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Aim of the study: The aim of the study was to analyse the methylation profile of the eight tumour suppressor genes (TSGs) *ARHI*, *CDH1*, *KCNQ1*, *MEST*, *p16INK4A*, *RASSF1A*, *SLC5A8* and *VHL* in noncancerous thyroid tissue adjacent to papillary thyroid carcinoma (PTC) and to assess whether it parallels the methylation level of the studied TSGs in the primary tumour.

Material and methods: Thyroid tissue samples were obtained from patients with PTC from the centre of the primary lesion and the adjacent noncancerous tissue, macroscopically unchanged (n = 11). Genomic DNA was modified with sodium bisulfite and methylation-specific polymerase chain reactions (MSPs) were performed. For each studied TSG methylated and unmethylated MSP primers were designed. Quality and quantity of MSP products were assessed in automated electrophoresis.

Results: Qualitative analysis revealed the presence of methylated and non-methylated alleles both in PTC and normal thyroid tissue for all genes, except one (*KCNQ1*). The highest methylation frequency was observed for *ARHI*, *CDH1*, *p16INK4A*, *MEST* and *RASSF1A*. Quantitative assessment confirmed a very high methylation level (MI values) for *ARHI*, *CDH1* and *RASSF1A*. Methylation levels of the studied TSGs were only slightly higher in the PTC group.

Conclusions: Alterations of TSG methylation levels in thyroid tissue may be considered as an early molecular event, specific not only for cancerous lesions. Epigenetic modifications of these genes may be of functional importance for thyroid carcinogenesis. On the other hand, it may be explained by the concept of field cancerization.

Key words: promoter hypermethylation, tumour suppressor genes, carcinogenesis, papillary thyroid carcinoma.

Methylation profile of selected TSGs in non-cancerous thyroid tissue adjacent to primary PTC

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Introduction

Tumour suppressor genes (TSGs) encode proteins involved in various crucial processes, such as cell proliferation, cell-cycle control, apoptosis, DNA damage detection and repair, cell adhesion, migration and invasion, senescence, and carcinogen detoxification [1]. The current definition of *bona fide* tumour suppressor specifies that a proven TSG loses its function in the development of a cancer and its *in vivo* inactivation enhances initiation, growth or progression of a tumour [2].

Thus far, many TSGs have been identified and confirmed to be associated with development and progression of many types of cancers. It is widely accepted that both functional copies of TSG are inactivated during carcinogenesis, via the "two-hit" model of tumour suppressor inactivation [3-5]. Initially, a mutational mechanism for the loss of TSG function was considered. Now it is complemented with a nonmutational TSG inactivation pathway, i.e. silencing through epigenetic mechanisms [5-9].

Epigenetic modifications are defined as heritable changes in gene expression without alteration in the DNA sequence [10]. A predominant mode of epigenetic alteration in cancer is gene silencing via promoter hypermethylation associated with addition of a methyl group (CH3) at the carbon 5 position of the cytosine ring in CpG dinucleotides [4, 10]. It is known that methylation of CpG islands interferes with active transcription by recruiting methyl-cytosine-binding proteins and histone deacetylases, which in a coordinated fashion modify nucleosomes to form transcriptionally repressive chromatin [4, 5, 8, 11].

A huge amount of data has been accumulated indicating epigenetic silencing of a number of TSGs in many human malignancies, e.g., in breast, prostate, colon, stomach, oesophagus, blood, central nervous system, and lung cancer [1, 5, 8, 9, 11]. Numerous studies have defined cancer-type specific methylation profiles [11-14]. Generally, aberrant DNA methylation is significantly associated with poorer tumour differentiation, tumour aggressiveness and poor prognosis [15].

On the other hand, accumulating evidence suggests that alterations of methylation are involved in the early and precancerous stages. It is hypothesized that precancerous conditions showing alterations of DNA methylation may progress rapidly and lead to more malignant cancers [6, 15-17].

In thyroid tumorigenesis hypermethylation of certain genes is associated with the inactivation of various signalling pathways [18-22]. But there is a small number of reports indicating that changes in DNA methylation profile can be observed in thyroid noncancerous tissue.

