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Aberrant methylation as a main mechanism of TSGs silencing in PTC

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1. ABSTRACT

In the present study the role of tumour suppressor genes (TSGs) hypermethylation and genetic instability of LOH/MSI type in thyroid tumorigenesis was assessed. Expression, methylation status and presence of LOH/MSI were analyzed for 8 TSGs selected from imprinted (IR) and non-imprinted (NIR) chromosomal regions in papillary thyroid carcinomas (PTCs) and nodular goitres (NGs). The results show that methylation-induced gene silencing occurs at an early step of thyroid carcinogenesis and involves multiple genes. Genetic changes of LOH/MSI type are less frequent. In PTC samples, the lack of significant differences in the frequency of LOH in IR and NIR suggests that it is not a key mechanism changing the pattern of gene expression. Co-methylation observed both in NG and PTC raises a possibility that, in thyroid tissue, methylation-induced silencing may occur not only in malignant transformation but also in functional context. We did not recognize any of the studied TSGs – in regard to aberrant methylation status or LOH/MSI

frequency – as a selective molecular marker in thyroid tumorigenesis.

2. INTRODUCTION

Papillary thyroid carcinoma (PTC), well-differentiated tumour, is the most frequent type – corresponding to more than 80% cases – of thyroid malignancy (1). Among different genetic factors, involved in the pathogenesis of PTC, genetic alternations, leading to the activation of mitogen-activated protein kinase signaling pathway (MAPK) were recognized as crucial (2-4). Genetic events involve genes coding for intracellular tyrosine kinase receptors (RET and NTRK1) and genes for signaling protein effectors RAS and BRAF. Most studies, concerning different populations, provide strong evidence that activating mutations in *RET, NTRK1, RAS* or *BRAF* gene are found in more than 70% of PTCs (3,5-8). Moreover, it has been confirmed that there is only little or no overlap between *RET/PTC* sequence*, NTRK1, RAS,* and *BRAF* mutations (3,9). Somatic point mutation of the *BRAF* gene, leading to V600E substitution, has been recognized as the most common genetic alteration in PTCs (36–69%) (2,5). Therefore, constitutive signaling along the RET-RAS-BRAF-MAPK pathway was confirmed as the key molecular event in PTC development (10).

Interestingly enough, it has been proved that PTCs with *RET/PTC, RAS* or *BRAF* mutations have different clinical phenotypes (11,12) and each of these mutations have different diagnostic and therapeutic value, according to the genetic heterogeneity of PTC (2,4,12).

Many studies, focused on altered gene expression in various types of human cancer have confirmed a significant role of epigenetic events as a one of the main mechanisms of gene regulation (13,14). Specific epigenetic modification of gene expression is promoter methylation of tumour suppressor genes (TSGs), causing their silencing *via* transcription repression and conferring the cell with selective advantages for tumour progression (15). This mechanism has been described in many human cancers (16,17). Although cytosine methylation in promoter regions of TSGs is crucial in genomic imprinting, little is known about the relationship between the changes in methylation pattern and cancer development. Neither has it been established so far, whether methylation constitutes the primary cause of imprinting or if it is just an accompanying event.

Recently, the phenomenon of TSG methylation, as the fundamental mechanisms of epigenetic inheritance, has been stressed in the oncogenesis and differentiation of malignant tumours of the thyroid gland (18-22). It is known that methylation-induced gene silencing affects multiple genes in thyroid tumours, including *PTEN, RASSF1A, TIMP3, DAPK, RARbeta2, SLC5A8, NIS, TSHR* (20-23). It is worth noting that aberrant DNA methylation patterns of CpG sequences may contribute to tumour transformation at an initial stage of thyroid carcinogenesis but, on the other hand, it is also a common phenomenon, often related to tumour progression (19-24).

Moreover, most of the studies, focused on thyroid carcinoma, have documented that genetic alterations, such as microsatellite instability (MSI) and loss of heterozygosity (LOH), constitute an integral part of thyroid carcinogenesis (25-27). Although, opposite to FTC (follicular thyroid carcinoma), LOH in PTC is a rather rare mechanism of loss of gene function (26,28). Until now there are some studies reporting a higher percentage of LOH frequency in specific chromosomal regions (1q, 4p, 4q, 5p, 7q, 7p, 9p, 9q and 16q) involving *loci* of important suppressor genes (25,29). Furthermore, some researches have proved that the occurrence of LOH/MSI in FTC, as well as in PTC, is connected with a more advanced stage of tumour development and invasion (26,29,30).

Taking into consideration the controversial results of many studies, concerning mainly the frequency and the importance of LOH in the mechanism of suppressor gene silencing and progression of PTC (25,29), it is worth continuing the research regarding these issues. Despite many research works being focused on epigenetic mechanism of TSGs silencing *via* aberrant promoter methylation and on LOH in thyroid tumours – including PTC – the knowledge concerning these mechanisms is not sufficient. The results of the recent studies, assessing the role of LOH in gene silencing in FTCs and FAs (follicular adenomas) have suggested different LOH frequency, depending on imprinted and non-imprinted regions (IR and NIR, respectively) of the genome (28,31). Therefore, the deletion of the only functional allele copy of imprinted TSG may be an important element in oncogenic transformation of the thyroid gland (31).

The aim of our study was to answer the question if DNA hypermethylation and/or LOH constitute the most important mechanisms of functional suppressor gene silencing in PTC tumorigenesis. We focused on selected suppressor genes located in IR (*i.e.*, *ARHI, MEST, KCNQ1* and *p16INK4A*) and NIR (*i.e.*, *RASSF1A, VHL, SLC5A8, CDH1*) to compare the involvement of those regions, as well as the role of epigenetic mechanism in PTC oncogenesis.

3. MATERIALS AND METHODS

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

3.1. Thyroid tissue samples

PTC tissue samples (100-150 mg) were obtained from 45 patients who had undergone surgery treatment of total thyroidectomy at the Department of General, Oncological and Endocrine Surgery, Medical University of Lodz, and at the Department of General and Oncological Surgery, Medical University of Lodz, Poland, during the years 2006 - 2009.

Total tumour tissue samples, immediately after resection, were collected into lysis buffer (Buffer RNAlater, Qiagen Sciences, USA), homogenized and frozen at -70°C until use.

Table 1. Clinical and histopathological characteristics of the studied patients with confirmed primary PTCs

PTC variant (number of cases)	Age (range)	Sex		Tumour stage	
		Women, (number of cases)	Men, (number of cases)	TNM (number of cases)	AJCC (number of cases)
PTC classic (27)	20 - 72	22	5	pT1N0M0 (1); pT1NxMx (2); pT1N0Mx (4); pT1aNxMx (2); pT1aN1Mx (2); pT1bNxMx (1); pT2N0M0 (1); pT2N0Mx (1); pT2NxMx (1); pT2aNxMx (1); pT2aN0M0 (2); pT2aN1aM0 (1); pT2bN1aM0 (1); pT2bN1Mx (1); pT2bNxMx (1); pT4N0Mx (1); pT4N1Mx (1); pT4aN1aM0 (1); pT4aN1Mx (1); pT4N1bM0 (1)	I (18) II (4) IIII (1) IVA (4)
PTC follicular (15)	26 - 80	11	4	pT1NxM0 (2); pT1aN0M0 (1); pT1aNxMx (1); pT2N1aMx (2); pT2aNxMx (1); pT2aN0M0 (1); pT2bN1aM0 (1); pT3NxMx (1); pT3aN0M0 (1); pT3N0Mx (1); pT4N1aM0 (1); pT4N1Mx (1); pT4aN0M0 (1)	I (7) II (1) III (5) IVA (2)
PTC tall-cell (3)	52 - 59	3	0	pT4N0M0 (1); pT4bN1Mx (2)	IVA (1) IVB (2)

Histopathological diagnoses for malignant thyroid lesions, according to WHO classification, were obtained from pathomorphological reports. PTC variants were as follows: PTC classic variant (27 cases: 22 females, 5 males; mean age 41.85 +/- 16.33), PTC follicular variant (15 cases: 11 females, 4 males; mean age 54.53 +/- 15.34), PTC tall-cell variant (3 cases: 3 females; mean age 56.67 +/- 4.04) (Table 1). The nodular goitre (NG) group consisted of 22 cases (20 females, 2 males; mean age 48.57 +/- 14.77).

Tissue sample was obtained from the centre of thyroid lesion and also from macroscopically unchanged tissue surrounding the lesion.

3.2. Isolation of total RNA and genomic DNA

Total RNA was extracted from fresh tissues, using RNeasy Protect Midi Kit (Qiagen, Hilden, Germany) and according to the manufacturer's recommendations. Quality and quantity assessments of total RNA samples were determined by mini-electrophoreses in polyacrylamide gel (Agilent 2100 Bioanalyzer, Agilent, USA), using RNA 6000 Pico/Nano LabChip kit (Agilent Technologies, USA).

Isolation of genomic DNA from thyroid tissue was performed using QIAamp DNA Mini Kit (Qiagen, Germany), according to the manufacturer's protocol. Quality and quantity of each DNA sample was spectrophotometrically assessed, measuring absorbance at wave length of 260/280 nm (NanoDrop Spectrophotometr ND-1000, ThermoScientific, USA).

3.3. Reverse transcription reaction

Complementary DNA (cDNA) was transcribed from 1000 ng of total RNA, using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) in a total volume of 20 microl per reaction. Reverse transcription (RT) master mix contained: 10x RT buffer, 25x dNTP Mix (100 mM), 10x RT Random Primers, MultiScribe™ Reverse Transcriptase, RNase Inhibitor and nuclease-free water. RT reaction was performed in a Personal Thermocycler (Eppendorf, Germany) in the following conditions: 10 minutes at 25°C, followed by 120 minutes at 37°C, then the samples were heated to 85ºC for 5 seconds, and hold at 4°C.

3.4. Gene expression analysis: TaqMan real-time PCR assay

Real-Time PCR reactions were performed in an ABI PRISM 7900HT Real-Time PCR System (Applied Biosystems, USA) using Micro Fluidic Cards (Applied Biosystems, USA). The cards with 384 wells were pre-loaded with selected assays (TaqMan® Gene Expression Assays, Applied Biosystems; see Table 2), containing unlabelled primers and fluorogenic TaqMan® MGB probes, selected on Applied Biosystems' web site (*www.appliedbiosystems.com*). TaqMan® probes were labelled with FAM™ (6-carboxy-fluorescein) at the 5' end as the reporter dye and with non-fluorescent quencher (TAMRA, 6-carboxy-tetramethylrhodamine) at the 3' end.

All nine selected TaqMan® Gene Expression Assays – 8 target genes and 1 reference gene (endogenous control) – were pre-loaded on micro fluidic cards, thus allowing to assess the expression levels of all genes (fourfold repeated) in 8 samples of cDNA run in parallel.

Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene as an endogenous control was used. All selected genes and corresponding TaqMan® Gene Expression Assays are listed in Table 2.

In the study design, macroscopically unchanged thyroid tissue served as calibrator sample.

