Assessment of *cyclin D1* gene expression as a prognostic factor in benign and malignant thyroid lesions

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Abstract

OBJECTIVE: Cyclin D1, encoded by *CCND1* (*cyclin D1*) gene with *locus* in chromosome 11q13, is a protein that plays the key role in the passage through the restriction point in G1 phase of cell cycle. The aim of the study were: 1) an assessment of *CCND1* gene expression level in benign and malignant thyroid lesions and 2) the evaluation of possible correlations between gene expression and the histopathological variants of papillary thyroid carcinoma (PTC), or tumour size, classified according to TNM definition of primary tumours (in case of PTC only) or patient's sex or age.

DESIGN: Thirty five (35) tissue samples were analysed: 24 cases of PTC, 4 cases of medullary thyroid carcinoma (MTC), 4 cases of follicular adenoma (FA) and 3 cases of nodular goitre (NG). In real-time polymerase chain reaction (real-time PCR), two-step RT-PCR (reverse transcriptase-polymerase chain reaction) in an ABI PRISM 7500 Sequence Detection System was employed. *Cyclin D1* gene expression level was assessed, calculating the mean relative quantification rate (RQ rate) increase for each sample.

RESULTS: The level of *cyclin D1* gene expression was significantly higher in malignant thyroid tumours (PTC, MTC), as compared with that in macroscopically unchanged thyroid tissue, FA and/or NG groups. However, the differences of RQ rate value between different PTC variants were statistically insignificant. No correlation was found between RQ values and patients' sex or age. On the other hand, the correlation was observed between RQ values and tumour size.

CONCLUSIONS: *Cyclin D1* gene expression in various thyroid lesions may be helpful in diagnostically doubtful cases. However, our results – mostly due to the small numbers of cases in the groups other than PTC – do not *yet* allow considering *cyclin D1* gene as a molecular prognostic marker.

ORIGINAL ARTICLE

INTRODUCTION

The cyclin D1 is encoded by the *CCND1* (*cyclin D1*) gene with *locus* in chromosome 11q13. It is expressed relatively ubiquitously, with exception of normal lymphoid and myeloid cells. The gene product is the 35-kDa protein that plays an important role in the passage through the restriction point in G1 phase, restarting the process of cell proliferation. Cyclin D1 levels are low in quiescent cells; however, they increase and reach the maximum level before S phase. Cyclin D1 is a regulatory unit of a holoenzyme that is formed by binding with cyclin-dependent kinases (CDKs), CDK4 and CDK6, depending on mitogenic stimulation. Cyclin-CDK enzyme phosphorylates the key substrates that facilitate the passage of proliferating cells through the restriction point in G1 phase. Retinoblastoma tumour suppressor protein – pRb (retinoblastoma protein) is one of the substrates for this enzyme. Phosphorylation of pRb inhibits its ability to restrain the activity of the family of transcription factors (E2F family), which induce the expression of genes important for cell proliferation [27,23,13].

Abnormalities, involving the components of cell cycle-regulatory machinery, are important events in cancerogenesis. The candidacy of *cyclin D1* gene as an oncogene has been studied, using primary rodent cell-transformation assay. *Cyclin D1* gene has been proved to function as an oncogene, contributing to cell transformation and suggesting a similar role in tumour progression *in vivo* [13]. Moreover, the authors have suggested that the genetic alterations, resulting in *cyclin D1* gene overexpression, observed in many human tumours, overcome the negative regulation of cell cycle and may cause transformation to malignant phenotype [13].

Indeed, *cyclin D1* gene overexpression has been found to occur in several primary tumours, including breast, oesophageal and hepatic carcinoma, head and neck squamous cell carcinoma, as well as melanoma [30,25,24,8]. *Cyclin D1* gene overexpression is particularly high in mantle cell lymphomas (up to 90%) [31] and breast cancers (45%) [7]. Moreover, the results of many studies indicate that *cyclin D1* gene overexpression is associated with more aggressive tumour phenotype, the higher risk of recurrence and poorer prognosis, lower survival rate and increased incidence of lymph node metastases [25,8,3,1].

The genetic basis of *cyclin D1* gene overexpression has not yet been determined but certain alterations have been identified, including pericentromeric chromosomal inversion in parathyroid adenomas [2] and translocation –t(11;14) in mantle cell lymphoma and multiple myelomas [29].

However, the most common alteration leading to *cyclin D1* gene overexpression, is its amplification. It has been found in a significant proportion of breast, oesophageal, lung, cervical, as well as colorectal carcinomas [25,24,7,3].

Cyclin D1 role in the pathogenesis of thyroid carcinoma has not yet been fully recognized. In most cases, immunohistochemical assessments of normal thyroid follicular cells (TFCs) did not show any nuclear immunoreactivity for cyclin D1 [26] but some authors have observed cytoplasmic cyclin D1 immunoreactivity in normal TFCs [21]. Cyclin D1 expression, being either nuclear or cytoplasmic or both, was found in FAs [26,21,10]. Noteworthy enough, nuclear cyclin D1 immunoreactivity was significantly higher in PTCs than in FAs [26,21]. An immunohistochemical study of fine needle aspiration specimens of PTCs revealed the expression of cyclin D1 in almost 80% of nuclei [18]. In various studies, the overexpression of cyclin D1 was identified in 31 to 60% of PTCs [21,18,16,4].

