Increased expression of mRNA specific for *c-Met* oncogene in human papillary thyroid carcinoma

Anna Cyniak-Magierska, Ewa Brzeziańska, Joanna Januszkiewicz-Caulier, Dorota Pastuszak-Lewandoska, Andrzej Lewiński

Department of Endocrinology and Metabolic Diseases, Medical University of Lodz, Polish Mother's Memorial Hospital – Research Institute, Lodz, Poland

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Corresponding author:
Andrzej Lewiński, MD, PhD
Department of Endocrinology and Metabolic Diseases
Medical University of Lodz
Polish Mother's Memorial Hospital – Research Institute
Rzgowska St. No. 281/289,
93-338 Lodz, Poland
Phone/fax: +48 42 2711343
E-mail: alewin@csk.umed.lodz.pl

Abstract

Introduction: Papillary thyroid carcinoma (PTC) is a differentiated type of thyroid cancer and the most common type of carcinoma of this gland in the Polish population. PTC originates from the thyroid follicular epithelial cell and its molecular pathogenesis is still poorly understood. The aim of the study was to estimate the expression of mRNA, specific for the *c-Met* oncogene, in the tissue of human PTC, and to evaluate the possible correlation between the level of *c-Met* oncogene expression and such parameters as: patient's age and gender, histopathological variants of the tumour and the assignment of patients to particular stages in the clinical staging system.
Material and methods: The level of RNA expression which was measured in macroscopically unchanged thyroid tissue served as controls. Reverse transcription-polymerase chain reaction (RT-PCR) and densitometry analysis were employed for mRNA expression measurements, with the glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) as a control housekeeping gene.
Results: Oncogene *c-Met* mRNA expression was evaluated for 18 cases of PTC (PTC follicular type – 8 cases, PTC classic type – 10 cases) and for the corresponding, macroscopically unchanged thyroid tissue. The mean expression of *c-Met* mRNA was significantly higher in PTC (three-fold), when compared to that found in macroscopically unchanged thyroid tissue.
Conclusions: These findings are consistent with the possibility that the *c-Met* oncogene plays a crucial role in the carcinogenic process in the human thyroid.

Key words: human papillary thyroid carcinoma, oncogene *c-Met*, mRNA expression.

Introduction

Papillary thyroid carcinoma (PTC) is a differentiated type of thyroid cancer and the most common type of carcinoma of this gland in the Polish population. PTC originates from the thyroid follicular epithelial cell [1].

The molecular pathogenesis of PTC is still poorly understood [2]. Among various genetic factors involved in the pathogenesis of PTC, chromosomal rearrangements or translocations of *RET* and *NTRK1* oncogenes appear to be of crucial importance [3, 4]. It is known that *BRAF* oncogene somatic mutation (*V599E*) is the most common genetic alteration in PTC and seems to be an alternative event to *RET/PTC* rearrangements or *RAS* mutations in the development of PTC. Moreover, no overlap was found between the changes in question of those genes [5, 6]. The process of constitutive activation of the RAS-RAF-MEK-MAP kinase pathway transmits a mitogenic

signal to the nucleus and promotes uncontrolled cell division, very frequently found in human carcinomas.

There are also several other mechanisms which may play role in the carcinogenic process in the thyroid. Increased expression of mRNA specific for the enzymes involved in pyrimidine and purine metabolism, e.g. thymidine kinase 1 (TK1), deoxycytidine kinase and thymidine phosphorylase (dCK), has been estimated in PTC [7].

In the molecular background of human PTC, the unregulated activation of intracellular tyrosine kinase (TK) seems to be of crucial value. The *c-Met* protooncogene, located in chromosome 7q31-34, encodes the Met protein, a TK receptor with high affinity for the hepatocyte growth factor (HGF) [8, 9].

The Met protein has motogenic, mitogenic and morphogenic properties and has been implicated in the invasion process of malignant cells in PTC [10].

Overexpression of the *c-Met* oncogene has been found in many carcinomas, such as: colorectal, ovarian and non-small-cell lung carcinoma [11]. A lot of studies performed using immunohistochemistry or Western blot analysis have reported increased levels of Met protein in PTC as compared with macroscopically unchanged thyroid tissue [12, 13], but only a few studies have shown high levels of mRNA specific for *c-Met* in PTC [14].

Activating mutations of the *c-Met* oncogene are also present in several types of cancer, particularly in human papillary renal carcinoma (in both its hereditary and sporadic forms) [15]. Recently, missense *c-Met* mutation T1010I in exon 14 was found in differentiated thyroid carcinoma (both papillary and follicular), but its molecular pathological role is not yet fully understood [16].

