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PCR amplification of CGRP II mRNA

Variable expression in tumoral and non-tumoral human thyroid

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Two genes code for calcitonin gene-related peptides (CGRPs). One expresses by tissue-specific alternate splicing calcitonin and CGRP I mRNAs, the other CGRP II mRNA. Calcitonin is the marker of sporadic or hereditary human medullary thyroid carcinoma (MTC). CGRP II expression is not well established in normal or tumoral thyroid. After amplification by polymerase chain reaction, CGRP I and II mRNAs were detected in six cases of MTC associated with other endocrine neoplasia (MEN IIa) and in two cases of isolated MTC. CGRP I was detected in all non-C cell tumoral thyroids (6 samples), CGRP II was barely detectable in three out of six cases. CGRP II could be a specific tumoral marker of MTC.

Medullary thyroid carcinoma; Calcitonin gene-related peptide; Calcitonin; Amplification; Tumoral marker; Hereditary cancer

1. INTRODUCTION

Medullary thyroid carcinoma (MTC), though a rare tumor (0.004%), occurs in familial and sporadic forms. It is a clinically heterogeneous disease, either isolated (MTC 'only') or associated with other endocrine neoplasia (multiple endocrine neoplasia) in the clinical syndromes of MEN IIa (MTC and pheochromocytoma, and/or parathyroid adenoma) and MEN IIb (MTC and dysmorphic habitus).

Calcitonin (CT) is the biological marker of MTC [1]. Measurement of basal and stimulated levels of the hormone represents the basis of the diagnosis, prognosis and detection of high risk individuals in the hereditary form of the disease [2]. However, the CT gene (CALC I) expresses a neuropeptide 'calcitonin gene related peptide' (CGRP) in addition to CT. Alternate splicing of the transcript of the CT/CGRP gene [3] leads to the formation of two specific mRNAs having a common 5'-terminal region and 3'-regions specific for CT or CGRP. Expression of these peptides is tissue specific, as CT is preferentially expressed in thyroid and CGRP in the central and peripheral nervous system [4]. A second CT-related gene (CALC II) codes for CGRP II but is a pseudogene for CT.

Expression of CT-related genes in normal and tumoral thyroid tissues is not well studied: CT is detected in all thyroid tissues whereas it is not known if CGRP I and II are co-expressed or not in tumoral tissues [5,6]. In the case of isolated MTC, CGRP I has been detected by radioimmunoassay [7]. In non-tumoral thyroid tissues CGRP I and CGRP II messengers were not detected by Northern blot analyses [8] whereas both peptides were detected by radioimmunoassay [9].

In studies of the expression of CT/CGRP gene in MTC patients we failed to detect CGRP mRNAs in samples of non-tumoral thyroid tissues using blot or Northern analysis. We have therefore used the polymerase chain reaction to amplify specifically transcripts of CALC I and CALC II genes. In all samples of medullary thyroid tumors studied CT, CGRP I and CGRP II were detected by ethidium bromide staining. In normal tissues, only CT was visualized by ethidium bromide fluorescence, hybridization of the amplified RNAs transferred to a nylon membrane with specific probe was positive for CGRP I in all control samples and barely detectable for CGRP II in 3 out of 6 control tissues. These results imply that expression of CGRP II is extremely low in non-tumoral C cells thyroid tissues and the tumorogenicity is apparently always accompanied by an increased production of CGRP II.

2. MATERIALS AND METHODS

2.1. *Tissue samples and isolation of RNA*

Medullary thyroid carcinomas were obtained from patients suffering from MEN IIa (5 familial and 1 apparently sporadic cases) and sporadic MTC 'only' (2 cases). Control tissues were obtained from non-tumoral thyroids (5 cases), and from an oxyphil cell carcinoma presenting an important C cell hyperplasia. Samples were rapidly frozen in liquid nitrogen. Total RNA was extracted by phenol/chloroform, purified by LiCl precipitation [10] and quantified by absorbance at 260 nm.