There are reports, describing the involvement of cyclin D1 expression in thyroid oncogenesis. However, while most of the authors approach the alterations of cyclin D1 expression as the early events in tumourigenesis [34,28,26,20,12,5], others find significant overexpression of cyclin D1 in more aggressive thyroid carcinomas, suggesting that cyclin D1 expression may play some role in tumour progression [36].

In some reports, the authors suggest that cyclin D1 expression may be a prognostic factor in PTC, indicating a poor prognosis [36,35]. Also, cyclin D1 immunohistochemistry may prove valuable in predicting metastatic potential in PTCs and PMCs (papillary microcarcinomas) of the thyroid gland [16,20,17].

Cyclin D1 gene overexpression at the mRNA level can be detected by a variety of methods, including Northern blotting, *in situ* hybridization or reverse transcription-polymerase chain reaction (RT-PCR). In the majority of research work, concerning the assessment of cyclin D1 expression, the immunohistochemical technique is used [36,35,26,18,16,4,34,20,12,17], revealing, however, certain limitations – low sensitivity level and high variability. The most recent studies employ a quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis of *CCND1* gene overexpression. This method allows accurate quantitation of mRNA expression level and its significance has been proved in the diagnostics of mantle cell lymphoma in blood marrow and tissue samples [31,14].

The aim of our study was the assessment of *CCND1* gene expression in benign and malignant thyroid lesions and the evaluation of possible correlations between the gene expression and the histopathological variant of examined PTCs (classic *versus* follicular), tumour size, grouped according to TNM definition of primary tumours (pT1, pT2, pT3, pT4), or patients' sex or age.

MATERIALS AND METHODS

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

Thyroid tissue samples and histopathological examination

Tumour tissue samples (100–150 mg) were obtained from 28 patients after total thyroidectomy for primary papillary thyroid carcinoma (PTC; 24 cases: 20 females, 4 males) or medullary thyroid carcinoma (MTC; 4 cases: 3 females, 1 male), at the Holy Family Municipal Hospital in Lodz and at the Centre of Oncology, the Maria Skłodowska-Curie Institute in Gliwice, Poland, during the years 2002–2005.

Four (4) cases of follicular adenoma (FA) and three (3) cases of nodular goitre (NG) were also included in the study.

There were 29 females and 6 males in the whole study cohort. The mean age of all the studied patients was 47.85 ± 17.86 years (range – 17 to 75 years).

Histopathological variants of PTC were as follows: PTC classic variant – 16 cases, PTC follicular variant – 5 cases, PTC tall-cell variant – 3 cases. Histopathological diagnoses for malignant thyroid lesions, according to WHO Classification of Tumours [9], were obtained from pathomorphological reports and presented in Table 1, together with TNM classification and AJCC stage groupings [33].

In the study design, macroscopically unchanged thyroid tissue served for the reference standard (calibrator).

Table 1. Age, sex, histopathological diagnosis of malignant thyroid tumours in the studied patients.

Case number	Age	Sex	Histopathological diagnosis	TNM staging system	American Joint Committee on Cancer (AJCC) grouping system
1	17	F	PTC, classic variant	pT2b N1a M0	I
2	47	M	PTC, classic variant	pT2a N1a M0	III
3	31	M	PTC, classic variant	pT2a N0 M0	I
4	16	F	PTC, classic variant	pT2a N0 M0	I
5	61	F	PTC, classic variant	pT2a N0 M0	II
6	30	M	PTC, classic variant	pT4 N1b M0	I
7	71	F	PTC, classic variant	pT2a N0 M0	II
8	48	F	PTC, classic variant	pT4 N1a M0	IVA
9	23	F	PTC, classic variant	pT1b Nx M0	I
10	24	F	PTC, classic variant	pT3 N0 Mx	I
11	47	F	PTC, classic variant	pT1a N0 Mx	I
12	38	F	PTC, classic variant	pT1 N0 Mx	I
13	52	F	PTC, classic variant	pT2 N0 M0	II
14	71	F	PTC, classic variant	pT1a N0 Mx	I
15	31	F	PTC, classic variant	pT1bNxMx	I
16	28	F	PTC, classic variant	pT1bNXMX	I
17	59	F	PTC, tall-cell variant	pT3 NxMx	III
18	52	F	PTC, tall-cell variant	pT4 N0 M0	I
19	49	F	PTC, tall-cell variant	pT2b Nx Mx	II
20	26	F	PTC, follicular variant	pT4 N1a M0	I
21	56	F	PTC, follicular variant	pT3a N0 M0	III
22	68	F	PTC, follicular variant	pT2b N1a M0	III
23	55	F	PTC, follicular variant	pT2a N0 M0	II
24	66	M	PTC, follicular variant	pT4a N0 M0	IVA
25	74	M	MTC	pT4N0M0	IVA
26	59	F	MTC	pT3a N0 M0	III
27	56	F	MTC	pT3a N0 M0	III
28	71	F	MTC	pT4 N1aM0	IVA

Tumour tissue samples, immediately after resection, were collected into lysis buffer (Buffer RLT, Qiagen Sciences, USA).