Each sample-specific PCR mix contained 50 microl cDNA (50 ng) and 50 microl TaqMan® Universal Master Mix (Applied Biosystems, USA). TaqMan Array card was centrifuged twice for 1 minute at 1200 rpm to fill the wells with PCR mixture. Then, it was sealed (TaqMan® Array Micro Fluidic Card Sealer, Applied Biosystems, USA) and placed in the Applied Biosystems 7900HT Fast Real-Time PCR System. The PCR conditions were as follows: after initial incubation at 50°C for 2 minutes and AmpliTaq Gold® DNA polymerase activation at 94.5°C for 10 minutes, real-time PCR amplification was processed in 40 cycles of 30 second denaturation at 97°C, followed by 1 minute elongation step at 59.7°C

The assay described in this report, involves the determination of a delta-delta C_T value. C_T (threshold cycle) indicates the number of cycles at which the

Table 2. TaqMan® Gene Expression Assays selected for the studied genes and applied in real-time PCR reactions.

Locus	Gene Name and Symbol	TaqMan® Gene Expression Assay ID	GenBank accession number
1p31	ADP-ribosylarginine hydrolase, ARHI	Hs00153890_m1	NM_001125.2
3p21.3	Ras association domain family 1, RASSF1A	Hs00200394-m1	NM_007182.4
3p26-p25	von Hippel-Lindau tumour suppressor, VHL	Hs01650959-m1	NM_198156.1
7q32	mesoderm specific transcript homolog (mouse), MEST	Hs00602654_m1	NM_177525.1
9p21	cyclin-dependent kinase inhibitor 2A, p16INK4A	Hs00233365_m1	NM_000077.3
11p15.5	potassium voltage-gated channel, KCNQ1	Hs00165003_m1	NM_000218.2
12q23.1-q23.2	solute carrier family 5 (iodide transporter), member 8, SLC5A8	Hs00377618_m1	NM_145913.3
16q22.1	E-cadherin (epithelial), CDH1	Hs00170423-m1	NM_004360.3
12p13	glyceraldehyde-3-phosphate dehydrogenase, GAPDH	Hs99999905-m1	NM_002046.3

Table 3. Summary of MSP assays: primers, product sizes and annealing temperatures.

Gene symbol	Primer length		Primer sequence (3' → 5')	Product length	Annealing temperature
ARHI	U[1]	F[3] – 26 bp R[4] – 25 bp	5'-TGTGTAGTTTTTAATGTATTTGTTGT-3' 5'-AACTAAAAAACCCAATTATATCATT-3'	164 bp	55.5°C
	M[2]	F – 25 bp R – 25 bp	5'-GTGCGTAGTTTTTAATGTATTCGTC-3' 5'-GACTAAAAAACCCGATTATATCGTT-3'	165 bp	60°C
CDH1	U	F – 25 bp R – 20 bp	5'- TAATTTTAGGTTAGAGGGTTATTGT -3' 5'- CACAACCAATCAACAACACA -3'	212 bp	60°C
	M	F – 22 bp R – 23 bp	5'- TTAGGTTAGAGGGTTATCGCGT -3' 5'- TAACTAAAAATTCACCTACCGAC -3'	206 bp	60°C
p16INK4A	U	F – 24 bp R – 22 bp	5'- TTATTAGAGGGTGGGGTGGATTGT -3' 5'- CAACCCCAAACCACAACCATAA -3'	154 bp	60°C
	M	F – 24 bp R – 21 bp	5'- TTATTAGAGGGTGGGGCGGATCGC -3' 5'- GACCCCGAACCGCGACCGTAA -3'	145 bp	60°C
KCNQ1	U	F – 21 bp R – 25 bp	5'- TATGTTGTTTTATGGGATTGG-3' 5'-ACTTCAACAAAAAAAACCTACCAAA-3'	198 bp	56°C
	M	F – 21 bp R – 21 bp	5'- GTACGTCGTTTTATGGGATCG-3' 5'- CAACGAAAAAAACCTACCGAA-3'	195 bp	57°C
RASSF1A	U	F – 23 bp R – 23 bp	5'-TTTGGTTGGAGTGTGTTAATGTG-3' 5'- CAAACCCCACAAACTAAAAACAA -3'	108 bp	55°C
	M	F – 20 bp R – 21 bp	5'- GTGTTAACGCGTTGCGTATC-3' 5'- AACCCCGCGAACTAAAAACGA-3'	111 bp	55°C
SLC5A8	U	F – 26 bp R – 22 bp	5'- TTGTTTTATATTTTTTTATTGGTTGT-3' 5'- TATAAAACCTCAAAAAATCAAC-3'	229 bp	52.5°C
	M	F – 24 bp R – 21 bp	5'- GTTTTATATTTTTTTATCGGTCGT-3' 5'- ATAAAACCTCGAAAAATCGAC-3'	226 bp	54.5°C
VHL	U	F – 25 bp R – 20 bp	5'- GTTGGAGGATTTTTTTGTGTATGT -3' 5'- CCCAAACCAAACACCACAAA -3'	165 bp	58°C
	M	F – 22 bp R – 17 bp	5'- TGGAGGATTTTTTTGCGTACGC-3' 5'- GAACCGAACGCCGCGAA-3'	158 bp	58°C
MEST	U	F – 25 bp R – 25 bp	5'- ATTTAATGTTGGAGGTTATTGTTGA-3' 5'-CATATTTCAAAAAACCAATTACACA-3'	251 bp	57°C
	M	F – 25 bp R – 23 bp	5'- TTATTTAATGTCGGAGGTTATTGTC-3' 5'- CATATTTCGAAAAACCGATTACG-3'	253 bp	58.5°C

Abbreviations: [1] unmethylated primers, [2] methylated primers, [3] forward primer, [4] reverse primer

fluorescent signal of PCR product reached the established threshold. The greater the delta-delta C_T value, the lower expression of the studied gene in the specimen. Fold-differences, representing relative expression results, are calculated using the equation: RQ (relative fold increase) = $2^{-\{\text{delta-delta}C_T\}}$.

3.5. Promoter methylation analysis

3.5.1. Sodium bisulfite modification of DNA

Genomic DNA (1.0 microg) was modified with sodium bisulfite, using the CpGenome™ DNA Modification Kit (CHEMICON International, Millipore, USA), according to the manufacturer's protocol. Concentration and purity of the modified DNA was spectrophotometrically estimated at 260/280 nm in NanoDrop Spectrophotometer ND-1000 (ThermoScientific, USA).

3.5.2. MSP primers and methylation-specific PCR

The primers for the methylation-specific PCR (MSP) were designed, using Methyl Primer Express® Software (*www.appliedbiosystems.com/methylprimerexpress*), according to the following criteria: (i) the length of the primers should be 18-26 bps; (ii) PCR product (150 – 250 bp long) should contain at least one CpG island flanking the 3'end; (iii) all used primers should have similar melting temperature, with difference no greater than 5°C; (iv) both unmethylated (UM) and methylated (M) primers should flank the same CpG sites and have similar length (UM primer can be 1,2 bp longer than M primer).

The set of primers for each studied gene was flanking the 1 kb 5' region upstream from the translation start point. All the used primer pairs flanked promoter sequences and, additionally, the first exon of each studied gene, according to the criteria described by Feltus *et al.* (32). Table 3 shows the sequences and length of MSP primers, the length of MSP products, as well as specific annealing temperature for the studied genes used in MSP assays.

Table 4. Summary of BSP primer sequences for *ARHI* and *CDH1* genes, annealing temperatures and PCR product sizes

Gene symbol	Primer length		Primer sequence (3' → 5')	Product length	Annealing temperature
ARHI	F[1]	24 bp	5'- TAGGTAAGGGAGAAAGAAGTTAGA-3'	192 bp	61.3 °C
	R[2]	26 bp	5'- AATACTACTATCCTAACAAAACCCTC -3'		
CDH1	F	19 bp	5'- GTTGTTGATTGGTTGTGGT-3'	277 bp	60.8 °C
	R	22 bp	5'- TACCTACAACAACAACAACAAC -3'		

Abbreviations: [1] forward primer, [2] reverse primer

The methylation status of each studied gene was determined by the conventional MSP method, described previously (33) with some modifications. Briefly, MSP was performed in triplicate for each sodium bisulfite modified DNA sample, using AmpliTaq Gold® DNA Polymerase Kit (Applied Biosystems, USA). Amplifications were conducted in a total volume of 25 microl in a Personal Thermocycler (Eppendorf, Germany). MSP master mix contained: 1000 ng DNA, 0.7 microM oligonucleotide primers (forward and reverse) (METAbion, Germany), 2.5 mM dNTPs mix, 2.5 microM $MgCl_2$, Hot Start AmpliTaq Gold® Polymerase (5U/microl), 10x Universal PCR buffer and nuclease-free water. PCR conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 35 cycles: denaturation at 95°C for 45 seconds, annealing at gene-specific temperature (52°C - 62°C) for 45 seconds and elongation at 72°C for 1 minute; the final elongation step was done at 72°C for 10 minutes.

In parallel reactions, the positive and negative MSP controls were performed for PCR standardization. CpGenome Universal Methylated DNA (enzymatically methylated human male genomic DNA; CHEMICON International, Millipore, USA) served as a positive methylation control. As a negative control, CpGenome Universal Unmethylated DNA was used from human fetal cell line (CHEMICON International, Millipore, USA). In order to control the specificity of primers and to confirm the completeness of DNA bisulfite conversion, an amplification reaction was conducted, using W primer, designed specifically for unmodified DNA. Additionally, blank samples with nuclease-free water were used instead of DNA as a control for PCR contamination.

The MSP products were electrophoretically separated on polyacrylamide (8% PAA) gel and concentrations (ng) of MSP products (U and M DNA alleles) were spectrophotometrically estimated, using DNA1000 LabChip Kit, on Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Afterwards, the Methylation Indexes (MIs) were assessed for each sample. The averaged MI, based on three repeats of amplification reaction, were calculated according to the formula MI = (M)/(M+U), where (M) stands for the methylated and (U) for unmethylated allele concentration, respectively.

3.5.3. BSP primers and bisulfite-specific PCR

Bisulfite-specific PCR (BSP) reactions for *ARH1* and *CDH1* genes were performed. Those two genes were selected due to the highest MI values, as compared with the other studied genes. BSP primers were designed using the Methyl Primer Express® Software (*www.appliedbiosystems.com/methylprimerexpress*), according to the following criteria: (i) the length of the primers should be 18-26 bp long; (ii) PCR product (200 – 350 bp long) should span the sequence with potential CpG sites; (iii) none of CpG can be present in the primer sequence, as there is one universal pair of primers designed for one gene. BSP primers were designed to span the regions involving the MSP sequences of each studied gene (34).

BSP reactions were performed in triplicate for each sodium bisulfite modified DNA sample, using AmpliTaq Gold® DNA Polymerase Kit (Applied Biosystems, UK). Amplification reactions were conducted in a Personal Thermocycler (Eppendorf, Germany) in a total volume of 25 microl master mix with BSP oligonucleotide primers (METAbion, Germany). PCR conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 40 cycles: denaturation at 95°C for 45 seconds, annealing at gene-specific temperature (60.8°C – 61.3°C) for 45 seconds and elongation at 72°C for 1 minute; the final elongation step was done at 72°C for 10 minutes.

The sequences of BSP primers for *ARH1* and *CDH1* genes, annealing temperatures and PCR product sizes are shown in
Table 4.

3.5.4. Direct sequencing

The BSP products were electrophoretically separated on 8% polyacrylamide (PAA) Tris-borate-ethylenediamine tetraacetate (TBE) gels, then were purified, using AutoSeq™ G-50 Columns (Amersham Biosciences, UK). Sequencing PCR was conducted in a total volume of 20 microl, using a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA), according to the manufacturer's protocol. Afterwards, the samples were purified, using Centri-Sep Spin Columns (Applied Biosystems, USA), dried in a Vacuum Concentrator 5301 (Eppendorf, Germany), resuspended in 20 microl of Hi-Di Formamide (Applied Biosystems, USA) and analyzed in a 3130 Genetic Analyzer (Applied Biosystems, USA).