The aim of the study was to estimate the expression of mRNA specific for the *c-Met* oncogene in the tissue of human PTC, and to evaluate the possible correlation between the level of *c-Met* oncogene expression and such parameters as: patient's age and gender, histopathological variants of the tumour and the assignment of patients to particular stages in the clinical staging system [17].

Material and methods

The procedures used in the study were approved by the Ethical Committee of the Medical University of Lodz, Poland.

Thyroid tissue samples (150 mg) were obtained from patients who had been submitted to surgery (total thyroidectomy) for PTC, at the Department of Oncological Surgery, Centre of Oncology, the Maria Sklodowska-Curie Memorial Institute, Gliwice, Poland, and at the Division of Surgery, the Holy Family Municipal Hospital, Lodz, Poland. Immediately after the collection, thyroid tissue was frozen at -70°C.

Papillary thyroid carcinoma (18 cases: 11 females, 7 males; the mean age of all studied patients was 52.9±19.4 years) was cytologically diagnosed before the surgery on the basis of fine needle aspiration biopsy (FNAB). Histopathological confirmation, according to WHO classification, was obtained from pathological reports (PTC types were as follows: PTC follicular type – 8 cases, PTC classic type – 10 cases).

Oncogene *c-Met* mRNA expression was measured in 18 pairs of PTC samples and the corresponding macroscopically unchanged thyroid tissue (which served as controls). Clinical and histopathological characteristics of the studied patients are summarized in Table I.

RNA isolation

Total RNA was extracted from thyroid tissue (150 mg) in guanidinium isothiocynate, by means of a commercially available kit [Total RNA Prep Plus (A&A BIOTECHNOLOGY, Gdynia, Poland)]. Tissue samples were stored at -70°C until analysis. RNA concentrations and purity in the final preparations were spectrophotometrically quantified by measuring absorbance at 260 and 280 nm (Ultraspec 2000 UV/Visible Specrophotometer Pharmacia Biotech, Sweden).

Reverse transcription-polymerase chain reaction (RT-PCR)

Reverse transcription was performed by random priming in a reaction mixture (20 µL) containing: 1000 ng of total RNA, 0.3 µL of *Random Primer Oligonucleotides* (3 µg/µL (Gibco BRL Co.), 2 µL of 0.1 M DTT (Dithiothreitol) (Gibco), 4 µL of 5 × *First--Standard Buffer* (250 mM Tris-HCl, pH 8.3; 375 mM of KCl; 15 mM of MgCl2) (Gibco), 1 µL of Reverse Transcriptase M-MLV (*Moloney Murine Leukemia Virus Reverse Transcriptase*) (200 U/µL) (Gibco), 2 µL of dNTP (2500 µM) (Gibco) and 1.0 µL of Inhibitor RNAse (10 U/µL) (Gibco).

Reverse transcription was performed in a TRIO-Thermoblock thermocycler (Biometra, Göttingen, Germany) at 37°C for 60 min. The following negative controls for RT were performed: RT reaction without any addition of RNA as the control of RT reagent contamination, and RT reaction without any addition of Reverse Transcriptase enzyme as the contamination control of genomic DNA.

The amplification was carried out in a Mastercycler personal (Eppendorf, Hamburg, Germany) in a total volume of 25 µL, containing: 1000 ng of cDNA (RT product), 2.5 µL of 10 × PCR buffer (1 × is 10 mM of Tris-HCL, pH 8.8: 50 mM of KCl, 1.5 mM of MgCl2, 0.1% Triton X-100) (Finnzymes Oy), 2.5 µL of dNTP (250 µM of each) (Gibco), 1 µL of *Taq* polymerase (DyNAzyme II DNA polymerase) (2U/µL) (Finnzymes Oy), and 25 pmol of each, i.e. 3' and 5' PCR primer.

The sequences of the synthetic oligonuclotide primers (TIB MOLBIOL, Gdynia, Poland) used for PCR amplification, the reaction conditions under which the amplifications were carried out, and the size of amplicons are presented in Table II.