Isolation of total RNA and reverse transcription (RT)

Total RNA was extracted from fresh tissues, using RNeasy Protect Midi Kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations.

RNA concentration was spectrophotometrically assessed by measuring absorbance at 260 and 280 nm (Ultraspec 2000 UV/Visible Spectrophotometer, Pharmacia Biotech, Sweden).

Reverse transcription was performed in a TRIO-Thermoblock thermocycler (Biometra, Goettingen, Germany), using 1μg of total RNA in the presence of oligo d(T)16 (50 μM) and MultiScribe™ Reverse Transcriptase (50 U/μL) in a total volume of 30 μL, including also: 10 × TaqMan RT Buffer, $MgCl_2$ solution (25 mM), dNTPs mixture (10 mM), RNAse Inhibitor (20 U/μL) and nuclease-free water (TaqMan Reverse Transcriptase Reagents, Applied Biosystems, Foster City, CA, USA).

The reactions were incubated for 60 minutes at 37 °C, heated for 5 min to 95 °C, and placed at 4 °C.

Analysis of the relative amount of cyclin D1 mRNA by real-time PCR

An established Relative Quantification PCR assay for cyclin D1 mRNA expression was used (in ABI PRISM 7500 Sequence Detection System, Applied Biosystems), according to the manufacturer's protocol. The PCR reactions for *cyclin D1* gene were run with 50 ng of cDNA in a total volume of 50 μL, using TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and the predesigned and labelled primer/probe set (Assays-on-Demand™ Gene Expression assay mix, Hs 00277039_m1). After initial incubation at 50 °C for 2 min to allow uracil-N-glycosylase (UNG) digestion and at 95 °C for 10 min to activate the AmpliTaq Gold®DNA polymerase, both of which are provided by the Universal PCR Master Mix, the samples were amplified through 40 bi-phasic cycles of 95 °C for 15 sec and 60 °C for 1 min (Table 2).

Macroscopically unchanged thyroid tissue served for calibrator. Amplification reactions were done in triplicate for each sample. Controls with no template cDNA were used with each assay.

Table 2. PCR conditions for the RQ experiment.

TIMES AND TEMPERATURES			
Initial setup		Each of 40 cycles	
		Denature	Anneal/Extend
HOLD	HOLD	CYCLE	
UNG activation 2 min, 50 °C	10 min, 95 °C	15 s, 95 °C	1 min, 60 °C

Expression levels of *β-actin* gene were measured, as endogenous control (reference gene), using the appropriate Assays-on-Demand™ Gene Expression product (Hs 99999903_m1, Applied Biosystems Foster City, CA, USA).

Both gene expressions were measured for each tumour sample in the same PCR reaction but in separate wells.

Assays-on-Demand™ Gene Expression product consists of 20 × mix of unlabelled PCR primers (18 μM for each) and TaqMan® MGB probe (5 μM) with FAM™ (6-carboxy-fluorescein) at the 5' end as the reporter dye and a non-fluorescent quencher (TAMRA, 6-carboxy-tetramethylrhodamine) at the 3' end. The two-minute, 50 °C step is required for optimal AmpErase® UNG activity when TaqMan® Universal PCR Master Mix (P/N 4304437) is used.

The fluorescence signal was measured in real-time in the extension phase of the PCR reaction and the measurement, proportional to the quantity of sample cDNA in the reaction, was plotted as an amplification curve against the cycle number. A threshold value of fluorescence in the exponential part of the amplification curve has been selected and – for each sample – the number of cycles has been measured, which is needed by the signal to reach the threshold (threshold cycle, C_T). The larger are the quantities of the starting material, the lower are C_T values.

Data analysis

Data analysis was performed with the Taq-Man SDS analysis software (Applied Biosystems), and the results were exported to Excel sheets for further processing.

Fluorescence emission data were determined as C_T values for each reaction and, for each sample, triplicate C_T values were averaged. The average C_T value for *β-actin* was subtracted from the average *cyclin D1* C_T value to yield the ΔC_T value ($\Delta C_T = C_T$ target $- C_T$ reference). Normalization to the reference gene (*β-actin*) has been necessary to account for the variabilities in sample quantity and quality and for the variabilities in PCR efficiency among samples. The assay, described in this report, involves the determination of $\Delta\Delta C_T$ value. This is calculated by: $\Delta\Delta C_T = \Delta C_T$ test sample $- \Delta C_T$ calibrator sample. The higher is the $\Delta\Delta C_T$ value, the lower is the expression of *cyclin D1* in the specimen. Fold-differences, representing relative expression results, are calculated, using the equation: relative fold increase $= 2^{-\Delta\Delta C_T}$.