3.6. Loss of heterozygosity and microsatellite instability analysis

3.6.1. Polymerase chain reaction with microsatellite markers

Twenty seven primer pairs were used to perform microsatellite analysis, based on PCR amplification of polymorphic microsatellite repeats: (T)n, (CA)n, (TTA)n and (TCTA)n in paired PTC and control (macroscopically unchanged thyroid tissue) DNA, derived from the same patients.

Table 5. Nucleotide sequences of microsatellite markers from the imprinted region (IR) of the genome.

Chromosomal region (gene) / marker	Nucleotide sequence of microsatellite marker (5'-3')	
	Sense	Antisense
1p31.2 (*ARHI*)		
D1S2137	ACATCTTTGGTTTGGATAGATG	CAAAACTGCACATTTTGCAC
D1S368	GGGCATTGTTTAGGGGTG	TAGTGGGCTTTACGTCTGC
7q32.2 (*MEST*)		
D7S1861	CCAAAAGTAATTTTTTAGGCAGC	AAAAAAAAGATGGAATGAGCTCC
D7S2519	GGAGGTTAAGATTTACAG	CACAAGTTCCTAAGTAGAG
D7S2519a	GGAGGTTAAGATTTACAG	GCTGTGGTGTATCCTGTG
D7S2544	TCCCCAGACCCCATTC	TCCTGTTCATCCTTCATTCC
D7S530	CGTTGCATTTTAGTGGAGCACAG	CAGCAGTAATGAAAGCAAAACACAG
9p21.3 (*p16INK4A*)		
D9S974	GAGCCTGGTCTGGATCATAA	AAGCTTACAGAACCAGACAG
D9S1604	CCTGGGTCTCCAATTTGTCA	AGCACATGACACTGTGTGTG
11p15.5 (*KCNQ1*)		
D11S4744	CTCTTATTATGAAGTGATTATCAG	TTCTGTTTACATGTGAATCATATT
D11S4726	CTTGGATTTGGCAGAGGTTTCTTAG	CACAGCACCTGGCCGATAACTATCT
D11S4088	GGGCAGAGGCAGTGGAG	GCATGTTTCGGGGGTG
D11S1318	CCCGTATGGCAACAGG	TGTGCATGTNCATGAGTG

In LOH/MSI analysis, 13 microsatellite markers mapped to known IRs, and 14 microsatellite markers selected from NIRs of human genome, were used (Tables 5 and 6). Genes important for cell cycle regulation, proliferation and adhesion are located in those regions. All primer sequences of the used markers and their cytogenetic localizations were found in NCBI database (*http:/www.ncbi.nlm.nih.gov/genome/sts/sts*) with supplementary mapping information, if necessary, provided in Cooperative Human Linkage Centre Database (*http://www.chlc.org*), the Genome Database (*http://www.gdb.org*). Each forward primer was labelled at 3'end with fluorescent dye: 6-FAM, NED, PET or VIC.

Amplification reactions with microsatellite markers were performed in a Personal Thermocycler (Eppendorf, Germany), in a total volume of 25 microl, including: 10x AmpliTaq Gold® 360 buffer (150 mM Tris-HCl, pH 8.3, 500 mM KCl), 360 GC Enhancer, 5U/microl AmpliTaq Gold® 360 DNA Polymerase, 25 mM $MgCl_2$, 10 mM dNTPs (Applied Biosystems, USA), 30-40 ng DNA, 0.5 microM each primer and nuclease-free water.

Each microsatellite marker was amplified at its own specific annealing temperature to optimalize the PCR reactions.

PCR conditions were as follows: initial denaturation at 95°C for 10 minutes, then 30 cycles of amplification with denaturation at 95°C for 45 seconds, primer annealing for 30 seconds at temperature specific for each marker, *i.e.*, in the range of: 47-50°C (for D3S1568, D3S3615, D3S1317, D3S3611, D7S2519, D7S2544, D11S4744, D11S4088, D11S1318, D12S1041, D12S1727, D16S3025), 51-59°C (for D1S2137, D1S368, D3S4351, D3S4188, D7S1861, D7S530, D9S974, D9S1604, D12S1101, D16S3314, D16S3206, D16S496) and 60-65°C (for D3S2832E and D11S4726), followed by elongation step at 72°C for 1 minute.

Afterwards, PCR product (0.5 microl) was mixed with 0.25 microl GS500-LIZ Size Standard (Applied Biosystems, USA) and formamide (Hi-Di™ Formamide, Applied Biosystems, USA) up to the final volume of 10 microl. The obtained mixture was denatured for 5 minutes at 95°C, then cooled on ice for 3 minutes, and separated by capillary electrophoresis in a 3130xl Genetic Analyzer (Applied Biosystems, Hitachi, USA).

3.6.2. Analysis of microsatellite loci

The results of gel capillary electrophoresis were analyzed, using the program for analysis of fragment sizes, *i.e.*, GeneMapper Software v 4.0, according to the manufacturer's protocol (Applied Biosystems, USA). Samples were considered informative, *i.e.*, heterozygous, when two distinct alleles were visible in the control DNA sample (from macroscopically unchanged thyroid tissue). Evaluation of LOH/MSI was performed by calculating the ratio of the fluorescence intensity of the alleles, originating from macroscopically unchanged tissue (N, normal, *i.e.*, control sample) to the fluorescence intensity of the alleles originating from PTC tissue (T, tumour). For each informative tumour-normal DNA pair (paired T and N samples), an Allelic Imbalance Ratio (AIR) was calculated, based on the maximum allele peak heights (fluorescence intensity), as follows: normal-allele 1: normal-allele 2/tumor allele 1: tumour allele 2 (N1:N2/T1:T2) (35). When AIR was less than 0.67 or greater than 1.35 it was considered indicative of LOH in tumour samples (according to the criteria of GeneMapper Software v 4.0). Tumour DNA was considered as harbouring MSI if one or more additional alleles were present in tumour DNA sample, as compared with the control DNA sample.

For each PTC sample Fractional Allele Loss (FAL) index was also calculated, reflecting the ratio of total number of chromosomal *loci* with LOH or MSI to the total number of informative *loci* examined (36).

3.7. Statistical analysis

The differences in relative expression levels (RQ values) for all the genes between the studied groups (PTC and NG) were statistically analyzed, using the non-parametric Mann-Whitney's test.

MI value, reflecting the methylation status of the individual genes, was defined as a total fraction of methylated alleles. The comparison of MI values between

Table 6. Nucleotide sequences of microsatellite markers from the non-imprinted region (NIR) of the genome.

Chromosomal region (gene) / marker	Nucleotide sequence of microsatellite marker (5'-3')	
	Sense	Antisense
3p21.3 (*RASSF1A*)		
D3S2832E	CCAGGGTCTGTGTGAATGTG	CCCACAGGAGGCATTCAG
D3S4188	CAGACTGGAATAGTGGCATAAGG	GAATGAATAAGTCCACATAAAACG
D3S1568	CCATGAACAGAACCTCCCTA	CCGCTGTCCTGCTGTAAG
D3S3615	TGGAAAGGTAAGCACAAGC	TCCTCCCAGGAAGCAC
3p25.3 (*VHL*)		
D3S4351	GTCTCTGAGGACGTCTGTCG	ACTTTGCAGGCTGTTGTGG
D3S1317	TACAAGTTCAGTGGAGAACC	CCTCCAGGCCATACACAGTCA
D3S3611	GCTACCTCTGCTGAGCAT	TAGCAAGACTGTTGGGG
12q23.2 (*SLC5A8*)		
D12S1101	CACATTGGAGAATGTGCTAAAC	TGATGGGAGAATACAGACCTCT
D12S1041	AACTGTGGAAAAAGGGGAAC	TGCAACAAACCACCATGG
D12S1727	AGTCACCACTGAAAATCCAC	GAGTGAGACCCCGTAAAAA
16q22.1 (*CDH1*)		
D16S3314	CCAAAAAGTGATTGCAGGGT	GGGGGTTAAGTTGAGGGGTA
D16S3206	ACTTCGGAGCTTGTCAGGAA	CTGGAAACTTGGCCTCTGAG
D16S496	GAAAGGCTACTTCATAGATGGCAAT	ATAAGCCACTGCGCCCAT
D16S3025	TCCATTGGACTTATAACCATG	AGCTGAGAGACATCTGGG

Table 7. Comparison of mean RQ values of the studied genes between PTC and NG groups.

Gene		PTC group (n = 45)	NG group (n = 23)
IR	*ARHI*	1.738	1.167
	p16INK4A	6.946	1.839
	KCNQ1	0.715	0.828
	MEST	0.173	0.095
NIR	*RASSF1A*	2.364	1.067
	SLC5A8	0.554	1.077
	VHL	0.988	1.127
	CDH1	1.207	1.071

PTC and NG groups, as well as between PTC variants (classic *vs*. follicular) were conducted, using Mann-Whitney's test.

FAL index values were statistically assessed between PTC variants (classic *vs*. follicular) using Mann-Whitney's test.

In PTC group, Kruskal-Wallis test, Mann-Whitney's test and Spearman's rank correlation coefficient were performed in order to correlate RQ, MI and FAL index values of the studied genes with clinical variables (age, gender), histopathological variants, and the particular tumour size, according to TNM and AJCC classification (37).

In order to compare RQ and MI values between IR and NIR genes in two separate groups (PTC and NG), Mann-Whitney's test was used. For comparisons of FAL index values among IR and NIR genes in PTC group, Mann-Whitney's test was also applied.

The relationship between RQ and MI values for each individual studied gene in PTC and NG groups was assessed by Spearman's rank correlation coefficient.

Spearman's rank correlation coefficient was used to assess the correlation between FAL index and RQ values for all 8 studied genes, as well as the correlation between FAL index and LOH/MSI in all the chromosomal regions in PTC group.

Statistical significance was determined at the level of p-value less than 0.05. The results are presented as mean or median +/- SEM and +/- SD values.

For calculations, the Statistica for Windows 7.0 program was applied.

4. RESULTS

4.1. Results of relative expression analysis of the studied genes

4.1.1. Results of relative gene expression analysis in PTC and NG groups

RQ value (expression level) for each studied gene was calculated, using the delta-delta C_T method, adjusted to *GAPDH* expression and relative to the expression level of calibrator (macroscopically unchanged thyroid tissue) for which RQ = 1. The exemplary results of relative expression for selected studied genes (*CDH1, p16INK4A* and *RASSF1A*) in PTC, as well as in NG group, are shown in Figure 1. The mean RQ values for all the studied genes in PTC and NG groups are listed in Table 7.

In PTC group, the obtained results (see Table 7) showed decreased mean RQ values for *KCNQ1, SLC5A8* and *VHL* gene. Increased mean RQ values were observed distinctly for *p16INK4A* gene and also for *ARHI* and *RASSF1A* genes. In case of *CDH1* gene, the mean RQ values were similar to calibrator expression level. *MEST* expression value was barely detectable.