The aim of our study was to assess the methylation profile of a panel of eight TSGs (*ARHI*, *CDH1*, *KCNQ1*, *MEST*, *p16INK4A*, *RASSF1A*, *SLC5A8*, *VHL*) in noncancerous thyroid tissue adjacent to the primary tumour, i.e., papillary thyroid carcinoma (PTC), and to determine whether it parallels the methylation level of the studied TSGs in PTCs.

Material 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 (100-150 mg) were obtained from patients who had undergone 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. For the analysis, two samples of thyroid tissue were obtained from each patient: from the centre of the primary lesion and the matching noncancerous thyroid tissue (macroscopically unchanged, procured at the most distant site from the resected specimen). Tissue samples, immediately after resection, were collected in lysis buffer (RNAlater buffer, Qiagen Sciences, USA), homogenized and frozen at –70°C until use. The collected biological material consisted of 11 pairs of tissue samples (PTC and adjacent noncancerous tissue, N_{PTC}), obtained from 8 women and 3 men, mean age 41.8 (range 25-66).

Regarding PTC tissue, some samples have already been analysed, but in a different context, and the results have been published in our previous study [23].

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 were spectrophotometrically assessed (NanoDrop Spectrophotometer ND-1000, ThermoScientific, USA).

Methylation status of the studied genes (*ARHI*, *CDH1*, *KCNQ1*, *MEST*, *p16INK4A*, *RASSF1A*, *SLC5A8*, *VHL*) was assessed by methylation-specific polymerase chain reaction (MSP) using bisulfite converted DNA. Genomic DNA (1.0 µg) was modified with sodium bisulfite, using CpGenome™ DNA Modification Kit (CHEMICON International, Millipore, USA), according to the manufacturer's protocol. Concentration and purity of the modified DNA were determined spectrophotometrically (NanoDrop Spectrophotometer ND-1000, ThermoScientific, USA).

Table 1. Gene-specific annealing temperatures in MSP reaction and length of the obtained MSP products

Gene	Annealing temperature		MSP product length	
	U (°C)	M (°C)	U (bp)	M (bp)
ARHI	55.5	60	164	165
CDH1	60	60	212	206
KCNQ1	*56*	*57*	*198*	*195*
MEST	57	58.5	251	253
p16INK4A	60	60	154	145
RASSF1A	55	55	108	111
SLC5A8	52.5	54.5	229	226
VHL	58	58	165	158

U – unmethylated
M – methylated

Primers for MSP were designed using Methyl Primer Express® Software according to the guidelines on the website www.appliedbiosystems.com/methylprimerexpress, and obtained from METAbion (Germany). Sequences and length of MSP primers have been described in our previous paper [23].

The conventional MSP method was performed according to Herman *et al*. [24] with some modifications. Briefly, MSP was run in triplicate for each sodium bisulfite modified DNA sample, using AmpliTaq Gold® DNA Polymerase Kit (Applied Biosystems, USA). Amplification reactions were conducted in a total volume of 25 µl and the MSP master mix contained 1000 ng of DNA, 0.7 µl (100 pmol) of each oligonucleotide primer (forward and reverse), 2.5 µl (2.5 mM) of dNTPs mix, 2 µl of 25 mM $MgCl_2$, Hot Start AmpliTaq Gold® DNA Polymerase (5 U/µl), 2.5 µl of 10 × AmpliTaq Gold buffer and nuclease-free water. MSP reactions were run for 35 cycles. Gene-specific annealing temperatures were determined experimentally and are presented in Table 1.

In each PCR reaction, positive and negative MSP controls were included. CpGenome Universal Methylated DNA (enzymatically methylated human male genomic DNA) served as a positive methylation control and CpGenome Universal Unmethylated DNA (human fetal cell line) was used as a negative control (CHEMICON International, Millipore, USA). Additionally, blank samples with nuclease-free water were used instead of DNA as a control for PCR contamination.

The obtained MSP products were separated on polyacrylamide gel (8% PAA) to visualize (in UV light, after ethidium bromide staining) unmethylated and methylated DNA alleles (quality assessment).

Additionally, concentrations (ng) of MSP products were spectrophotometrically estimated, using DNA1000 LabChip Kit, in an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Based on the quantitative results, methylation indexes (MIs) were calculated for each sample, according to the formula MI = (M)/(M + U), where (M) stands for the methylated and (U) for the unmethylated allele concentration.