Statistical analysis

The data were statistically analyzed, using Kruskal-Wallis' test to compare RQ values among all the four studied groups (PTC, MTC, FA, NG). For two independent groups (PTC, classic variant and PTC, follicular variant), the parametric t-test was used, followed by non-parametric U Mann-Whitney's test. The t-test, Pearson's test, χ^2 test, Spearman's rank correlation coefficient and U Mann-Whitney's test were performed in order to correlate the level of expression of *cyclin D1* gene

(RQ rate values) with examined parameters (age, gender, PTC histopathological variant and tumour size, classified according to TNM definition of primary tumours – pT1, pT2, pT3, pT4).

Statistical significance was determined at the level of $p<0.05$. The results are presented as means ± SEM and means ± SD values.

Statistica for Windows 7.0 program was applied for calculations.

RESULTS

The specimens were amplified in the ABI PRISM 7500 Sequence Detection System in reaction, containing primers and probes for *cyclin D1* gene and a control gene, *β-actin*. The Sequence Detection System software, provided with the instrument, analyses the fluorescence data, generated during the reaction, and calculates the cycle number at which fluorescence crosses the threshold value (C_T). Figure 1A and 1B illustrate typical assay results (amplification curves), generated from tumour specimen (Figure 1A) and macroscopically unchanged thyroid tissue (Figure 1B). Triplicate amplifications of the sample produced nearly identical, overlapping curves, from which C_T values were calculated.

The assay, described in this paper, was based on the determination of a $\Delta\Delta C_T$ value ($\Delta\Delta C_T$ method) for each sample (the greater was the $\Delta\Delta C_T$ value, the lower was the expression of *cyclin D1*) and on calculating the difference in *cyclin D1* expression level between tumour sample and calibrator (RQ value). The results for the studied groups are presented in Table 3.

$\Delta\Delta C_T$ values for PTC, classic variant, ranged from –0.455 to –6.571; for PTC, follicular variant the results

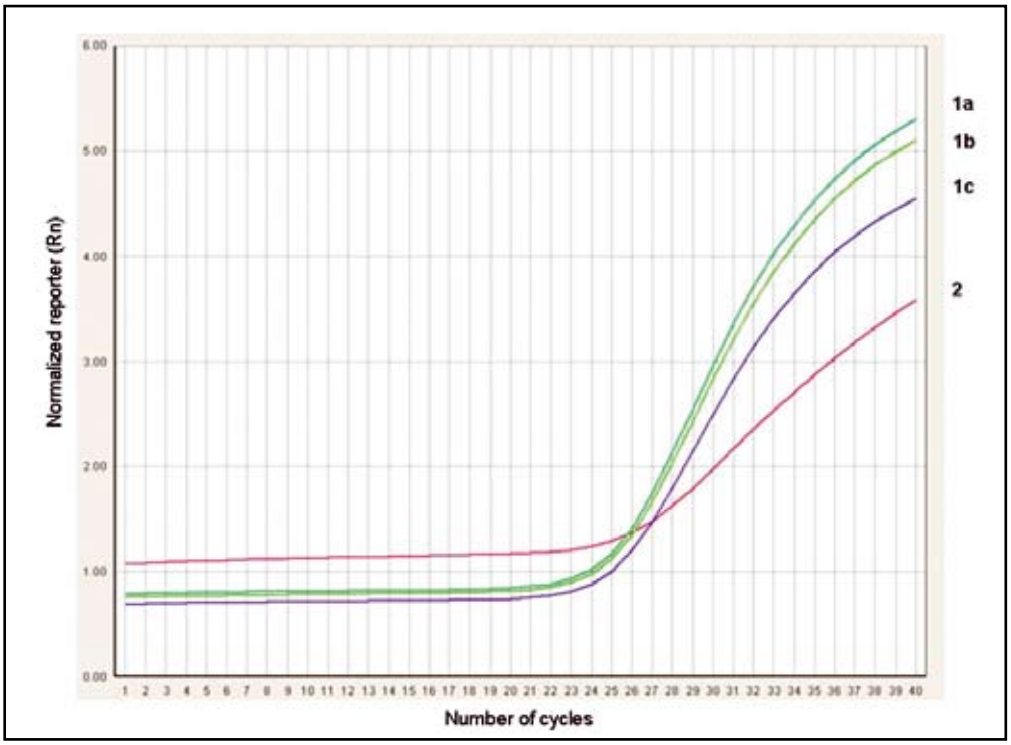

Figure 1A. Representative amplification curves for *cyclin D1* and *β-actin* genes in tumour specimen (PTC). Note the low C_T value for *cyclin D1*, indicating strong overexpression in PTC case, as compared with the macroscopically unchanged thyroid tissue (Figure 1B).
1a, 1b, 1c – amplification curve for *cyclin D1* gene (in triplicate).
2 - amplification curve for *β-actin* gene (endogenous control).
C_T value (mean) for *cyclin D1* gene – 23.878.
C_T value (mean) for *β-actin* gene – 25.697.