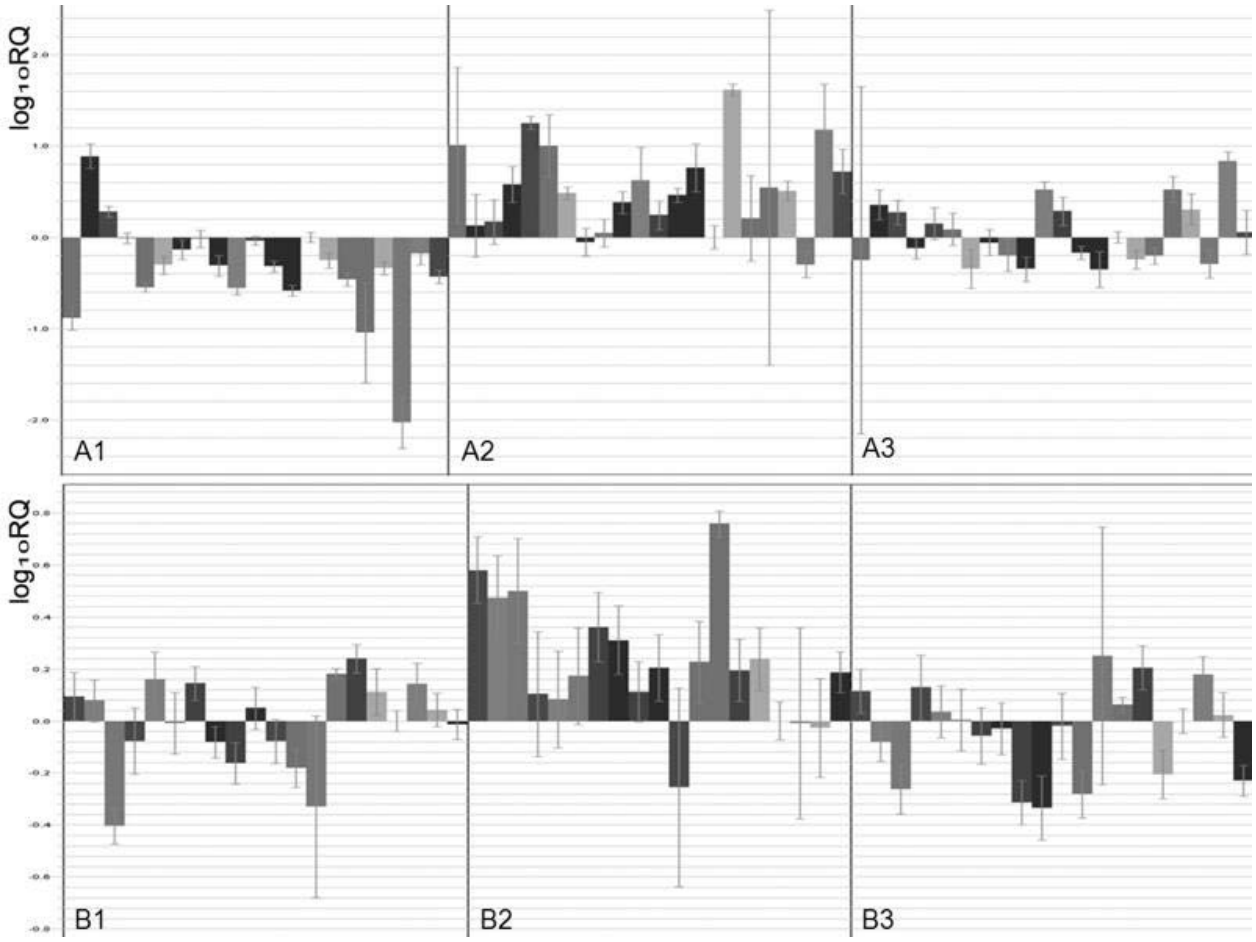

Figure 1. An example of relative expression (log_{10}RQ) level of *CDH1* (1), *p16INK4A* (2) and *RASSF1A* (3) in PTC (A) and NG (B) samples. Each bar represents an individual sample.

In NG group, the mean RQ values (see Table 7) of 7 genes (*ARHI, CDH1, p16INK4A, KCNQ1, RASSF1A, SLC5A8, VHL*) were at the level close to calibrator; *p16INK4A* showed the highest mean RQ value. Regarding *MEST* gene, similarly to PTC samples, its expression was slightly visible. Therefore, that gene was excluded from the subsequent comparisons.

The percentage of samples with a significant decrease of expression (*i.e.*, RQ less than or equal to 0.7) in a studied cohort was also calculated, both in PTC and NG group to compare the results obtained for each studied gene (Figure 2).

The obtained results showed a higher percentage of significantly reduced expression (RQ less than or equal to 0.7) in PTC samples, as compared to NG samples for all the studied genes. The results indicated that 4 studied genes, *i.e.*, *VHL, CDH1, SLC5A8* and *KCNQ1*, showed significantly reduced expression in a high percentage (47% - 80%) of the studied samples in PTC group, comparing to NG samples (17% - 34%) (see Figure 2). In case of the remaining genes (*RASSF1A, p16INK4A* and *ARHI*) a significantly decreased expression in PTC group was observed in 6% - 10% of the studied samples, while in NG group the same genes revealed significantly decreased RQ values in 0% - 20% of the studied samples.

4.1.2. Statistical analysis of gene expression

4.1.2.1. Comparison of RQ levels between PTC and NG groups

Mann-Whitney test was used to analyze the differences between RQ level and the type of lesion, *i.e.*, PTC *vs.* NG. Statistically significant differences (p-value less than 0.05) were found for 4 genes: *p16INK4A, KCNQ1, SLC5A8* and *VHL*. In case of *p16INK4A,* RQ values were higher for PTC group, while the other genes (*KCNQ1, SLC5A8* and *VHL*) showed higher RQ values for NG group. The examples of statistical results for *p16INK4A* and *KCNQ1*genes are shown in Figures 3 and 4, respectively.

4.1.2.2. Comparison of RQ levels in PTC group

An analysis of RQ values of all the studied genes between three variants of PTC (classic, follicular and tall-cell) with Kruskal-Wallis test revealed no statistically significant differences. However, when tall-cell PTC group was excluded because of the small number of cases, Mann-

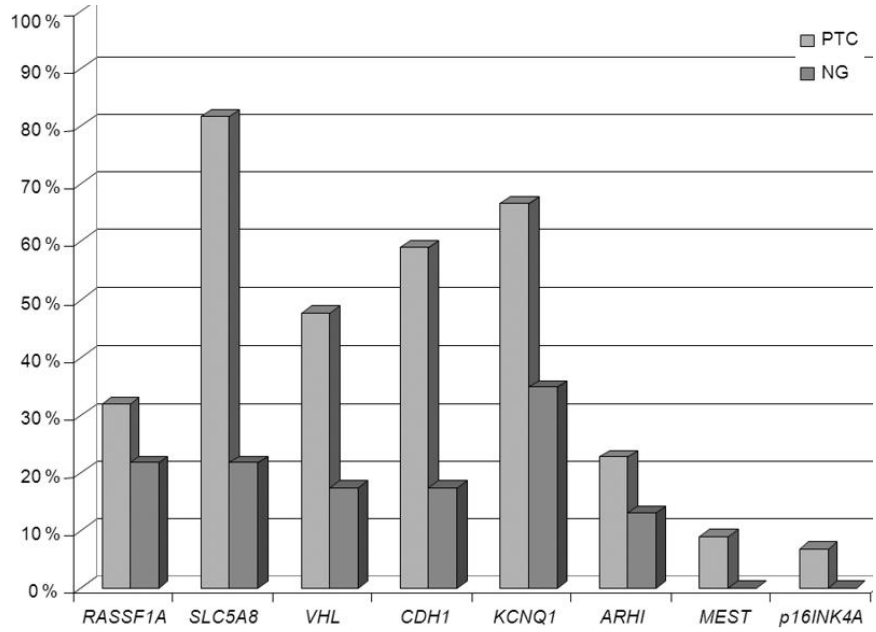

Figure 2. A comparison of sample percentage with significantly decreased RQ values for all the studied genes between PTC and NG groups.

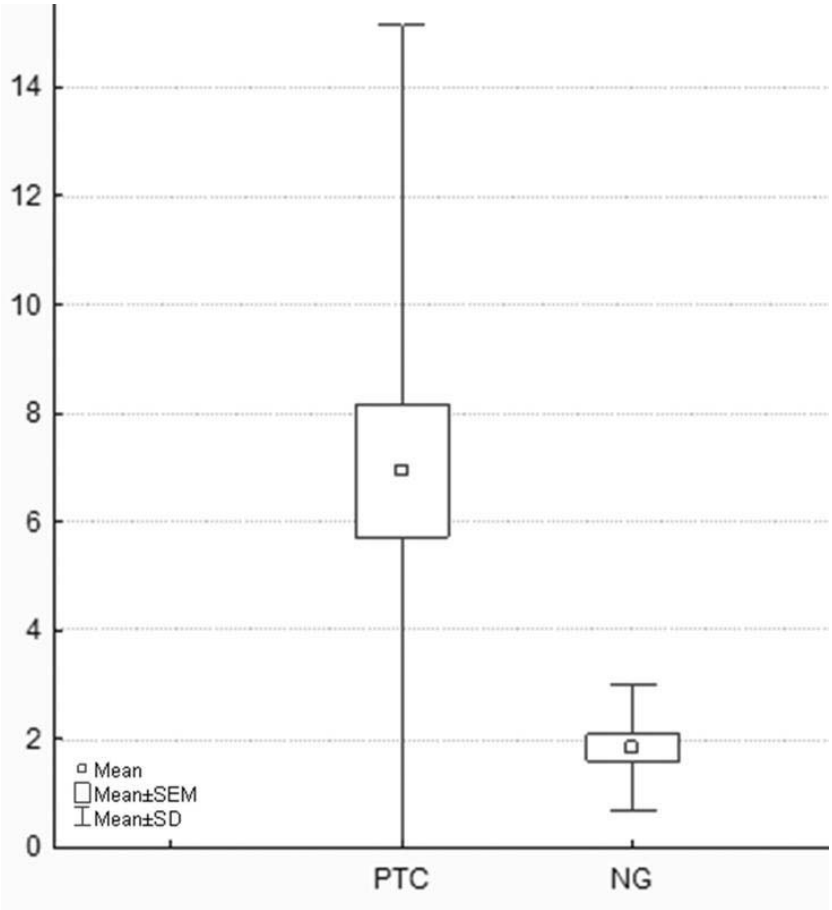

Figure 3. Box-and-whisker plots, representing the expression (mean RQ levels) of *p16INK4A* gene in PTC and NG groups (Man-Whitney test, p-value less than 0.05).