The differences in methylation levels (MI values) of the studied genes between cancerous and noncancerous thyroid tissue were statistically analysed using non-parametric Mann-Whitney test and Student's t-test, and presented as mean or median ± SEM and ± SD values. Statistical significance was determined at the level of p-value less than 0.05. For calculations, Statistica for Windows 7.0 program was applied.

Results

Qualitative assessment of methylation frequency

Analysis of methylation status of the studied TSGs (*ARHI*, *CDH1*, *KCNQ1*, *MEST*, *p16INK4A*, *RASSF1A*, *SLC5A8*, *VHL*) revealed the presence of both methylated and nonmethylated alleles in the majority of tissue specimens (PTC and

Table 2. Frequency (percentage) of cases that revealed promoter hypermethylation in the studied groups: tumour (PTC) and adjacent non cancerous thyroid tissue (NPTC)

	ARHI (%)	*CDH1* (%)	*KCNQ1* (%)	*MEST* (%)	*p16INK4A* (%)	*RASSF1A* (%)	*SLC5A8* (%)	*VHL* (%)
PTC	100	100	33.33	80	100	100	0	42.86
N_{PTC}	100	71.43	33.33	80	90.91	90.91	20	28.57

Table 3. Frequency (percentage) of simultaneous TSG methylation in tumour tissue (PTC) and adjacent non cancerous thyroid tissue (NPTC)

Tissue	Number of simultaneously methylated genes							
	1 (%)	2 (%)	3 (%)	4 (%)	5 (%)	6 (%)	7 (%)	8 (%)
PTC	100	100	90.91	81.82	63.64	27.27	9.09	0
N_{PTC}	100	100	90.91	72.73	45.45	18.18	9.09	0

adjacent noncancerous tissue, N_{PTC}). The frequencies of methylated alleles in the studied tissue groups, for all TSGs, are given in Table 2.

The highest frequency of methylated alleles in both tumour and adjacent tissue samples was observed for *ARHI*, *CDH1*, *p16INK4A* and *RASSF1A* genes. Only one gene, *SLC5A8*, was not methylated in PTC and very infrequently in N_{PTC}. Relatively low methylation frequency was found for *KCNQ1* (in both tissue groups) and *VHL* (especially in non-cancerous thyroid tissue). The remaining gene, *MEST*, showed a similarly high level of methylation in both PTC and NPTC. In each tumour and adjacent tissue at least two genes were methylated, as listed in Table 3. Methylation of multiple genes was common, both in cancerous and non-cancerous tissue. There were no samples with no methylated gene at all.

We also analysed the concordance of methylation of the studied TSGs between tumour and matched noncancerous thyroid tissue. The results are summarized in Table 4. In most cases, clear overlapping of TSG hypermethylation could be seen between paired samples. The number of cases with hypermethylated genes in tumour and no methylation in paired noncancerous tissue was small. Most genes were never or rarely methylated in noncancerous tissue in the absence of methylation in the paired cancerous tissue. Two of the studied genes – *SLC5A8* and *KCNQ1* – were unmethylated in most of the matched samples (Table 4).

Table 4. Frequency (percentage) of concomitant TSG methylation in paired samples ("+", presence of gene methylation; "–", lack of gene methylation)

Gene	N_{PTC}+/PTC+ (%)	N_{PTC}–/PTC+ (%)	N_{PTC}+/PTC– (%)	N_{PTC}–/PTC– (%)
ARHI	100	0	0	0
CDH1	71.4	28.6	0	0
MEST	80	0	0	20
p16INK4A	90.9	9.1	0	0
KCNQ1	33.3	0	0	66.7
RASSF1A	90.9	9.1	0	0
SLC5A8	0	0	20	80
VHL	14.3	28.6	14.3	42.8

Quantitative assessment of methylation level

Based on spectrophotometric estimation performed in the Agilent 2100 Bioanalyzer, fluorescence units (FU) of MSP products were quantified (ng/μl), according to DNA size marker (DNA ladder, Agilent Technologies, USA). The assessed concentrations of methylated and unmethylated alleles served for MI value calculations, for each gene and in each tissue sample (Table 5).