Table I. Clinical and histological characteristics of studied patients

Case No.	Age	Sex	PTC type	TNM Staging System, according to the American Joint Committee on Cancer	TNM
1.	16	M	PTC – follicular type	I	$pT_{1b}N_0M_0$
2.	29	F	PTC – follicular type	I	$pT_{4a}N_{1a}M_0$
3.	26	F	PTC – follicular type	I	$pT_{4a}N_{1a}M_0$
4.	47	M	PTC – classic type	III	$pT_{2a}N_{1a}M_0$
5.	56	F	PTC – follicular type	III	$pT_{3a}N_0M_0$
6.	68	F	PTC – follicular type	III	$pT_{2b}N_{1a}M_0$
7.	74	F	PTC – classic type	I	$pT_{1a}N_0M_0$
8.	68	M	PTC – classic type	II	$pT_{2a}N_0M_0$
9.	31	M	PTC – classic type	I	$pT_{2a}N_0M_0$
10.	41	F	PTC – follicular type	I	$pT_3N_0M_0$
11.	55	F	PTC – follicular type	II	$pT_{2a}N_0M_0$
12.	77	F	PTC – classic type	II	$pT_{2a}N_0M_0$
13.	69	M	PTC – classic type	I	$pT_{1a}N_0M_0$
14.	66	M	PTC – follicular type	IVA	$pT_{4a}N_0M_0$
15.	30	M	PTC – classic type	I	$pT_{4a}N_{1b}M_0$
16.	71	F	PTC – classic type	II	$pT_{2a}N_0M_0$
17.	68	F	PTC – classic type	III	$pT_{1a}N_{1a}M_0$
18.	61	F	PTC – classic type	II	$pT_{2a}N_0M_0$

Table II. Nucleotide sequences of PCR primers, conditions of amplification reactions and size of amplicons for *c-Met* and *GAPDH* genes [26]

Primer sequence	PCR conditions	Product size (bp)
c-Met Sense 5'-TAC TTG TTG CAA GGG AGA AGA CTC CT-3' Antisense 5'-GGG ACC AAG CCT CTG GTT CTG ATG C-3'	– Initial denaturation 95°C, 5 min – Denaturation 96°C, 1 min – Annealing 56°C, 1 min – Elongation 72°C, 1 min – Final elongation 72°C, 8 min 30 cycles	470
GAPDH Sense 5'-CGG AGT CAA CGG ATT TGG TCG TAT–3' Antisense 5'-AGC CTT CTC CAT GGT GGT GAA GAC-3'	– Initial denaturation 95°C, 5 min – Denaturation 96°C, 1 min – Annealing 56°C, 1 min – Elongation 72°C, 1 min – Final elongation 72°C, 8 min 30 cycles	307

The following negative controls for PCR were performed: a reaction with RT reaction product without any addition of RNA as the control of PCR reagent contamination, and a PCR reaction with RT reaction product without any addition of Reverse Transcriptase enzyme as the control for genomic DNA contamination.

The amplification of the control housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) served as an internal standard.

The identity of amplification products with the presumed sequences was proven by digestion with restriction enzyme *Hae III* (Eurogentec, Brussels, Belgium) to obtain 362 bp and 108 bp fragments using manufacturer's buffers and protocol.

Analysis of RT-PCR products

RT-PCR products were identified using 8% polyacrylamide (PAA) Tris-borate-ethylenediamine tetraacetate (TBE) gels and visualized by ethidium bromide staining (0.5 mg/mL). Densitometric analyses were conducted using the Scan Pack 3.0 system (Biometra Göttingen, Germany). Results are expressed in ng units and are presented as the ratios of *c-Met* (pg) expression to *GAPDH* gene (ng) expression.

Statistical analysis

The data were statistically analyzed using *t*-test for average values in independent groups, followed by U Mann-Whitney test. Statistical significance was determined at the level of $p<0.05$. The results are presented as mean ± SEM and ± SD values. Pearson's test, χ^2 test and U Mann-Whitney test were performed in order to correlate the level of expression of the *c-Met* gene in patients with PTC with clinicopathological parameters (age, gender, PTC histopathological variant and assignment to a particular stage in the clinical staging system [17]).

For calculations, Statistica (StatSoft, Poland) for Windows 7.0 programme was applied.