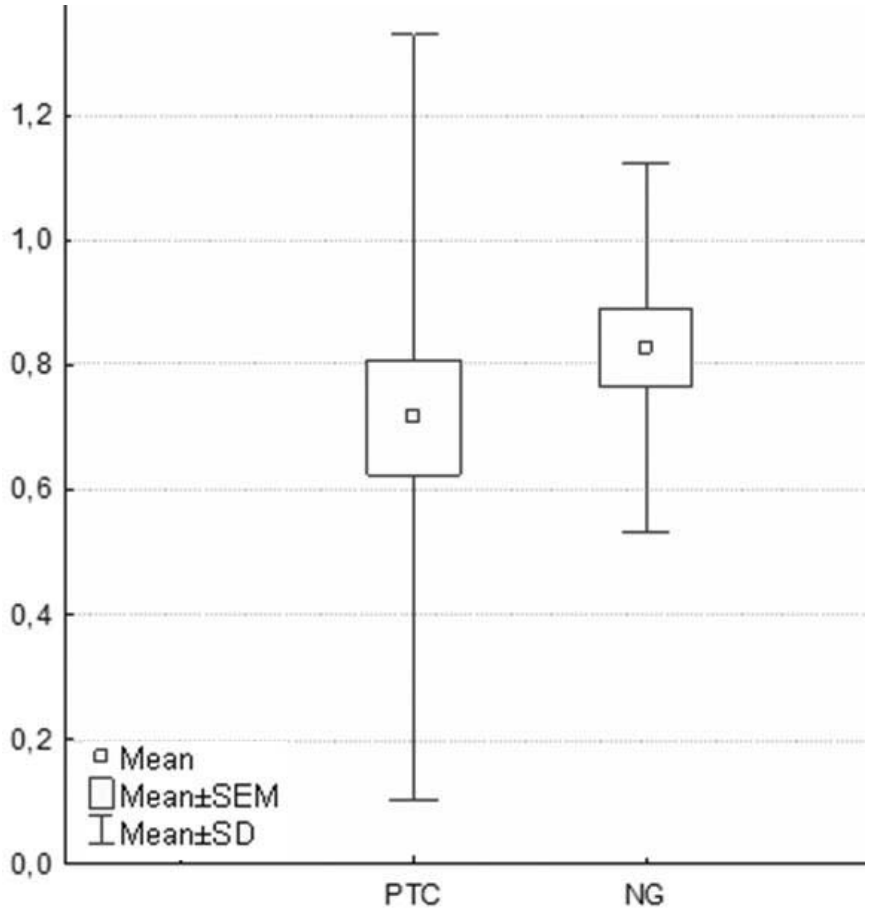

Figure 4. Box-and-whisker plots, representing the expression (mean RQ levels) of *KCNQ1* gene in PTC and NG groups (Man-Whitney test, p-value less than 0.05).

Whitney test showed statistically significant differences in RQ levels for *KCNQ1* between classic and follicular PTCs (lower values in classic variant; p-value less than 0.05).

In order to analyze RQ levels in PTC group in relation to patients' age, Kruskal-Wallis test was used for 3 age categories (under 40, 40-65, and over 65 yrs). Among all the studied genes, only *ARHI* gene showed statistically significant differences between the three age groups in PTC samples – the lowest RQ values for the youngest (*i.e.*, under 40 yrs) patients (p-value less than 0.05).

There were no statistically significant differences between RQ levels and patients' gender (Mann-Whitney test, p-value more than 0.05) in PTC group. The results of Kruskal-Wallis test, confirmed by Spearman's rank correlation coefficient, showed no statistically significant differences between RQ levels and tumour size (p-value more than 0.05), according to TNM or AJCC classification.

4.1.2.3. Comparison of RQ levels between IR and NIR genes in PTC and NG groups

In PTC group, there were no statistically significant differences between the genes belonging to NIR (*RASSF1A, SLC5A8, VHL* and *CDH1*) *vs* IR (*p16INK4A, ARHI, KCNQ1* and *MEST*), concerning RQ levels (Mann-Whitney test, p-value more than 0.05), despite clear differences in the mean values of samples, *i.e.*, lower mean RQ value was observed for NIR genes and higher for IR genes. However, it should be stressed that statistical distributions for both groups (NIR and IR) were significantly different from normal distribution (p-value less than 0.05).

In NG group, Mann-Whitney test revealed statistically significant differences (p-value less than 0.05): higher RQ values were observed for NIR genes.

4.2. Results of methylation status analysis of the studied genes

4.2.1. Gene methylation status in PTC and NG groups

Promoter methylation analysis of the studied genes, performed as MSP reactions, distinguished unmethylated (U) from methylated (M) DNA alleles after electrophoretic separation. Both U and M alleles were found in the studied groups (PTC and NG). The presence of M alleles was observed in PTC tissue for the following genes: *ARHI, CDH1, p16INK4A, KCNQ1, RASSF1A, VHL*

Table 8. Methylation status, expressed as mean MI values, of the studied genes in PTC and NG groups.

Gene		Mean MI values			
		PTC group	*PTC v. classic*	*PTC v. follicular*	NG group
		(n=21)	(n=13)	(n=8)	(n=10)
IR	*ARHI*	0.847	0.829	0.877	0.951
	p16INK4A	0.137	0.163	0.098	0.159
	KCNQ1	0.378	0.457	0.250	0.000
	MEST	0.281	0.250	0.332	0.293
NIR	*RASSF1A*	0.342	0.389	0.266	0.412
	SLC5A8	0.004	0.002	0.008	0.000
	VHL	0.093	0.136	0.000	0.022
	CDH1	0.555	0.551	0.563	0.431

and *MEST*, with exception of *SLC5A8*. In NG specimens M alleles were detected for *ARHI, CDH1, p16INK4A, RASSF1A,* and *MEST* genes. The presence of U alleles was found both in PTCs and NGs for all the studied genes, apart from *ARHI*.

Based on spectrophotometric estimation (Agilent 2100 Bioanalyzer), fluorescence units (FU) of MSP products were calculated as nanograms (ng), according to DNA size marker. An example of spectrophotometric assessment on Agilent 2100 Bioanalyzer is shown in Figure 5. MI values were calculated for each analysed sample (Table 8).

In PTC group, the highest mean MI values were observed for *ARHI* and *CDH1* genes, the lowest mean MI values: for *SLC5A8* (barely detectable) and *VHL* genes. Similarly was in NG group: *ARHI* and *CDH1* showed the highest mean MI values, while *VHL* revealed very low methylation level and, in case of *SLC5A8* and *KCNQ1* genes, no promoter methylation was found (Figure 6; see also Table 8).

Mean MI values were generally higher for the genes localized in the imprinted region, as compared with the non-imprinted region, both in PTC and NG groups (Figure 7). The same was true for PTC variants: the mean MI values were higher for IR genes.

4.2.2. Sequencing analysis of ARHI and CDH1 in PTC group

Two genes, characterized by the highest mean MI values, *i.e., ARHI* and *CDH1*, were additionally submitted to the direct sequencing analysis. The observed changes in DNA were due to cytosine to thymine (C/T) substitutions (see Figure 8). The differences were detected not only in MSP primer binding site, but also within the amplicon.

4.2.3. Statistical analysis of promoter methylation status
4.2.3.1. Comparison of MI values between PTC and NG groups

Mann-Whitney test showed no statistically significant differences, regarding MI values between PTC and NG groups (p-value more than 0.05). The same test revealed no differences between classic and follicular PTCs (tall-cell PTCs were excluded, due to the small number of cases). Also, there were no statistically significant differences in MI levels regarding patients' sex (Mann-Whitney test, p-value more than 0.05) or age (3 age categories: under 40, 40-65, and over 65 yrs; Kruskal-Wallis test, p-value more than 0.05). The results of Kruskal-Wallis test, confirmed by Spearman's rank correlation coefficient, showed no statistically significant differences between MI levels and tumour size, according to TNM or AJCC classification (p-value more than 0.05).

4.2.3.2. Comparison of MI values between IR and NIR genes in PTC and NG groups

In PTC group, Mann-Whitney test showed statistically significant differences (p-value less than 0.05) between IR and NIR: mean MI values were higher for IR genes (Figure 9). In NG group, no statistically significant differences were observed between IR and NIR (Mann-Whitney test, p-value more than 0.05)

4.2.3.3. Relationship between RQ and MI values in PTC and NG groups

There was no statistically significant relationship between RQ and MI values within PTC group (Spearman's rank correlation coefficient, p-value more than 0.05) or within NG group (Spearman's rank correlation coefficient, p-value more than 0.05).

4.3. Results of LOH/MSI analysis
4.3.1. Results of LOH/MSI analysis in PTC and NG groups

LOH/MSI analysis was performed, using 27 microsatellite markers. The informativity of the markers was assessed to be in the range of 84-100%, mean 94.71+/-6.21%. All the studied DNA samples, derived from PTCs (20 cases), were informative for one or more studied *loci*. LOH/MSI changes were observed for 8 out of 27 (30%) microsatellite markers.

In PTC group, the most frequent genetic instability (LOH/MSI) was observed for D9S1604 marker, spanning the chromosomal region 9p21.3 (25%; 3/12 informative *loci*). Additionally, for D9S974 marker – localized also at 9p21.3 – the frequency of LOH in the studied DNA samples was 8% (1/13 informative *loci*). Genetic instabilities of LOH/MSI type were also observed for D16S3025 and D16S3206 markers, mapped to 16q22.1, with the frequency of 21% (4/19 informative *loci*). LOH/MSI frequency for D3S1568 (3p21.3) and D12S1041 (12q23.2) markers was 6% (1/17 informative *loci*). The same LOH/MSI frequencies of 8% (1/12 informative *loci*) were observed for D1S368 (1p31.2) and D7S2519 (7q32.2) markers. Representative examples of LOH/MSI in PTC are shown in Figure 10.

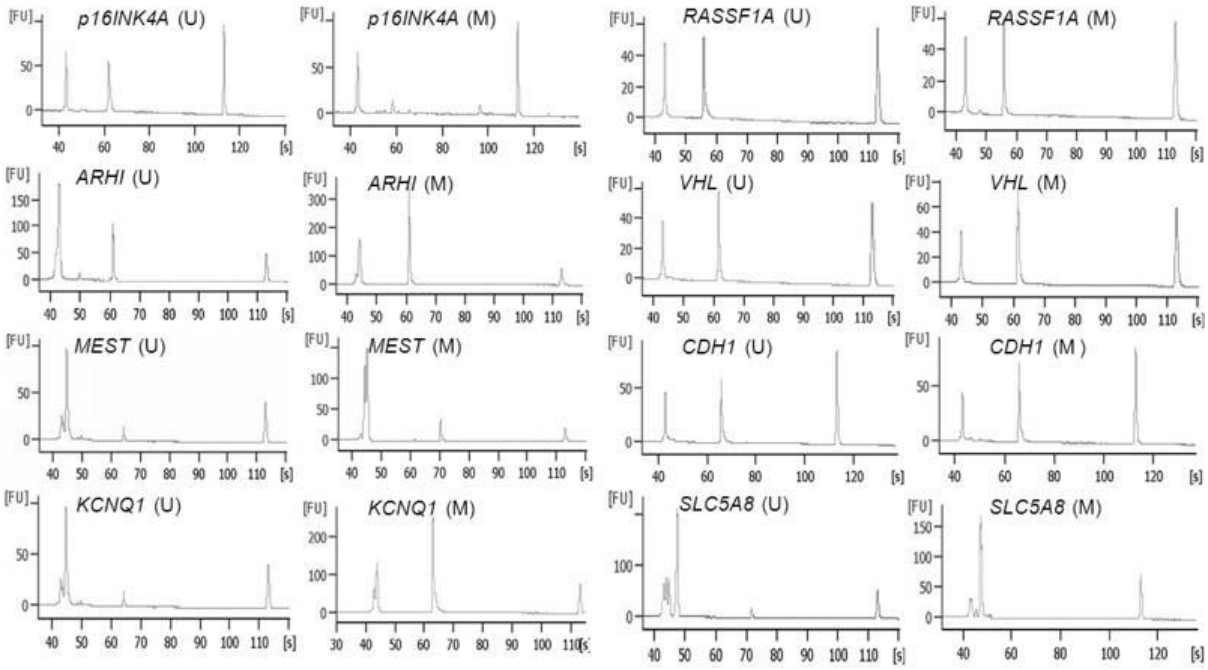

Figure 5. Exemplary results of each gene methylation status in one PTC sample (Agilent 2100 Bioanalyzer software). *Note*: U, unmethylated product; M, methylated product.