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Table I

Primers used in the PCR reactions

RNA corresponding	Primers used 5′ → 3′	Product size	Exons spanned	Ref.
CT	CCGCCTCTCTGATCCAAGCC CCAGGTGCTCCAACCCC	392 bp	1-2-3-4	[11]
CGRP I	CCGCCTCTCTGATCCAAGCC GGTGGCTGACGGGGCCTAGA	709 bp	1-2-3-5-6 (CALC I)	[12,13]
CGRP II	CGGCCGCTCGCGCTGCCCTG GGTGGAGCTGCATGATCAAC	737 bp	1-2-3-5-6 (CALC II)	[14]

2.2. *Amplification method*

2.2.1 *Reverse transcriptase reaction*

The first cDNA strand was synthesized from 10 μg of denaturated total RNA, by extension of a 3′-poly(dT) primer (10 pmol), with 200 units of MoMuLV reverse transcriptase (BRL) in a final volume of 20 μl containing 1× reverse transcriptase buffer (BRL), 20 units of RNase inhibitor (Boehringer) and 1 mM of each dNTP.

The reaction was performed at 37°C for 40 min, then stopped on ice.

2.2.2. *Polymerase chain reaction (PCR) protocol*

80 μl× PCR buffer (Cetus) containing 20 pmol each of upstream and downstream specific primers and 2.5 units of AmpliTaq DNA polymerase (Cetus) were added to 20 μl reverse transcriptase reaction mixture.

Amplification was performed on a programmable heater (Perkin Elmer) for 25 cycles. The reaction began with a denaturation step, 94°C for 2 mn followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min (60°C for CGRP II amplification) and elongation at 72°C for 2 min (6 min for the last cycle).

2.3. *Analysis of results*

After agarose gel electrophoresis, PCR products were quantified by two methods.

(i) The gel, containing ethidium bromide, was photographed under UV light using positive/negative instant pack film (Polaroid 665) and the negative analysed by densitometry using a densitometer module (Transidyne General Corp.).

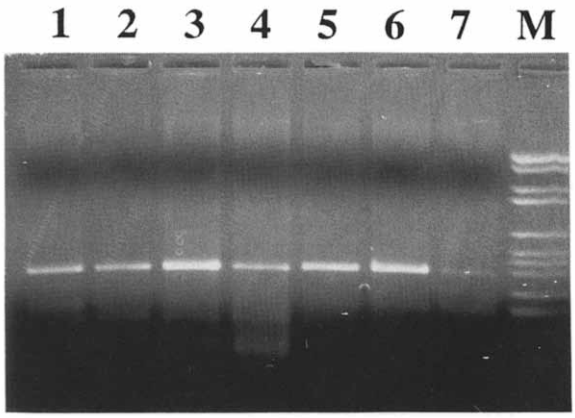

Fig. 1. Agarose gel analysis of PCR products. Total RNA extracted from the tumor was reverse transcribed and amplified with specific CT mRNA primers. One-fifth of the reaction was submitted to gel electrophoresis. (Lanes 1-6) Tumoral tissues; (lane 7) control thyroid. Molecular weight markers (M) consisting of the 2.17 kb, 1.76 kb, 1.23 kb, 1.03 kb, 0.65 kb, 0.51 kb, 0.45 kb, 0.39 kb, 0.29 kb, 0.22 kb and 0.15 kb *Bgl*I and *Hin*fI restriction fragments of pBR 328. The 1.2% agarose gel contained 0.5 μg/ml ethidium bromide.

(ii) The DNA was blotted on Gene Screen membrane (New England Nuclear, Boston, MA) and hybridized with specific probes using an already-described protocol [15]. The probes corresponded respectively to 116 base pairs of CALC I exon 4 coding for CT and 126 base pairs of CALC I exon 5 coding for CGRP I. They were labeled by random primer reaction [16] using [α-^{32}P]dCTP to a specific activity of $2-5\times10^8$ cpm/μg DNA.