Table 3. The expression level of *cyclin D1* gene, calculated by the ΔΔCT method in the studied groups.

Diagnostic groups	Mean CT for *cyclin D1* gene	Mean CT for *β-actin* gene	Mean ΔCT	Mean ΔΔCT	Mean RQ	Range of RQ values
Calibrator	29.562	26.355	3.207	0.000	1.000	-
PTC	26.852	27.303	-0.451	-3.602	22.819	1.809–100.663
MTC	29.849	32.614	-2.765	-4.696	57.697	3.520–164.715
FA	28.922	28.436	-0.486	-1.268	3.089	0.870–5.829
NG	24.985	26.123	-1.373	-0.567	1.567	1.152–2.343

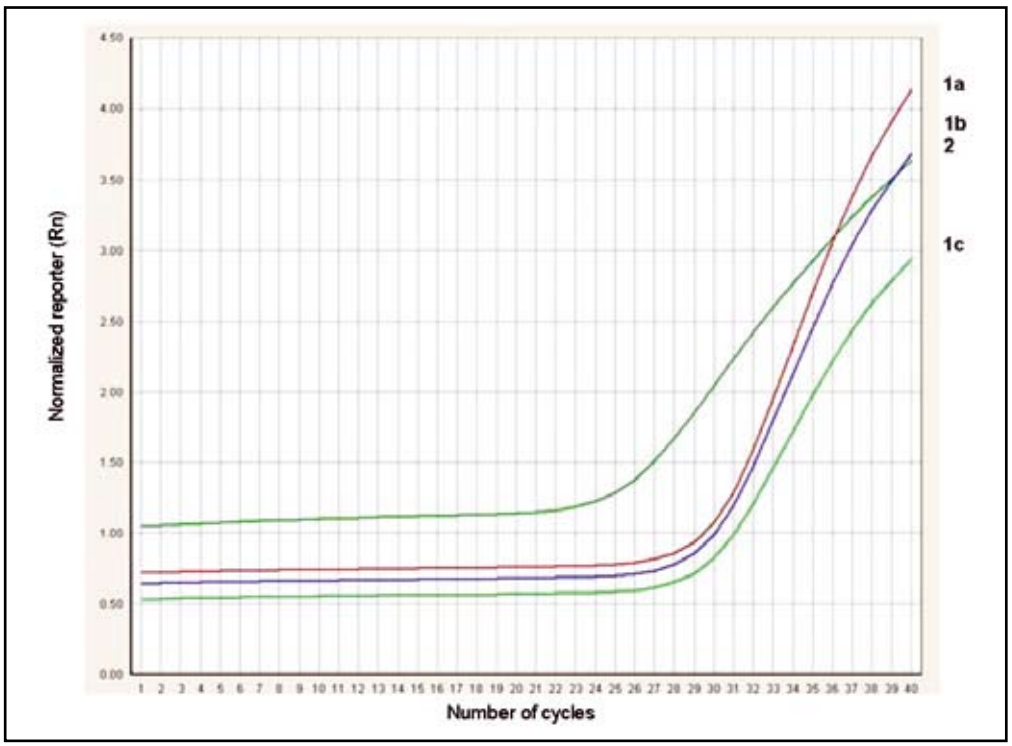

Figure 1B. Representative amplification curves for *cyclin D1* and *β-actin* genes in macroscopically unchanged thyroid tissue.
1a, 1b, 1c – amplification curve for *cyclin D1* gene (in three repeats).
2 - amplification curve for *β-actin* gene (endogenous control).
C_T value (mean) for *cyclin D1* gene – 28.983.
C_T value (mean) for *β-actin* gene – 25.322.

were from -3.305 to -5.480; and for PTC, tall-cell variant from –2.638 to –5.197. As a whole group, PTCs showed the mean $\Delta\Delta C_T$ value of –3.602 and the mean fold-difference in *cyclin D1* gene expression between PTC samples and calibrator (RQ value) was 22.819.

$\Delta\Delta C_T$ values for MTCs ranged from -1.730 to -7.266. These results yielded the mean RQ value of 57.697.

$\Delta\Delta C_T$ values for FAs ranged from +0.200 to -2.5430 and the calculated mean RQ value was 3.089.

$\Delta\Delta C_T$ values for NGs ranged from -0.204 to -1.228 and the calculated mean RQ value was 1.567.

The obtained results (RQ values) show elevated levels of *cyclin D1* gene expression in thyroid tumours, as compared to normal thyroid tissue. In thyroid carcinoma samples (PTCs and MTCs), the expression of *cyclin D1* gene was clearly higher and only negligibly increased in FAs and NGs (Table 3).