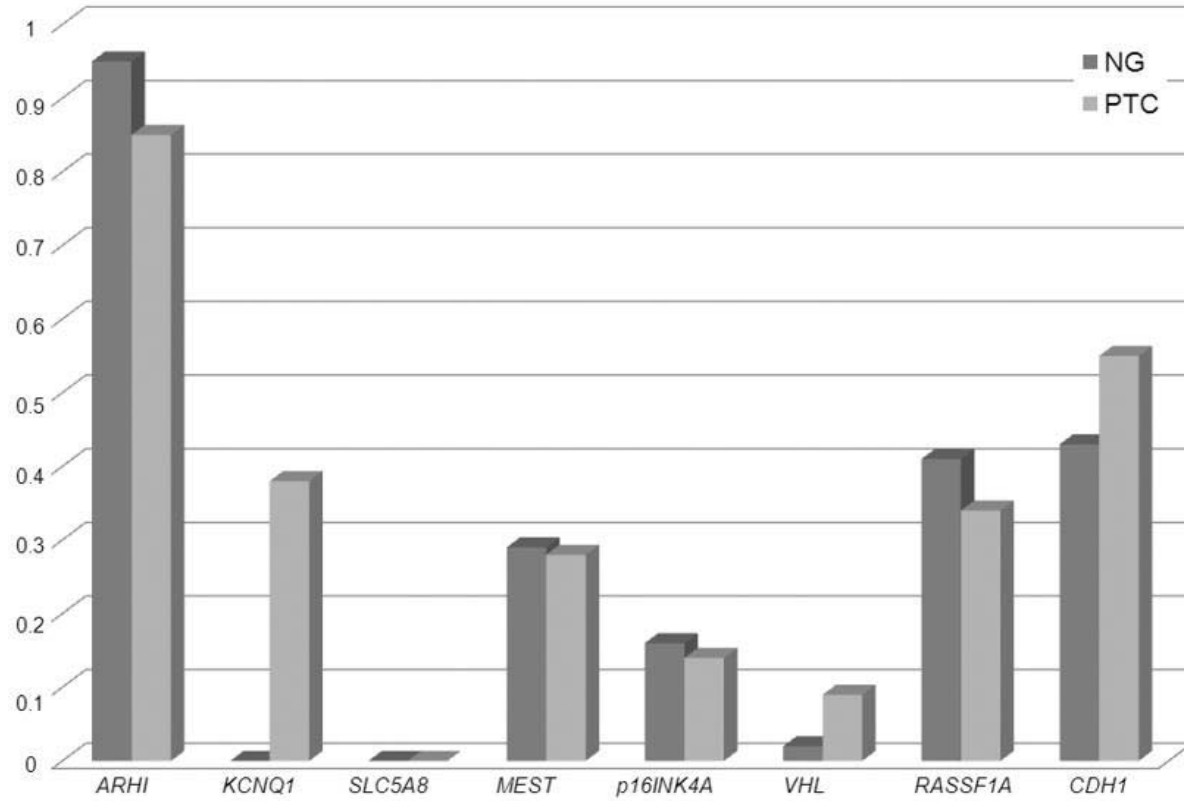

Figure 6. A comparison of mean MI values between PTC and NG groups for all the studied genes.

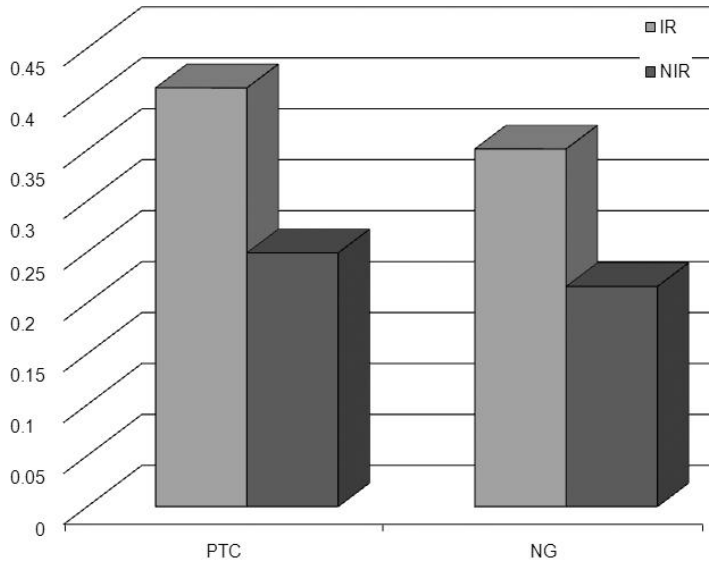

Figure 7. A comparison of mean MI values of IR and NIR genes between PTC and NG groups.

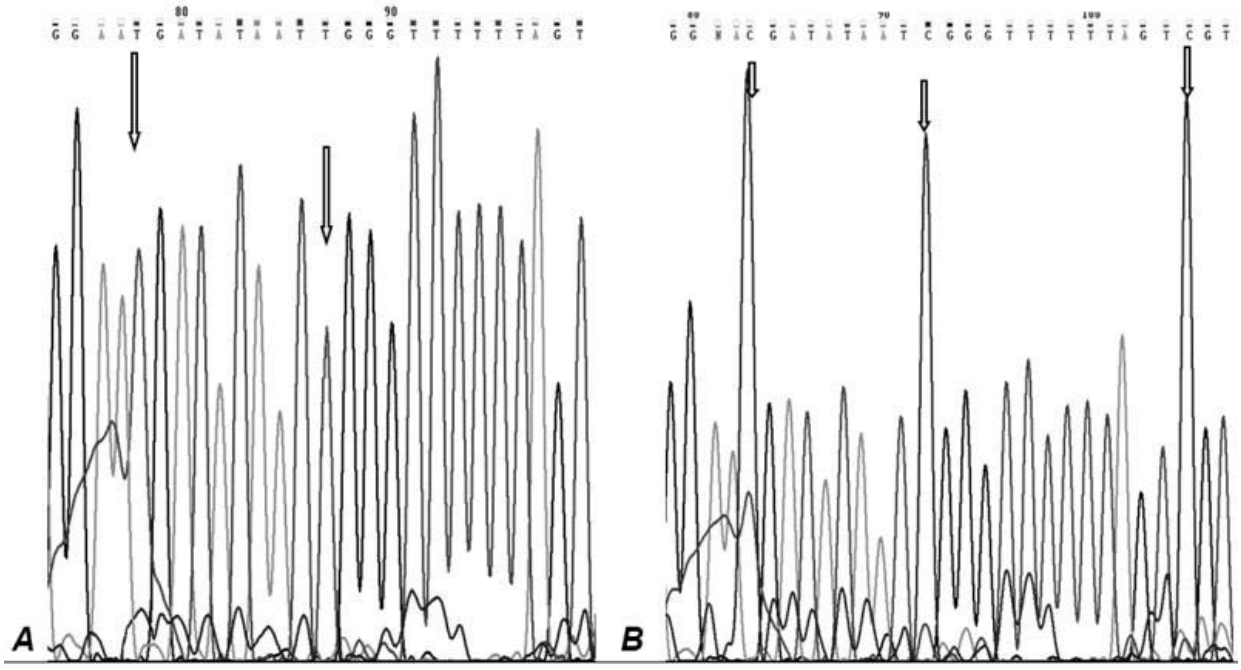

Figure 8. Examples of the sequenced promoter region of *ARHI* gene (3130xl Genetic Analyzer). (A) Unmethylated control DNA from cell culture; the arrows point to thymines, indicating there were no methylated cytosines in DNA sequence; (B) DNA from PTC sample (classic variant); the arrows point to cytosines, indicating there were methylated cytosines in DNA sequence.

In NG group, no LOH/MSI was found in any of the analyzed chromosomal regions.

The obtained results indicate that in PTC samples, LOH/MSI frequency was in the range of 0–25% (mean 9.6+/-9.45%), depending on the chromosomal region. The highest frequency of LOH/MSI was observed at 9p (25%) and 16q (21%); no LOH/MSI was found at 11p.

The frequency of genetic instability (LOH/MSI) in PTC group was also assessed as FAL index, ranging from 0 to 0.33 (mean 0.05+/-0.08). Figure 11 shows FAL indexes for all the studied PTC samples.

The frequency of LOH/MSI was also referred to two different genomic regions under the study: IR and NIR. The corresponding microsatellite markers are listed in Tables 3 and 4.

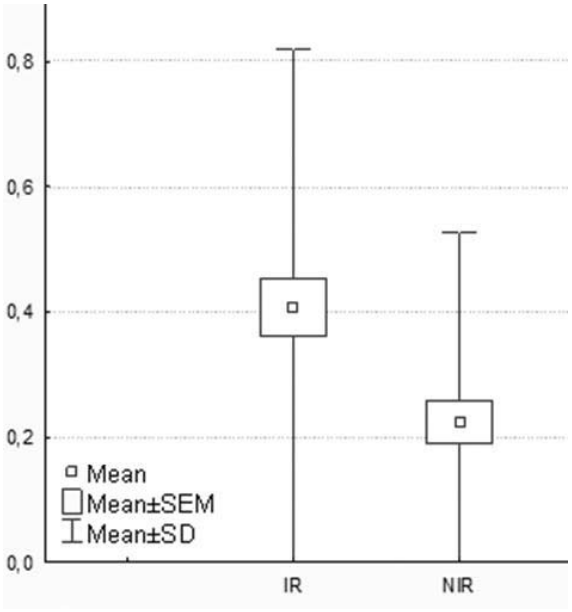

Figure 9. Box-and-whisker plots, representing mean MI values of IR and NIR genes in PTC group (Man-Whitney test, p-value less than 0.05).