Table 5. MI values (range, mean and median) in PTC and NPTC groups

Gene	*n*	PTC			N_{PTC}		
		Range	Mean	Median	Range	Mean	Median
ARHI	8	0.620-1.000	0.880	0.993	0.102-1.000	0.751	0.816
CDH1	7	0.005-0.912	0.471	0.581	0.000-0.953	0.290	0.228
KCNQ1	6	0.000-1.000	0.167	0.000	0.000-1.000	0.332	0.000
MEST	10	0.000-0.515	0.205	0.172	0.000-0.507	0.199	0.166
p16INK4A	11	0.009-0.630	0.232	0.152	0.000-0.989	0.192	0.075
RASSF1A	11	0.065-0.751	0.365	0.334	0.000-0661	0.355	0.412
SLC5A8	10	0.000	0.000	0.000	0.000-0.418	0.042	0.000
VHL	7	0.000-0.998	0.251	0.000	0.000-0.391	0.056	0.000

Based on the median values, we estimated the percentage of samples with high promoter methylation (MI ≥ median value) and with low promoter methylation (MI < median value) for each gene. Among the 8 studied genes, 3 of them – *KCNQ1*, *SLC5A8* and *VHL* – revealed a very low methylation level in most paired samples. Especially, in the case of *SLC5A8* 100% and 90% of specimens were completely unmethylated in PTC and NPTC respectively. Similarly, *VHL* showed methylation only in two cases (2/7) of PTC (although with a high level of MIs) and in one case of NPTC. Regarding *KCNQ1*, the number of samples with low promoter methylation was two-fold higher than the number of samples with high promoter methylation (66.7% vs. 33.3%). For the remaining genes, both groups of samples (highly and low methylated) were similar, regarding the number of cases, ranging from 42.8% to 57.14%, with a small predominance of highly methylated specimens. The highest diversity was observed for *p16INK4A* in N_{PTC} tissues: a high level of promoter methylation was found in 72.7% of samples and low promoter methylation in 27.3% of specimens.

Statistical analysis

Statistical analysis comparing MI values between tumours (PTC) and adjacent noncancerous tissue (N_{PTC}) for each TSG was performed. Non-parametric Mann-Whitney test revealed no significant differences regarding each TSG between the two studied tissue groups ($p > 0.05$). These results were confirmed by Student's t-test.

Methylation profile, i.e., total methylation level of all studied TSGs, in the PTC group in comparison with the N_{PTC} group was not significantly different ($p > 0.05$, Student's t-test). The relevant "box and whisper" plot is shown in Fig. 1.

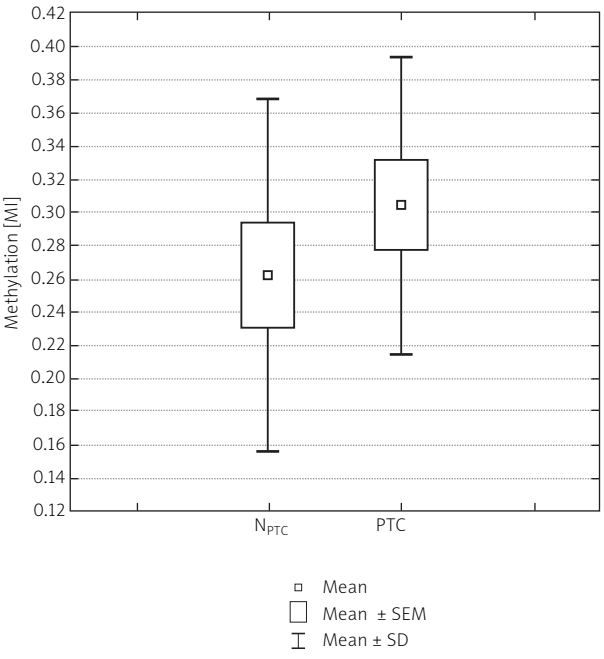

Fig. 1. Comparison of methylation profile (total methylation level of all studied TSGs) between PTC and N_{PTC} group

Regarding the groups classified according to high and low methylation levels (for each gene), there were no statistically significant differences between both groups, PTC vs. N_{PTC} ($p > 0.05$, two tailed Fisher exact test). The results were confirmed by φ^2 correlation.

The results for PTCs, regarding patients' age and sex, as well as tumour staging according to TNM and AJCC classifications, have been published in our previous study [23].

Discussion

Now it is widely accepted that loss of tumour suppressor function leads to the initiation and progression of human cancer. Inactivation of TSG can result from both genetic mechanisms such as mutations or epigenetic modifications such as promoter hypermethylation. The results of many studies indicate that, particularly in the case of TSGs with a low incidence of mutations, epigenetic inactivation is a more frequent event than genetic alteration, especially that only a few genetic changes are known to be responsible for cancer development, particularly in the earlier, precancerous stages [5, 6, 25].