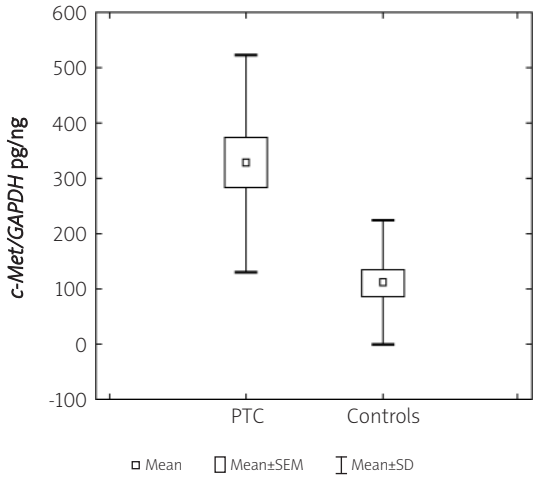

Figure 1. Relative expression of mRNA encoding for *c-Met* in macroscopically unchanged thyroid tissue (controls; $n = 18$) and in human papillary thyroid carcinoma (PTC; $n = 18$). Gene expression is expressed as the amount of amplified cDNA. Results are expressed in (ng) units and are presented as the ratios of *c-Met* (pg) expression to *GAPDH* gene (ng) expression. Bars represent the mean ±SD, ±SEM, $P<0.05$ *vs.* controls

Results

Significant differences in *c-Met* mRNA expression were observed between PTC and macroscopically unchanged thyroid tissue (*t*-test, $p<0.0003$). The mean relative expression for *c-Met* was significantly, three-fold higher in human PTC (Figure 1).

Moreover, in the group of 18 pairs – PTC and the corresponding macroscopically unchanged thyroid tissues obtained from the same patient, in most cases expression of the *c-Met* gene was higher in PTC than in the control tissue (Figure 2).

Interestingly enough, in five specimens of macroscopically unchanged thyroid tissue, there was no expression of mRNA specific for the *c-Met* gene. In two pairs, PTC and macroscopically unchanged thyroid tissue, the level of expression of *c-Met* mRNA was higher in controls than in PTC samples. In our study, no significant correlation was found between *c-Met* expression and such characteristics as patient's age at diagnosis (Pearson test, $p=0.126$) and patient's sex (χ^2 test, $p=0.493$). We compared expression levels of the *c-Met* oncogene in different types of PTC (follicular type – 8 cases, and classic type – 10 cases). Interestingly enough, no statistically significant differences were found among histopathological variants of the tumour (*t*-test, $p=0.901$; U Mann-Whitney test $p=0.789$).

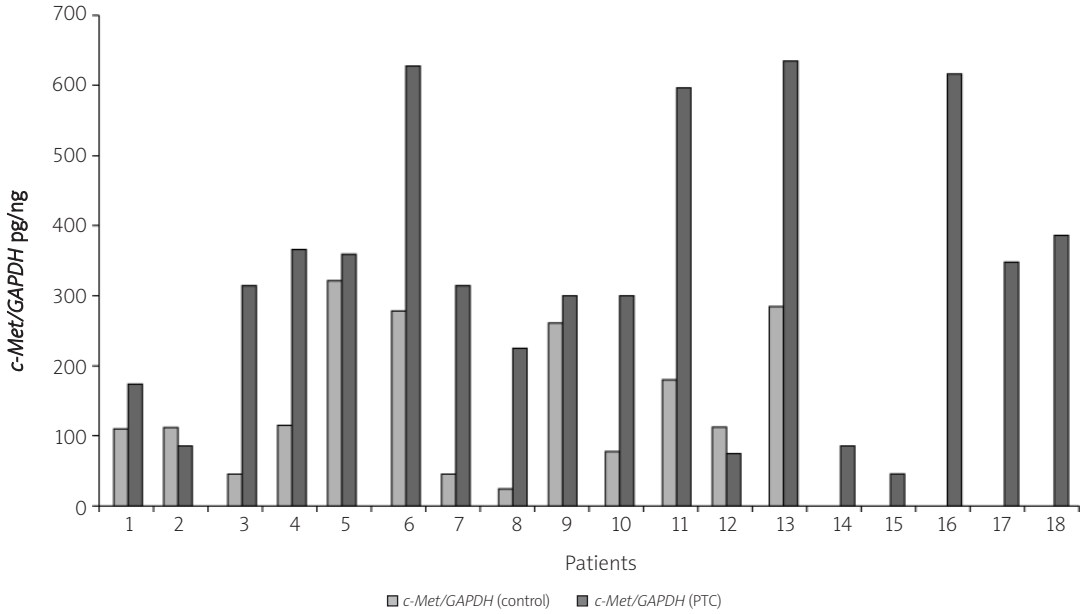

Figure 2. Relative expression of mRNA encoding for *c-Met* in macroscopically unchanged thyroid tissue (controls) and in human papillary thyroid carcinoma (PTC). Gene expression is expressed as the amount of amplified cDNA. Results are expressed in ng units and are presented as the ratios of *c-Met* (pg) expression to GAPDH gene (ng) expression