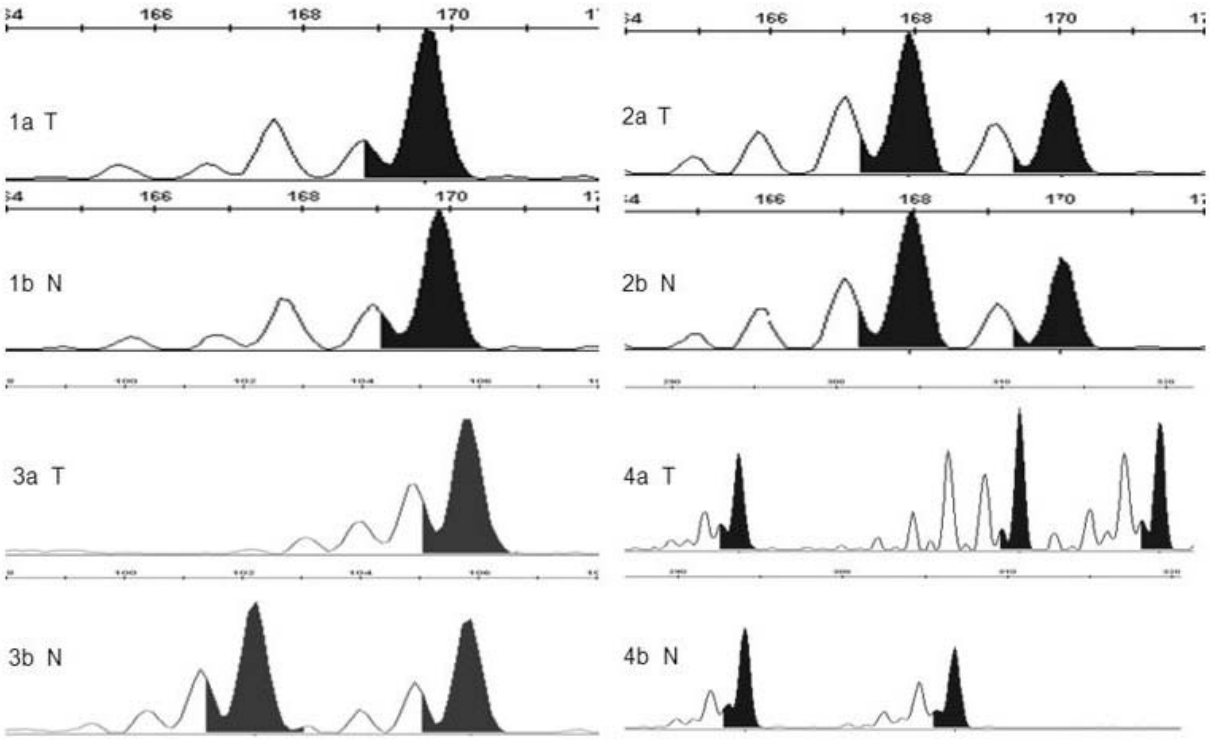

Figure 10. LOH/MSI analysis in PTC samples (3130xl Genetic Analyzer, GeneMapper Software v. 4.0). 1a, homozygous DNA from patient with diagnosed tall-cell PTC (D3S1317 marker); 1b, homozygous DNA from macroscopically unchanged tissue from the same patient; 2a, heterozygous DNA from patient with diagnosed follicular PTC (D3S1317 marker); 2b, heterozygous DNA from macroscopically unchanged tissue from the same patient; 3a, LOH in DNA from the patient with diagnosed classic PTC (D16S3025 marker); 3b, heterozygous DNA from macroscopically unchanged tissue from the same patient; 4a, MSI in DNA from the patient with diagnosed classic PTC (D1S368 marker); 4b, heterozygous DNA from macroscopically unchanged tissue from the same patient; N, normal (macroscopically unchanged tissue); T, tumour tissue.

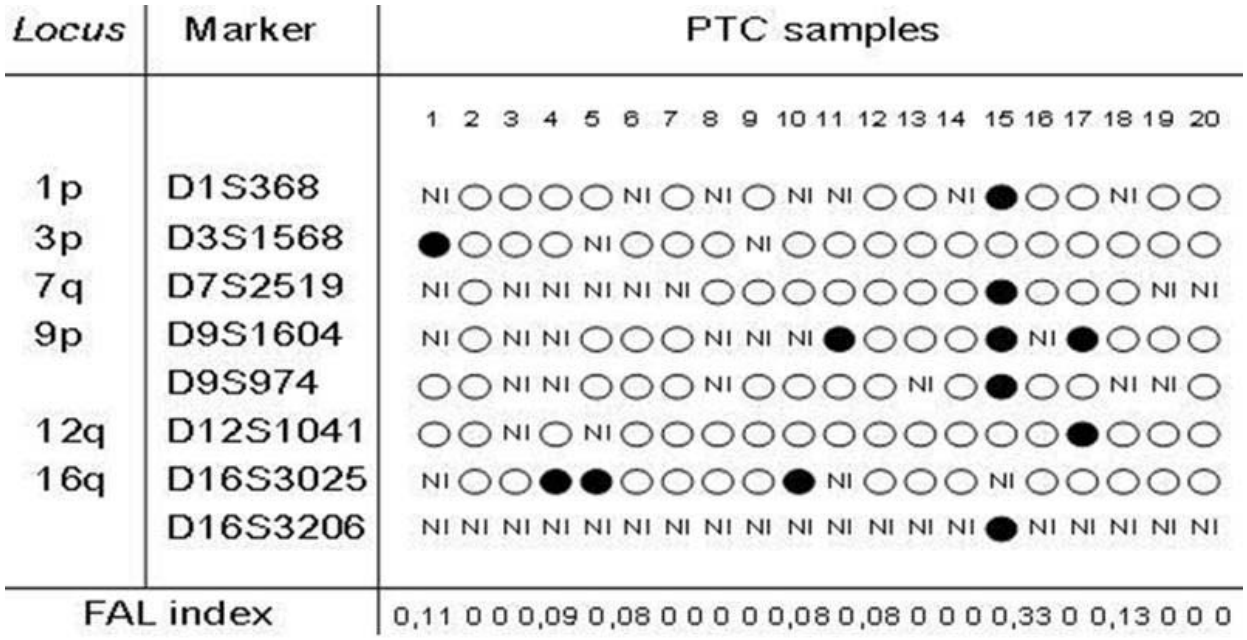

Figure 11. The assessed LOH/MSI frequency (FAL index) in PTC DNA samples for 8 studied microsatellite markers, mapped to: 1p, 3p, 7q, 9p, 12q and 16q. *Note*: white circle (○), informative *loci* without LOH/MSI; black circle (●), presence of LOH/MSI; NI, non-informative allelotype (homozygote).

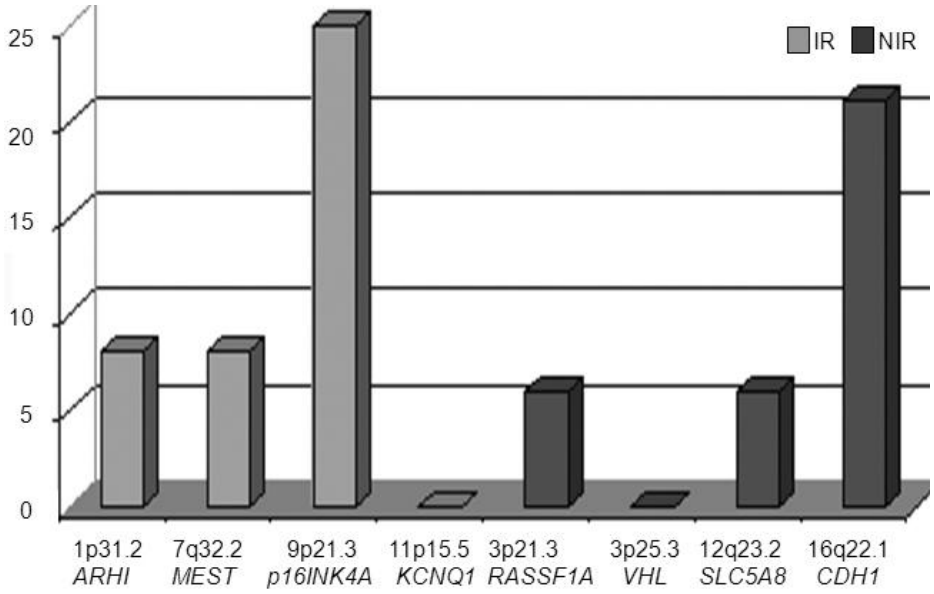

Figure 12. LOH/MSI frequency (%) in PTC in IR (involving *ARHI, p16INK4A, MEST* and *KCNQ1 loci*) and NIR (involving *RASSF1A, VHL, SLC5A8* and *CDH1 loci*).

The results of LOH/MSI analysis in PTC samples within IR revealed additional alleles at 1p (D1S368 marker), 7q (D7S251 marker) and 9p (D9S1604 marker). Those changes confirmed the presence of MSI. LOH was also found in IR for two microsatellite markers, mapped to 9p (D9S1604 and D9S974 markers). Similarly to IR, also within NIR both LOH (for D3S1568, D12S1041, D16S3025 and D16S3206 markers) and MSI (for D16S3025 marker) were observed. The total percentage of LOH/MSI was assessed for IR and NIR separately. The values were the same, *i.e.*, 5%, for both groups.

The frequency of LOH/MSI, regarding gene *loci* within IR (*ARHI, p16INK4A, MEST* and *KCNQ1*) and NIR (*RASSF1A, VHL, SLC5A8* and *CDH1*) in PTC, is shown in Figure 12.

4.3.2. Statistical analysis of LOH/MSI frequency

4.3.2.1. Comparison of FAL index values in PTC group

Mann-Whitney test showed no statistically significant differences (p-value more than 0.05), regarding FAL index values between PTC v. classic and PTC v. follicular. Different FAL index values between women and men were of no statistical significance (Mann-Whitney test, p-value more than 0.05). There was no statistically significant correlation between FAL index levels and patients' age (Mann-Whitney test and Spearman's rank correlation, p-value more than 0.05), as well as between FAL index values and tumour size, according to TNM or AJCC classification (Kruskal-Wallis test, p-value more than 0.05). Regarding the genes located within IR and NIR, Mann-Whitney test showed no statistically significant differences in FAL index values between the two groups (p-value more than 0.05). Moreover, no significant differences were observed in FAL index values between 3 age categories (under 40, 40-65, and over 65 yrs) (Kruskal-Wallis test, p-value more than 0.05).

4.3.2.2. Relationship between FAL index and RQ values in PTC group

Spearman's rank correlation coefficient was used to assess the relationship between FAL index and RQ values for all 8 studied genes. It showed a statistically significant correlation between decreased RQ/increased FAL index and increased RQ/decreased FAL index values in case of *VHL* gene (p-value less than 0.05).

4.3.2.3. Relationship between FAL index values and LOH/MSI frequency in PTC group

No statistically significant relationship between FAL index and LOH/MSI (Spearman's rank correlation coefficient, p-value more than 0.05) was found.

5. DISCUSSION

Despite there are many reviews regarding genetic factors involved in initiation and progression of thyroid cancers, including PTC (3,5,38), our understanding of causes underlying neoplastic transformation of thyroid cells is still limited. However, it is already known, that PTC tumorigenesis represents the final product of genetic, as well as epigenetic events.

Many experimental works, focused on the epigenetic background of cancer, have proved that methylation of CpG dinucleotides in TSG promoter region is associated with transcriptional silencing of the methylated gene. Aberrant promoter methylation is a frequent and important mechanism of TSGs silencing in different types of neoplasms, including also thyroid lesions, and is being described for the still growing number of TSGs (19,22,23,39,40).

In the present study, we have analyzed promoter methylation status of 8 selected tumour suppressor genes, which are thought to be important in thyroid gland tumorigenesis. According to our data, TSG aberrant promoter methylation is a common event in PTC and also in NG, as the studied samples revealed the presence of co-methylation for at least 3 out of 8 TSGs. It is consistent with the data previously reported by other authors, who have showed that an altered epigenetic pattern of genomic DNA is a frequent and significant process in different thyroid lesions, *i.e.*, in hyperplasia's, adenomas, as well as in cancers (19,20,41,42). Nowadays, it is widely assumed that aberrant promoter methylation occurs at an early step of thyroid tumorigenesis (19,20,41).

One line of our investigation was to find which of the genes under study (*ARHI, MEST, CDH1, VHL, KCNQ1, p16INK4A, SCL5A8, RASSF1A)* had a distinct promoter hypermethylation in cancer tissue (PTC), and which of them could be recognized as a selective genetic marker of thyroid tumorigenesis. Another important issue of our study was the observation if the methylation status of the individual gene correlated with its mRNA expression level. Moreover, we elucidated the involvement of LOH in gene silencing mechanism. Finally, we considered the expression, methylation and LOH data of each studied gene in the aspect of its genome localization, *i.e*., in the imprinted or the non-imprinted region.