Table 4. Statistically analyzed results for two variants of PTC – classic and follicular (t-test). Student t-test, p – level of significance

Variable	Mean PTC, classic variant	Mean PTC, follicular variant
RQ	21.53769	26.22860
No. of samples	16	5
SD	31.93534	13.74556
t-test statistics	-0.314879	
p-value	0.756211	

Kruskal-Wallis test, applied for RQ comparison between the four studied groups (PTC, MTC, FA, NG), showed statistically significant differences (p=0.0050) among them (Figure 2). The multiple comparison test, used for pairs of the studied groups, revealed significant differences only between the PTC group (statistically higher RQ values) and the FA group (p=0.024889) and NG group (p=0.032153). In cases of other pairs, the differences in RQ levels were not significant (p>0.05). Similarly, the results of the parametric t-test did not show any significant differences among the individual variants of PTC (p=0.756211) (Table 4 and Figure 3). In the whole studied group of PTCs, a correlation analysis of RQ value with patients' sex and age, histopathological variant or tumour size revealed a positive correlation only for the last one (p=0.001938) (Figure 4). There was no significant relationship between the *cyclin D1* gene expression level and the patients' age or sex (p> 0.05).

DISCUSSION

The molecular pathogenesis of thyroid cancerogenesis is still poorly understood [22]. Cytogenetic and molecular analyses have identified genetic alterations in protooncogenes, which may play some role in the development and progression of thyroid tumours. Chromosomal rearrangements (*RET*/PTC or *Trk*) or translocations of *RET* and *NTRK1* oncogenes appear to be of crucial importance in the development of PTC [22,6]. There are also several other molecular mechanisms, such as point mutations of *RAS* oncogenes, which may play

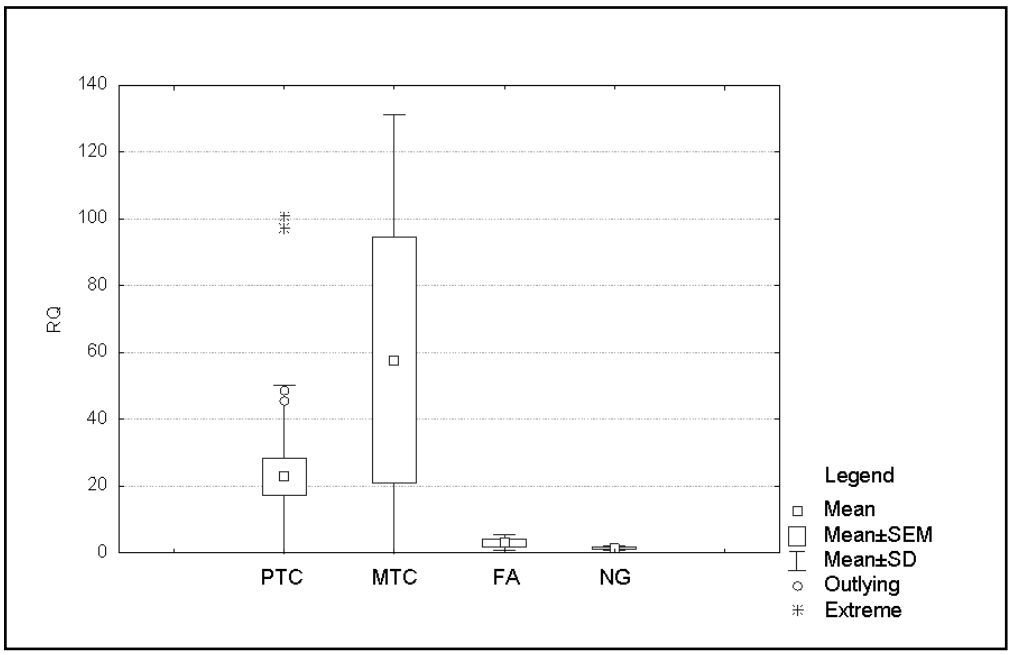

Figure 2. Box-and-whisker plots, representing the expression of *cyclin D1* gene in the studied groups (PTC, MTC, FA, NG). Results are calculated as RQ values. Whiskers represent means±SD (standard deviation) for particular groups. Boxes represent means±SEM (standard error of mean). The results were statistically analyzed using Kruskal-Wallis' test, p=0.0050.

