regions. This could be explained in part by the very short probes (~100 nt) in the case of QRT-PCR as compared with larger (>1.5 kb) DNA fragments on the array, sometimes covering more than 500 kb genomic regions.

This study revealed that the Tre-2 oncogene locus was amplified in 86.7% of the GPTC and in 69.2% of the BPTC groups (Table 4). There were no differences in occurrence within the GPTC group with or without metastasis. Genetic elements of Tre-2 are found in chromosomes 5, 18 and 17. In our study, the QRT-PCR probes amplified the 17p13 chromosome region. Many researchers have demonstrated that this oncogene is consistently transcribed in various human cancer cells [46]. The expression of Tre-2 in normal somatic cells, however, has not yet been reported. Tre-2 oncogene seems to encode a non-functional Rab (GAP). Martinu et al. identified this oncogene as a novel regulator of the Arf6-regulated plasma membrane recycling system [47]. Masuda-Robens et al. reported that Tre-2 co-precipitated specifically *in vivo* with the active forms of Cdc42 and Rac1 [48], which play fundamental roles in transformation and actin remodelling, and thus in cancer formation and progression.

Table 5 DNA copy number alterations in six chromosomal regions in three groups of PTCs (GPTC1: PTC cases without metastasis; GPTC2: patients with only regional metastases; BPTC: patients with a bad disease outcome)

	Thymosin beta 10			AKAP13			Tre-2 oncogene			TRAK2			Fibroblast growth factor 7			Eukaryotic initiation factor 4EBP3		
	Deletion	No change	Amplification	Deletion	No change	Amplification	Deletion	No change	Amplification	Deletion	No change	Amplification	Deletion	No change	Amplification	Deletion	No change	Amplification
GPTC1	0.0%	56.3%	43.8%	12.5%	75.0%	12.5%	0.0%	12.5%	87.5%	31.3%	56.3%	12.5%	12.5%	75.0%	12.5%	37.5%	50.0%	12.5%
GPTC2	0.0%	42.9%	57.1%	7.1%	50.0%	42.9%	0.0%	14.3%	85.7%	35.7%	50.0%	14.3%	21.4%	57.1%	21.4%	35.7%	64.3%	0.0%
BPTC	0.0%	38.5%	61.5%	23.1%	61.5%	15.4%	0.0%	30.8%	69.2%	46.2%	46.2%	7.7%	30.8%	61.5%	7.7%	30.8%	61.5%	7.7%

Deletion of the EIF4EBP3 gene loci could be detected in 36.7% of the GPTC group and 30.8% of the BPTC group, indicating no discriminative marker potential between the groups. Protein coding for EIF4EBP3 is the negative regulator of protein biosynthesis and the initiation of translation; it therefore represses translation in this region.

The TRAK2 gene was under-represented in almost half of the cases, regardless of their disease outcome (33.3% of the GPTC and 46.2% of the BPTC group). Recent CGH study on thyroid cancer revealed similar genomic alterations in a genomic region with close proximity of the TRAK2 gene (2q33.3) [37]. TRAK2 is a member of a coiled-coil family of proteins and has roles in protein and organelle anterograde transport in excitable cells [49, 50]. The under-representation of this kinesin-associated protein gene may result in a dysfunction of the trafficking of organelles and possibly a role in cancer cells.

The FGF7 locus displayed both DNA gains and losses in a few samples in both groups, but amplification of the FGF7 locus was found in more cases in the BPTC group (30.8% vs. 16.7%). Yashuara et al. detected FGF7 over-expression in advanced ovarian neoplasms, and a possible significant role of this over-expression in the development of these neoplasms is implied [51]. Furthermore, the inhibitory role of FGF7 is suggested on the induction of apoptosis in breast neoplasms [52], through the up-regulation of bcl-2; and FGF7 may play a role in limiting mechanically induced apoptotic processes at the epithelial wound edge [53]. It has been demonstrated that FGF family members play roles in embryonic development, cell growth, morphogenesis, tissue repair, tumour growth and invasion. Besides the role of FGF7 in some neoplastic samples, the occurrence of gene amplification in our patient population was low, and it therefore cannot be used as a potential marker for discrimination.