According to our investigation, the highest level of promoter methylation was found for *ARHI* and *CDH1*genes. However, the mean MI levels of those genes were similar in PTC and NG tissues and, therefore, neither *ARHI* nor *CDH1* could be regarded as a molecular marker of cancerous transformation toward the PTC. The majority of the remaining TSGs under our study showed analogous trend towards similar level of methylation in both groups (PTC and NG). Our results are consistent with the report of Hoque *et al*. (20) who did not confirm significant differences in methylation status of the selected various genes between non-malignant and malignant thyroid lesions, either, even regarding those genes which had seemed to be promising thyroid tumour markers (18,22,43).

Our findings regarding *CDH1*gene, *i.e.*, its high methylation status and corresponding decreased expression, are in accordance with the observations of other investigators, who showed epigenetic silencing of *CDH1* gene by promoter hypermethylation in benign and malignant thyroid tumours (20,44,45). Those results and our data prove that epigenetic promoter hypermethylation of *CDH1* plays an important role in gene silencing during thyroid tumorigenesis, including PTC development.

Contrary to *CDH1*, the other highly methylated gene in our study cohort, *i.e.*, *ARHI*, showed a significantly reduced expression level only in a small fraction of PTC and NG specimens. Our observations are consistent with those reported by Weber *et al.* (41) who found that maternally imprinted *ARHI* gene is frequently and clonally underexpressed in FTC but not in PTC or FA. Moreover, the mechanism of decreased *ARHI* expression in FTC is driven by large deletions or histone modifications (acetylation and methylation) (41,46). On the other hand, it is known that an increased *ARHI* mRNA expression does occur in thyroid tumours, and it may be associated with the gain of gene copy number (47). However, little is known about regulation of *ARHI* expression in PTC. Interestingly

enough, the increased expression of *ARHI* in our study correlated with patients' age. Lower gene expression in younger patients needs further observations, especially as we did not find similar correlation in other reports.

According to the published reports, epigenetic inactivation of *RASSF1A* gene is a frequent event, both in malignant as well as in benign thyroid tumours (18-20,48,49), although, among different types of thyroid carcinomas, the lowest hypermethylation level of *RASSF1A* is observed in PTC (18,19). Relatively low methylation level and corresponding low expression, as observed in our study, can be connected with the possible concomitant presence of *BRAF* mutations which are common in the early step of PTC tumorigenesis. According to Hu *et al.* (22), the silencing of *RASSF1A via* hypermethylation is exclusive with *BRAF* mutation. Although we did not evaluate the presence of *BRAF* mutation (especially the key mutation V600E) in the present study, our previous report showed a rather significant – as compared with other population – frequency (48%) of $BRAF^{V600E}$ mutation in the Polish population (7).

It was also proved that the presence of *BRAF* mutation in PTC coexisted with a low expression of *SLC5A8* (22,50). Our data show a significantly reduced expression of *SLC5A8* in a majority of PTC samples and, concomitantly, a very low methylation status in PTC (and no methylation in NG). Therefore, our results suggest that promoter hypermethylation is not an underlying mechanism of *SLC5A8* silencing. It is postulated that the methylation-associated silencing of *SLC5A8* might be secondary to the constitutive activation of the MAPK pathway, resulting from the $BRAF^{V600E}$ activating mutation (50), although a high frequency of *SLC5A8* methylation in thyroid carcinoma is also described, especially in the classical variant of PTC (22,50).

The decreased expression of another gene under our study, *VHL*, and its concomitant low promoter methylation level indicate that factors, other than methylation, are the cause of *VHL* silencing in thyroid tumours. Our data, regarding the methylation status of *VHL* gene in thyroid lesions, are innovative, as there are no such published reports. Only *VHL* expression pattern was assessed in thyroid carcinomas and it was found to be altered at the late stage of thyroid tumorigenesis, associated with the loss of differentiation and higher degree of aggressiveness (51).

In case of *KCNQ1* gene, our data confirm its promoter methylation in PTC group. Due to the lack of methylation in NG samples, it seems that *KCNQ1* methylation is characteristic for malignant thyroid lesion. Similarly to *VHL* gene, there are no reports on the role of aberrant methylation level of *KCNQ1* in thyroid tumours. Our findings show a significantly decreased expression of *KCNQ1* gene in a high percentage of PTC specimens and statistically significant difference with NG group, highlighting the importance of aberrant methylation pattern of *KCNQ1* promoter in PTC development. Moreover, we documented that in PTC group, RQ values for this gene were significantly lower in the classic variant as compared with the follicular variant. Therefore, we suppose that RQ value of *KCNQ1* may have a diagnostic value, differentiating both PTC types, from which follicular variant is regarded to have poorer prognosis. However, the usefulness of our finding needs to be confirmed in further experiments. Nevertheless, our data prove the important role of down-regulation of *KCNQ1* in PTC development, although other than epigenetic factors are probably dominant.

We found interesting data concerning low methylation status and high expression level of *p16INK4A* gene. Promoter methylation of *p16INK4A* was low in PTCs and, similarly, in NGs. The obtained results were surprising, especially in view of other reports, describing a high frequency and high levels of *p16INK4A* methylation in many types of cancer (38,52-54). Moreover, *de novo* methylation of the 5' CpG island of this gene in primary thyroid tumours was confirmed (55). Based on our findings, we hypothesize that loss of imprinting (LOI) resulting in the loss of parental-origin-specific differential allele expression of this gene may be a possible event in thyroid cancer; especially, that it is known that LOI usually leads to activation of the normally silent copy (56). On the other hand, according to Kjellman *et al.* (57), the most frequently detected imbalance in nearly 30% PTCs is the gain of 9q33-qter, in proximity of *p16INK4A locus*, that also can result in increased expression of this gene.

Regarding *MEST* gene, our results indicate low methylation level, both in PTC and NG groups. There are no published reports on *MEST* methylation status in thyroid lesions. Its hypermethylation is described in some carcinomas, e.g., in glioblastoma multiforme, osteosarcoma (58,59), but also LOI of this gene is documented (60). The latter process may take place in thyroid tumorigenesis and it would have underlied the low methylation level of *MEST* in our study.

The analysis of *MEST* expression was problematic, as its mRNA expression was barely detectable. If not promoter hypermethylation, other factors must be responsible. Such a causative event may be the genetic instability of LOH type.

The data on LOH in 7q – containing, among others, *MEST locus* – are controversial in thyroid tumours: some authors report the presence of LOH in this region only in FTC and ATC, not in PTC (31,61), others prove frequent LOH in *MEST locus* in PTC with metastasis (29). Moreover, the presence of LOH in goitres and hyperplasias suggests that LOH at 7q21 may represent a very early event in thyroid tumorigenesis (61). Therefore, chromosomal *locus* 7q21 appears to be relevant in thyroid tumour development and progression and our data indicate the role of LOH in *MEST* silencing in PTC, resulting from the deletion of the only functional copy of this imprinted gene.

In case of chromosomal region 16q22.1, with *CDH1 locus*, we also observed a high frequency of LOH, consistently with previous reports (25,62). As we found a

significantly reduced expression of this gene in a high percentage of the samples and high promoter methylation – although a little lower than described in other reports – we hypothesize that a concomitant presence of both mechanisms plays some role in *CDH1* silencing.

The data, obtained by several other groups, show that there are numerous deletions in chromosomal region 9p21 (*p16INK4A locus*) in PTC (25,29). We did detect very high frequency of LOH in *p16INK4A locus* in PTC tissues and, due to the low level of the observed promoter methylation, it seems that LOH might play an important role in silencing of *p16INK4A*. However, we should not neglect the significance of either LOI or of the gain of copies in this region, that may also influence the expression profile of *p16INK4A* and may explain its increased expression in our study cohort.

The lack or infrequent deletions in the remaining chromosomal regions under our study, *i.e.*, 11p15.5 (*KCNQ1 locus*), 1p32-36 (*ARHI locus*) and 3p (*VHL* and *RASSF1A loci*), implicate that LOH does not underlie the decreased expression of the studied genes, localized within these analyzed regions. Regarding regions 1p and 3p, the obtained results are consistent with those previously reported by others: these regions were found to contain significant deletions in FTC and ATC but not in PTC (29,63). In case of chromosomal region 11p15.5, LOH was observed both in FTC and PTC, moreover 11p15 was suggested to have some predictive value in PTC (25). Our results do not confirm the presence of LOH at *KCNQ1 locus*.

It seems that in papillary thyroid carcinoma – similarly to other types of human cancers – aberrant promoter methylation plays an important role in tumorigenesis, being responsible for TSG silencing. However, the pattern of co-methylation, that we observed across the studied genes, evident both in NG and PTC, also raises a possibility that, in thyroid tissue, methylation-induced silencing may occur not only in malignant transformation but also in functional context. In order to confirm this hypothesis, a more complete interpretation of our findings will be needed, regarding the actual function of the studied genes in the thyroid gland. In case of imprinted genes, LOI may be the cause of their increased expression, permitting oncogenic gene function. Alternatively, LOH at imprinted *locus* may result in the deletion of the only functional gene copy, resulting in gene silencing, although, this pathogenic pathway seems to be a rare event in case of PTC.

It should be stressed that our data support the role of methylation-induced gene silencing as the significant pathogenetic mechanism in the development of PTC but the level of background methylation, found in routine tissue samples, shows that the genes, which we studied, lack discriminatory power for diagnostic purposes. This may be improved by discovering more thyroid cancer-specific markers or by more selective sampling of tissue, e.g., by implementing microdissection procedures or using cytological material, rather than tissue specimens. Finally, we have demonstrated that, in PTC transformation, the accumulation of epigenetic and genetic events contributes to an altered gene expression and clonal transformation of follicular epithelial cells. Our study has confirmed that LOH in IR, as well as in NIR, may play some role in the initiation and development of PTC in the early step of malignant transformation. However, high methylation level of IR genes in PTC, as observed in our study, suggests that this mechanism dominates over LOH in thyroid tumorigenesis.

6. ACKNOWLEDGEMENT

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Abbreviations: PTC: Papillary thyroid carcinoma; MAPK: mitogen-activated protein kinase signaling pathway; TSG: tumour suppressor gene; MSI: microsatellite instability; LOH: loss of heterozygosity; FTC: follicular thyroid carcinoma; FA: follicular adenoma; IR: imprinted region; NIR: non-imprinted region; NG: nodular goitre; RT: reverse transcription; PCR: polymerase chain reaction; cDNA: complementary DNA; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; MSP: methylation-specific PCR; BSP: bisulfite-specific PCR; bp: base pair; MI: Methylation Index; PAA: polyacrylamide; TBE: Tris-borate-ethylenediamine tetraacetate; AIR: allelic imbalance ratio; FAL: fractional allele loss; RQ: relative quantitation; LOI: loss of imprinting.

Key Words: Papillary thyroid carcinoma, Nodular goitre, Tumour suppressor gene, Imprinted region, Non-imprinted region, Methylation pattern, Methylation index, Methylation-specific PCR, Bisulfite-specific PCR, Real-time PCR, Gene expression, Loss of heterozygosity, Microsatellite instability, Fractional allele loss

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