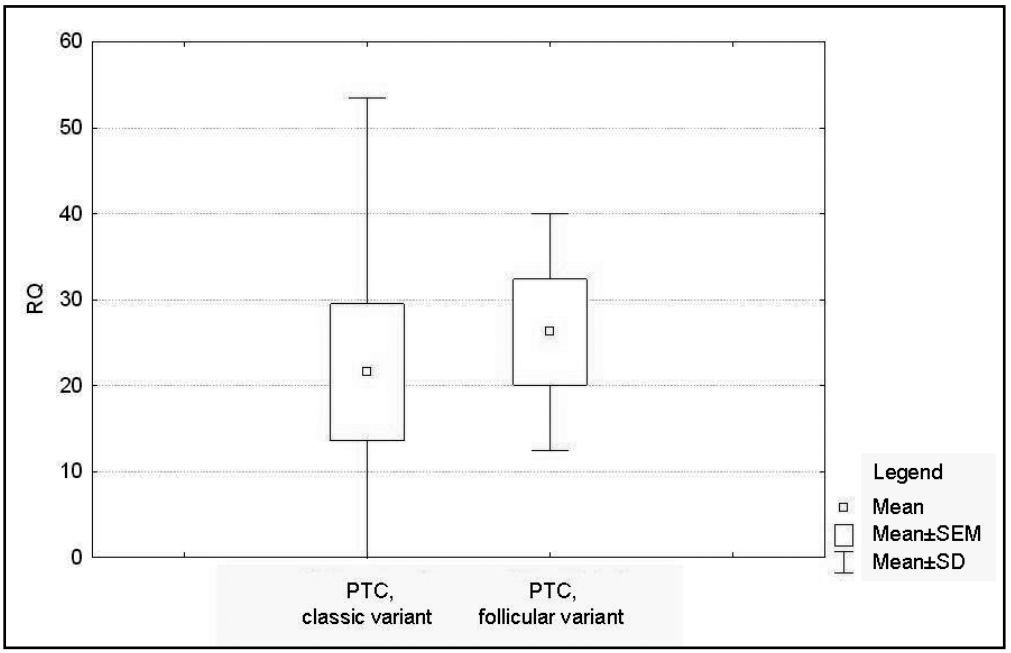

Figure 3. Box-and-whisker plots, representing the difference in the expression of *cyclin D1* gene between PTC - classic variant and PTC - follicular variant. Results, calculated as RQ values, do not show any significant differences between the studied groups (t-test, p=0.756211). Whiskers represent means±SD (standard deviation) for particular groups. Boxes represent means±SEM (standard error of mean).

some role in cancerogenic processs in the thyroid. Tissue culture-based experiments evidenced that *cyclin D1* gene functions as a collaborative oncogene that enhances the oncogenic transformation of other oncogenes (i.e. *RAS, Src, E1A*) in cultured cells [11].

In our study, we examined whether the *CCND1* (*cyclin D1*) protooncogene was involved in thyroid cancerogenesis.

The majority of reports, concerning cyclin D1 involvement in thyroid cancer pathogenesis, have been obtained in studies performed at the protein level, using the immunohistochemical approach [36,35,26,18,16,4,34,20,12, 17]. Real-time PCR is a recently developed technique. It is an extremely sensitive, precise and reproducible method for detection of nucleic acids and for their quantitative assessment. Unfortunately, there are only few reports on cyclin D1 mRNA expression in thyroid tumours, using the real-time PCR method [26,5]. In our study, we performed real-time relative quantification PCR assay for cyclin D1 mRNA expression, based on fluorescent TaqMan methodology and using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems).

The assay, used for the study, is based on the determination of a $\Delta\Delta C_T$ value for each sample and on calculat-

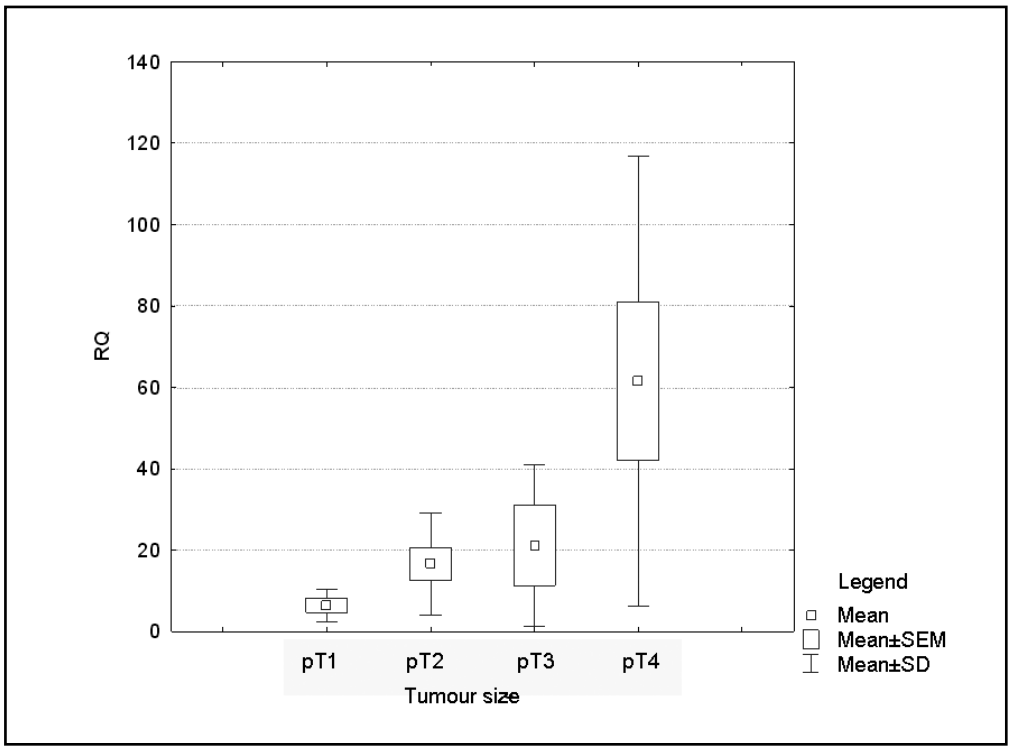

Figure 4. Box-and-whisker plots, representing the difference in the expression of *cyclin D1* in PTCs, depending on the tumour size (with regards to TNM definition of primary tumours; pT1, pT2, pT3, pT4**).** Results are calculated as RQ values. Whiskers represent means±SD (standard deviation); boxes represent means±SEM (standard error of mean). The results were analyzed using Spearman's rank correlation coefficient (0.569350, p=0.001938).

ing the fold-difference in *cyclin D1* expression between tumour sample and normal thyroid tissue (RQ value). The higher is the $\Delta\Delta C_T$ value, the lower is the expression of *cyclin D1*.