Discussion

In 1992, Di Renzo et al. reported that Met protein was strongly overexpressed in PTC. The overexpression of the protein was not associated with either amplification or rearangements of the *c-Met* oncogene and the Met protein did not show structural alterations [12]. The development of immunohistochemical techniques and reagents active on paraffin-embedded material allowed a wide spectrum of immunohistochemical investigations of thyroid carcinomas. In normal thyrocytes, Met protein is either not expressed or expressed at a very low level. In contrast, high Met expression is a frequent abnormality in PTC. It was found that about 90% of PTC cases, including all histological types, overexpressed Met protein [18]. However, recently, Nardone et al. observed higher expression of Met protein in tall cell variant of PTC, which may explain the invasive behaviour of this histological type of PTC [19].

In the present study, we evaluated *c-Met* oncogene expression in PTC and macroscopically unchanged thyroid tissue by means of the RT-PCR method. We found no mRNA expression specific for the *c-Met* gene in five specimens of macroscopically unchanged thyroid tissue, while in most studied cases the expression of *c-Met* was higher in PTC than controls (macroscopically unchanged thyroid tissue).

Our results showing the presence of *c-Met* RNA in normal thyrocytes are in concordance with the results obtained by other authors [20]. Fluge at al. also observed *c-Met* oncogene overexpression in some cases of PTC by the hybridization method, using complex cDNA probes specific to *c-Met* RT-PCR products [20].

Recently, the gene expression profile of PTC has been studied using oligonucleotide microarrays method [14, 21]. Among the overexpressed genes, *c-Met* oncogene has already been recognized to be involved in PTC. Surprisingly, Wasenius et al. documented a significant difference between the methods (RT-PCR *vs.* cDNA expression array), assessing expression of the *c-Met* oncogene. In cDNA array analysis, half of the studied PTC cases showed increased *c-Met* oncogene expression, whereas in RT-PCR analysis as many as 90% of PTC samples showed about six-fold higher *c-Met* expression than unchanged thyroid tissue [14].

The overexpression of *c-Met* oncogene may be secondary to the changes observed in other genes involved in the development of PTC. Ivan et al. have shown that introduction of activated *RAS* and *RET* oncogenes in thyrocytes results in overexpression of Met protein [22]. This hypothesis seems to be interesting because it suggests the possibility of a relationship between Met protein expression and activation of the RAS or RET signalling pathway (through MAP kinase) in PTC. In order to test the hypothesis that *c-Met* expression is induced by the activation of other genes known to play a major role in the MAP kinase signalling pathway, we decided to search for *RAS*, *RET* and *BRAF* mutations in the examined series of PTC at our laboratory.

We proved that two cases of PTC had contained an activating *RAS* oncogene mutation. The first mutation was found at codon 61 of N-*RAS*, the second at codon 31 of K-*RAS* [23]. Recently, we have found 6 out of 18 cases of rearrangements in *RET* and NTRK1 oncogenes (rearrangements in *NTRK1* gene – 2 cases, and rearrangements in *RET* gene – 4 cases) [3]. Accidentally, in the case of one patient with a mutation at codon 61 of the N-*RAS* gene, we detected V600E mutation in gene *BRAF* [24]. As mentioned before, it is suggested in many studies that *BRAF* and *RAS* mutations do not overlap in PTC. Recently it has been shown that *RAS* mutation does not have any additional effect on the proliferation of thyroid follicular cells and the mutation in the *BRAF* oncogene is a dominant factor in activating the MAPK signalling pathway [25]. Considering the role of the *c-Met* gene in PTC, our results may suggest that the overexpression of *c-Met* is secondary to mutations of *RAS* or *RET* oncogenes.

In turn, Wasenius et al. [16] have recently reported a change of the sequence in the *c-Met* oncogene. It is missense mutation T1010I, which replaces an isoleucine residue for a threonine residue in the Met receptor tyrosine kinase. This change has been found in papillary, follicular and medullary thyroid carcinomas, and it is the first report concerning *c-Met* oncogene sequence changes in the thyroid [16]. Missense *c-Met* mutations have recently been discovered in papillary renal carcinoma, gastric carcinoma, glioma, childhood hepatocellular carcinoma and small cell lung carcinoma [11]. Although the mechanism of increased expression of the *c-Met* oncogene in human PTC is probably complex, the one associated with either somatic or inherited mutations seems to play an essential pathological role.

Conclusions

To sum up, increased expression of mRNA specific for *c-Met* is involved in neoplastic processes in human thyroid, namely in the pathogenesis of PTC.

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