Image EST (Acc. No. R78712) is mapped to the middle of the AKAP13 gene. No significant differences were detected in the AKAP13 gene regions between the BPTC and GPTC cases. DNA gains were observed in 2 cases (15.4%) in the BPTC group with distant metastases, and in 6 cases (26.1%) in the GPTC group. However, when the GPTC cases were divided into two groups (with or without local metastasis), significant differences in gene amplification pattern could be identified (Table 5). Amplification of the AKAP13 region was detected in 42.9% and 15.4% of those with local or distant metastasis, respectively, while 12.5% amplification was detected in the PTC cases without metastasis. AKAP13 anchors both protein kinase A and 14-3-3, and thus inhibits the Rho-GEF activity of the AKAP-Lbc signalling complex [54]. At least 3 transcript variants of this gene encode different isoforms containing a *dbl* oncogene homology (DH) domain and a pleckstrin homology (PH) domain. Shibolet et al. concluded that AKAP13

presumably plays a role in TLR2-mediated NF-kappaB activation [55]. Furthermore, a significant connection was observed between the AKAP13 Lys526Glyn variant and an increased risk of the development of breast cancer; and this marker might have a role in the susceptibility to other [56]. Additionally, Unger and his co-workers found similar genomic alterations in a genomic region with close proximity of the AKAP13 gene (15q26.1) [37]. Moreover, our findings suggest a possible connection between the amplification of AKAP13 and metastasis, which could be studied in more detail (with gene and/or protein expression) with PTC and other cancer samples with or without metastatic forms.

In the TB10 region, 61.5% of the BPTC group with distant metastases and 50.0% of the GPTC group exhibited amplification (Table 4). When we focused on the metastatic cases, we found that 43.8% of the cases with no metastasis displayed amplification, while 60% of those with regional or distant metastasis had amplification in the TB10 region (Table 5). It has previously been shown that an altered TB10 gene expression can function as a possible tool in the diagnosis of thyroid neoplasias [57, 58], since TB10-positive staining is found in all human thyroid carcinomas, and particularly in the anaplastic histotypes. Some observations suggest that TB10 plays a significant role in the cellular processes controlling apoptosis [59]. Maelan et al. presumed that TB10 has a physiological role in the sequestration of G-actin and in cancer cell motility [60]. In a study by Takano et al. [58], TB10 mRNA was highly over-represented in human and experimental thyroid tumours and its expression was profuse in undifferentiated thyroid carcinomas [58, 61]. Other authors have shown that the TB10 gene is highly expressed in human thyroid carcinoma cell lines and tissues, whereas its expression was not observed in the normal thyroid. These results seem to be closely related to our results in connection with genomic rearrangement. Determination of the level of expression of the TB10 gene may be a useful marker for the diagnosis and prognosis of a large variety of human cancers [62]. Over-expression of TB10 has been reported in thyroid, pancreatic, gastric, breast, ovarian, uterine, colon and oesophageal cancer, melanoma and germ cell tumours. Lee et al. described that the up-regulation of TB10 significantly inhibited vascular endothelial growth factor-induced endothelial cell proliferation, migration, invasion and tube formation *in vitro* by interfering with the Ras function [63]. Santelli et al. demonstrated that TB10 protein synthesis suppression reduces the growth of human thyroid carcinoma cells [64]. Although there have been several studies implicating TB10 as a specific marker on the basis of gene expression data, our study is the first to report on genomic amplification. Previous and our present results imply that TBs could possibly influence carcinogenesis and particularly metastatic potential of tumours [65] and it would be worthwhile to screen this locus in more samples and in other cancer types in order to further validate this marker.

In conclusion, we have detected rearrangements of six regions of the genome in a significant number of PTCs; some PTCs have already been described as having an altered gene expression and a possible role in the development and progression of tumours. In order to apply these genomic rearrangements as reliable diagnostic markers, further studies involving several hundred samples are needed. However, our results already indicate that the synchronous detection of the amplification of TB10 and AKAP13 has the potential to become a good diagnostic (or possibly prognostic) marker for the development of metastases in PTC patients. Our results lead us to suggest that when it is intended to detect genetic markers such as genomic rearrangements or gene expression markers, it is worthwhile to initiate further studies on finding novel genomic markers that could differentiate cases within the GPTC group: cases with and cases without metastasis.

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Conflict of interest statement We declare that we have no conflict of interest.

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