3. RESULTS

3.1. *CT mRNA*

Ethidium bromide staining of agarose gel electrophoresis of amplified products using CT mRNA specific primers revealed a single, strongly stained band in all six MEN IIa patients corresponding to the expected size (392 bp) (Fig. 1: lanes 1-6) and in both cases of isolated MTC (data not shown). No such signal was observed in non-MTC samples with the exception of a C cell hyperplastic sample (Fig. 1: lane 7) in which a moderately stained band was observed. After transfer of the gels to Gene Screen membrane and hybridization to a specific CT probe a faint band of the same size was observed in the autoradiographies of all the control samples. Fig. 2 shows the autoradiography of three control cases and an MTC case.

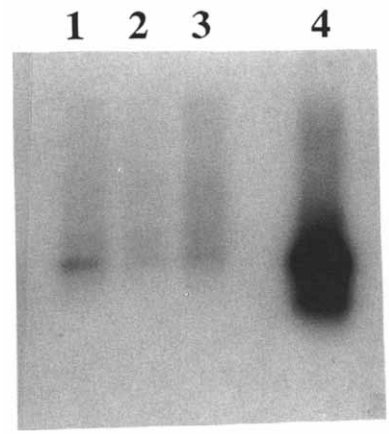

Fig. 2. Autoradiogram after hybridization, with CT-specific probe, of a Southern blot with PCR products amplified with specific CT mRNA primers. (Lanes 1-3) Control tissues, each sample represents 1/4 of the starting material; (lane 4) MTC tissue, 1/40 of the PCR reaction was analysed.

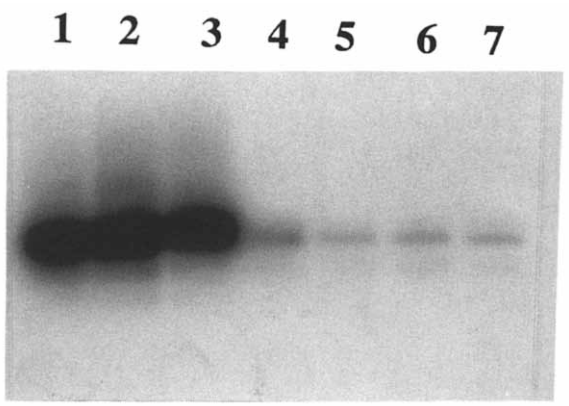

Fig. 3. Autoradiogram after hybridization with CGRP probe of a Southern blot of PCR products amplified with specific CGRP I mRNA primers. (Lanes 1-3) MTC tissues, 1/40 of the reaction was analysed; (lanes 4-7) control tissues, 1/4 of the reaction was analysed.

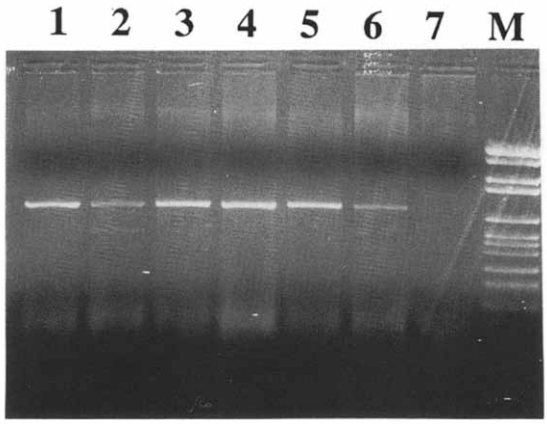

Fig. 4. Agarose gel analysis of PCR products. Reverse transcribed mRNAs were amplified with specific CGRP II mRNA primers. One-fourth of the reaction was submitted to gel electrophoresis. (Lanes 1-6) MTC tissues; (lane 7) control tissue. Molecular weight markers (M) = the same as in Fig. 1.

3.2. *CGRP I mRNA*

Using primers specific to CGRP I mRNA we detected PCR products in the eight cases of MTC by ethidium bromide fluorescence (data not shown). After transfer and hybridization, we detected CGRP I mRNA in all control cases. Fig. 3 shows the autoradiography of three MTC cases and four control cases.

3.3. *CGRP II mRNA*

A specific band was observed in the eight cases of MTC. Fig. 4 shows PCR products using primers specific to CGRP II mRNA of six cases of MEN IIa visualized with ethidium bromide. CGRP II mRNA after Southern blot and hybridization was detectable (OD >0.01) in three out of six control samples.