The aim of our study was to assess the methylation status of 8 tumour suppressor genes – *ARHI*, *CDH1*, *KCNQ1*, *MEST*, *p16INK4A*, *RASSF1A*, *SLC5A8*, *VHL* – in noncancerous thyroid tissue adjacent to the primary tumour (papillary thyroid carcinoma) and compare it with the methylation level of the studied TSGs in PTC tissues, in pairs.

The results obtained in our study indicated that promoter hypermethylation of TSGs in thyroid tissue undergoing malignant transformation was a frequent event. Among the 8 studied TSGs the highest methylation rate, i.e. 100% of methylated specimens in the PTC group, was found for 4 genes: *ARHI*, *CDH1*, *p16INK4A* and *RASSF1A*; in the case of *MEST* 80% of PTC samples were methylated. The same genes displayed similarly high frequency of promoter methylation in paired noncancerous samples (see Tables 2 and 4). In the case of each of them, 50% and even more DNA specimens revealed high promoter methylation levels (according to median MI values) both in PTC and in N_{PTC}.

Regarding the remaining 3 studied TSGs, i.e., *KCNQ1*, *SLC5A8* and *VHL*, the frequency of promoter methylation was low in both cancerous and noncancerous tissue groups, affecting only 2-3 cases. We did not observe any methylated alleles of *SLC5A8* in PTC specimens.

There are several published reports describing epigenetic modifications in thyroid carcinomas. In a panel of analysed TSGs, promoter hypermethylation of *CDH1*, *p16INK4A*, *RASSF1A* and *SLC5A8* in malignant thyroid tumours was confirmed [21]. Methylation frequency of those genes observed in PTCs ranged from 22% to 83% for *CDH1* [18, 26-28], 25-41% for *p16INK4A* [29-32], 15-60% for *RASSF1A* [18, 33-35] and 33-90% for *SLC5A8* [20, 36], depending on the study and the technique used for analysis. In the majority of experiments the most frequently used method for methylation analysis was methylation-specific PCR (MSP). It is a sensitive technique, although qualitative, with a high risk of false-positive results. As proved in some studies, the quantitative analysis of methylation showed better results for the discrimination of malignant thyroid tumours [18, 32].

The quantitative method used in our study enabled us to calculate MI values based on concentrations of methylated and unmethylated alleles. Expressing MI × 100%, we found that the methylation levels of 3 genes in our study – *ARHI*, *CDH1* and *RASSF1A* – generally fell within the range of frequencies reported in the literature.

However, our findings with regard to methylation of *SLC5A8* differed from those reported [20, 36], as we did not observe its promoter hypermethylation in PTC. We did not find it in the adjacent thyroid tissue either, which has not been studied so far. Moreover, in our previous study, we did not observe *SLC5A8* methylation in nodular goitre (NG) [23]. Our results could support the findings of other authors indicating *SLC5A8* methylation at a later stage during thyroid carcinogenesis and its association with poor pathological characteristics of PTC, predicting increased progression and aggressiveness [20, 36]. Additionally, the results of the study performed by Schagdarsurengin *et al*. [37] indicated preferential *SLC5A8* methylation in undifferentiated thyroid carcinomas (UTC) as compared to other thyroid lesions (PTC; follicular thyroid carcinoma, FTC; medullary thyroid carcinoma, MTC; follicular adenoma, FA; NG).