The results, obtained in our study, show that an elevated level of *cyclin D1* gene expression can already be observed at the NG stage and, to a greater extent, at the FA stage of tumourigenesis. It is to be recalled that FAs are benign neoplasms and some of them are capable of malignant transformation and progression. The overexpression of the *CCND1* gene, observed in both the benign and malignant thyroid lesions, suggests that cyclin D1 plays an early role in thyroid tumourigenesis, what has been confirmed by many research groups [34,28,20,12,5]. Such results were obtained by means of the immunohistochemical technique, as well as the real-time PCR assay. Using the TaqMan real-time PCR method, Bieche *et al.* [5] found the overexpression of *CCND1* gene in 33.3% of benign thyroid tumours and in 41.7% of malignant thyroid tumours. Nakashima *et al.* [26] observed overexpression of cyclin D1 mRNA in 45.5% of FAs and 54.5% of PTC by TaqMan real-time PCR (thyroid tumours from a radiocontaminated area). Using immunohistochemical methods, Nakashima *et al.* [26] found cytoplasmic or nuclear (or both, cytoplasmic and nuclear) cyclin D1 immunoreactivity in FAs and the only nuclear cyclin D1 immunoreactivity – in PTCs (however, much stronger). The authors suggest that cyclin D1 may be up-regulated at an early phase and accumulates in the cytoplasm, translocates to the nuclei of tumour cells and, subsequently, promotes malignant transformation during thyroid tumourigenesis.

The other study, performed in thyroid PMC that may be considered a precursor of PTC, has also revealed that cyclin D1 expression may be increased at an early stage of thyroid cancerogenesis and may promote the tumour growth [20].

In immunohistochemical assessment of thyroid PMCs of different size, Lantsov *et al.* [20] showed that cyclin D1 expression was significantly higher in PMC of size over 5 mm in diameter than in PMC of size below 5 mm in diameter, while no significant difference was found between PMC with diameter over 5 mm and PTC.

Cyclin D1 immunoreactivity in NGs was observed by other investigators but it was not classified as overexpression [10] and it was much lower than in PTCs [18]. In MTCs, the immunohistochemical study, performed by Sporny *et al.* [32], showed that the mean value of the cyclin D1 expression index in MTCs was higher than in PTCs.

Statistical analysis of our present results has also revealed significant differences, regarding RQ values among the PTC group and the FA group ($p<0.05$) and the NG group ($p<0.05$). Comparing *CCND1* gene expression between PTCs and MTCs, the value of RQ was higher in the MTC group, although not to any statistically significant extent ($p>0.05$).

Recent reports suggest that *cyclin D1* gene expression may be associated with some clinical features of PTC course, among others with poor prognosis [36,35]. Papil-

lary thyroid carcinomas, as well as PMCs of the thyroid gland that overexpress cyclin D1, are characterized by a significantly higher incidence of lymph node metastases, compared with tumours that do not show any increased expression of cyclin D1 [16,20,17,15]. It appears that cyclin D1 overexpression, found at the level exceeding 60%, predicts lymph node metastasis in clinical course of PTCs [16]. The cited group of investigators showed that over 90% of metastasizing PMCs expressed cyclin D1, compared to 8.8% of non-metastasizing ones [17]. It is even suggested that the benign behaviour of most autopsy-derived thyroid PMCs may be associated with the lack of cyclin D1 overexpression [19].

However, Kim *et al.* [18] found no difference in cyclin D1 expression between PTCs with regional lymph node metastasis and PTCs without metastasis. Similarly, Basolo *et al.* [4] observed that cyclin D1 mRNA overexpression was inversely correlated with the lymph node status. Neither was there any correlation with the gender or tumour status, but the protein product was higher in the tumours from patients aged below 40 [4].

In our study, the applied statistical analysis did not reveal any significant relationship between the *cyclin D1* gene expression level and the patients' age or sex ($p>0.05$). On the other hand, there was a positive correlation between RQ rate value and tumour size, that might confirm the possible association between *cyclin D1* gene expression and poor prognosis of thyroid lesions.

In summary, the level of *cyclin D1* gene expression in various thyroid lesions may be helpful in diagnostically doubtful cases. However, the results, obtained in our study – mostly due to the small numbers of cases in the groups other than PTC – do not y*et* allow considering *cyclin D1* gene as a molecular prognostic marker.

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