The results of PCR reaction are summarized in Table II.

The CT/CGRP I ratio was not statistically different between the various groups, using parametric and non-parametric test (Anova $P = 0.74$; Kruskal-Wallis $P = 0.93$) in contrast the CGRP II/CGRP I ratio was significantly different between the various groups (Anova $P = 0.0002$; Kruskal-Wallis $P = 0.008$) being maximal in isolated MTC and minimal in control tissues.

4. DISCUSSION

Though the structure and sequence of the CT-related genes are well established in man, modifications in the expression of these genes in the course of MTC are not well documented. This is probably due to the rareness of the disease (1 out of 25 000 subjects) and to the existence of multiple clinical forms. We have chosen to study MEN IIa, the most frequent familial form of the disease and in two cases of isolated sporadic MTC.

In order to amplify specifically the mature form of the mRNAs we used oligo(dT) as a primer for the reverse transcriptase reaction and specific primers for the Taq polymerase in the terminal 5'- and 3'-regions of the cDNA. The length of the transcripts we amplified was the same as that of the respective mRNAs. We detected CT transcripts in all control samples studied, thus confirming the presence of a C cell population.

Our results establish the presence of mature CGRP I and CGRP II mRNA in all medullary carcinoma of the thyroid. We detected CGRP I transcripts in all control tissues studied, demonstrating for the first time that in these tissues, the expression of the CT/CGRP gene leads to the production of specific mRNAs coding for CGRP I.

The CALC II gene expression is more complex, as extremely low levels were detected by hybridization, after amplification, in only 3 out of 6 samples from non-

Table II

Quantification of CT, CGRP I and II mRNA, after PCR amplification of total RNA extracted from control and tumoral thyroid human tissues

	Men IIa	Isolated MTC	Control tissues
CT	900.3 ± 97.4 (6)	505 ± 120 (2)	17.8 ± 11.2 (6)
CGRP I	183.2 ± 24.5 (6)	102 ± 51 (2)	2 ± 0.6 (6)
CGRP II	245.5 ± 46.8 (6)	246 ± 66 (2)	0.6 ± 0.4 (6)
CT/CGRP I	5.9 ± 1.7 (6)	7.4 ± 4.9 (2)	8.9 ± 3.3 (6)
CGRP II/CGRP I	1.4 ± 0.2 (6)	3 ± 0.9 (2)	0.2 ± 0.1 (6)

Values reported are means ± SE of relative DNA quantity (arbitrary units), number in parentheses indicates the number of samples.

tumoral thyroid. This low level of CGRP II gene expression in these three samples could simply reflect a mild C cell hyperplasia secondary to disturbances in follicular cell metabolism. This hypothesis is in part supported by the detection of higher levels of CGRP II mRNA in one oxyphil thyroid cancer, in which we authenticated C cell hyperplasia by specific staining of the C cells (data not shown).

Analysis of the ratios of CALC I and II genes expression between tumoral and non-tumoral tissues is of particular interest. For CT and CGRP I mRNAs the ratios are statistically not different indicating that the critically regulated step (splice choice) could be independent of the level of CT/CGRP I gene expression in human tumoral and non-tumoral C cells and agree with the results in rat MTC [17]. We have also compared CALC I and CALC II genes expression, the ratios of CGRP II/CGRP I between clinical groups are different, indicating that the regulation of the expression of these related genes could be different in normal and neoplastic C cells. Similar results were observed in cultured cells of murine MTC in response to dexamethasone treatment [18].

In conclusion, CT and CGRP I are both produced by normal C cells and their increase in malignant tumors is probably a reflection of an increase in the number of C cells. Furthermore the ratio of CT mRNA to CGRP I mRNA in our samples is in favour of the maintenance of normal splicing mechanisms in the tumoral cells we have studied. On the other hand, the increased levels of CGRP II mRNA expression in the tumoral samples could be due to an activation of its gene during C cell proliferation. If this is the case, CGRP II should be considered as a second tumoral marker in MTC.

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