Regarding *ARHI* and *MEST*, which were found to be frequently methylated in our study, their roles and epigenetic as well as genetic mechanisms of inactivation in thyroid neoplastic transformation require further analyses, due to the paucity of studies and their controversial results [23, 38-41]. However, our observations of similar frequencies and levels of methylation in paired samples for each of those genes indicated that the same molecular mechanism of regulation associated with carcinogenesis might occur in the earliest stage of thyroid tumourigenesis, although different in the case of each gene, as described below. According to the results of numerous studies, *ARHI* silencing in cancers can be caused by loss of heterozygosity (LOH), DNA methylation, histone deacetylation and transcriptional regulation [42, 43]. The functional effect of TSG inactivation also depends on gene localization, i.e., within the IR (imprinted) or NIR (non-imprinted) genome region. During tumourigenesis maternally imprinted *ARHI* can lose its function with a "single hit", via inactivation of one functional allele. The results obtained in our study, i.e., 100% frequency of methylation observed in the studied samples and very high MI values (ME = 0.99), indicated that the functional paternal allele of the gene could be hypermethylated, especially that the study performed by Weber *et al*. [39] showed infrequent LOH at the *ARHI* locus in PTC samples. In the case of the other imprinted gene involved in our study, MEST, high frequency of methylation observed in PTC and NPTC samples revealed the presence of methylated alleles in all, except two, specimens. However, MI values did not exceed 0.50 (ME < 0.50) and in most cases were even lower. This means that the only functional allele of *MEST* possibly remained unmethylated and, moreover, the imprinted allele could be subjected to LOH or loss of imprinting (LOI). LOI at the *MEST* locus has been described in some carcinomas, including breast and lung cancers [44-46], and the same molecular event could possibly be involved in thyroid tumourigenesis. On the other hand, although data on LOH involving *MEST* in thyroid tumours are controversial [38, 40, 41], the results of our previous study indicated the possible role of LOH in *MEST* silencing in PTC [23].

Summing up, we found frequent promoter methylation of 5 tumour suppressor genes, i.e., *ARHI*, *CDH1*, *p16INK4A*, *MEST* and *RASSF1A*, and 3 among them (*ARHI*, *CDH1* and *RASSF1A*) showed high methylation levels (MI values), both in PTC and N_{PTC}. So far, TSG methylation in thyroid lesions has not been analysed widely in tissue adjacent to the primary tumour. However, promoter methylation of some genes, including *CDH1*, *p16INK4A* and *RASSF1A*, was also found in benign thyroid tumours (follicular adenomas), as well as in nodular goitres [18, 23, 26, 29, 32-35], indicating epigenetic modifications as an early event in thyroid tumourigenesis. Moreover, several studies have revealed that the methylation pattern of some TSGs (including *CDH1*, *p16INK4A* and *RASSF1A*) was nearly identical when comparing cancerous and noncancerous tissues [18, 32]. Our results confirmed promoter methylation of *CDH1*, *p16INK4A* and *RASSF1A* as an early event in malignant thyroid transformation.

The presence of epigenetic modifications of TSGs in noncancerous tissue adjacent to the primary tumour may be explained by the concept of field cancerization. Originally, it referred to genetic changes occurring in normal tissue, explaining the local relapses [47-49]. The presence of a field (areas) composed of genetically altered cells is a risk factor for future carcinogenesis. Now, it has become obvious that epigenetic alterations also play a role in this phenomenon. An epigenetic field defect has been observed in several carcinomas, including head and neck cancers, oesophageal, lung, urothelial and stomach cancer [50-52]. So far, however, such changes have not been well documented in thyroid lesions. According to our knowledge, only one report regarding TSG methylation analysis in malignant thyroid tumours in comparison with adjacent thyroid tissue has been published so far [28]. The results did not show any differences in the methylation status of 4 genes (*CDH1*, *DAPK*, *ATM* and *TSHR*) between cancerous and noncancerous thyroid tissue. The authors hypothesized that it might represent field cancerization, supported by the prevalence of multifocal thyroid carcinomas. The results of our study are similar: they indicate a nearly identical methylation profile of individual TSGs in PTC and adjacent tissue. Only a few specimens of NPTC have revealed no methylation for a given TSGs methylated in PTC.

In conclusion, in our study promoter methylation of multiple genes, especially of *ARHI*, *CDH1*, *p16INK4A* and *RASSF1A*, was common, both in cancerous and adjacent thyroid tissue. This finding makes it impossible to establish a panel of methylated TSGs specific for thyroid papillary carcinoma, although it improves our understanding of PTC biology. Epigenetic modifications of those genes may be of functional importance for thyroid carcinogenesis, especially in its very early stage. Loss of function of the studied genes, via promoter hypermethylation, may lead to gain of uncontrolled growth advantage or apoptosis inhibition.

The findings of our study expand the knowledge on the molecular alterations occurring before the development of

thyroid cancer. The obtained results might be the basis for a future gene-targeted therapy of preneoplastic lesions in the thyroid gland. Additionally, they could have great value in risk assessment, early cancer detection and monitoring of disease progression.

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