Loss of heterozygosity and microsatellite instability in the 3p24.2~3pter region in papillary thyroid carcinoma

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Abstract

Introduction: The aim of this study was to analyse the loss of heterozygosity (LOH) and microsatellite instability (MSI) at the *thyroid hormone receptor beta* (*THRB*) *locus* (3p24.3) and in the 3p24.2~3pter region in human papillary thyroid carcinoma (PTC).
Material and methods: Polymorphic cleaved amplified polymorphic sequence (CAPS) markers were used for cleaved amplified polymorphic sequence analysis to examine the *THRB locus* and microsatellite markers D3S1435, THRB, D3S1597 and D3S1304 were used to perform the innovative, PCR-based WAVE® Nucleic Acid Fragment Analysis to evaluate LOH/MSI presence in the 3p24.2~3pter region.
Results: Twenty-three (23) PTC samples were used. Interestingly enough, only one [1] among 18 informative cases (1/18) presented with LOH at the *THRB locus*, while none of the same 18 cases revealed any trace of MSI. Moreover, we also observed a single polymorphism at the *THRB* gene, restricted to one of the used CAPS markers.
Conclusions: Our results suggest that LOH and MSI in the 3p24.2~3pter region are not characteristic features in sporadic PTC.

Key words: papillary thyroid carcinoma, genetic instability, WAVE System.

Introduction

Many types of human cancer, both familiar and sporadic, have frequently been associated with genetic instability, characterized by chromosomal aberrations and/or DNA mutations. Studies on DNA alterations have revealed that a loss of heterozygosity (LOH) and/or microsatellite instability (MSI) occur in cancer cells with variable frequency [1-5].

Previous molecular genetic studies have identified LOH/MSI alterations as a specific pattern for cancer type, depending on tumour hot spots in DNA and the mechanism of carcinogenesis [1].

In some reports, their authors suggest that several different deleted regions – 1q, 2p, 2q, 4p, 7q, 10q, 8p, 9p, 9q, 11p, 16q, 17p, 22q – and, rather rarely – chromosome 3p, are involved in carcinogenesis of PTC [6-11]. However, it is known that LOH is more frequent in follicular thyroid carcinoma (FTC) than in papillary thyroid carcinoma (PTC) [12]. Moreover, a high rate (65%) of allelic losses has been reported at several chromosome regions in PTC [8, 11], while a low rate of allelic losses (20-33%) in PTC has been observed by other authors [7, 9].

In a previous study of thyroid carcinomas, MSI was not found [13], but subsequent examinations did not confirm that observation. MSI has been reported with variable frequency (6-36%) in malignant as well as in benign thyroid lesions [1, 8, 14-16]. Moreover, MSI was observed less frequently than LOH in cancer tissue [8, 13].

Recently, some controversial results have been obtained regarding the role of 3p genes in thyroid gland carcinogenesis [6, 7, 11, 16]. The last study by Rodrigues-Serpa et al. [16] confirmed the existence of one distinct region on 3p chromosome (3p25.3~p24.2) in PTC tissue, where LOH was observed in 40% while MSI in 6.6% of studied cases. It was supposed that, at least, one of the tumour suppressor genes (TSG) involved in PTC carcinogenesis had to be present at the 3p region – in the vicinity of the *thyroid hormone receptor beta* (*THRB*) and *Von Hippel Lindau* (*VHL*) genes [6, 16]. Because no mutations were found in the *VHL* gene (3p25 *locus*) in PTC cases with LOH at 3p25.3~p24.2 region [6], we decided to evaluate the LOH/MSI incidence in the vicinity of this gene.

Our study concentrated on the region covering the *locus* of the *THRB* gene and encoding the nuclear thyroid hormone receptor (TR).

It is known that the *THRB* gene mediates the effects of the thyroid hormone on gene expression [17], which suggests that the product of this gene may act as a tumour suppressor gene by promoting cell differentiation and inhibiting cell proliferation [18]. In favour of this hypothesis, some recent studies have proposed *THRB* gene involvement in the area of genetic deletion in various types of human cancers, but the role of this gene in tumour initiation and progression is still unclear [2, 4, 19, 20]. In the present study, we tested the hypothesis whether the loss of genetic material in the 3p chromosomal region at the *THRB locus* and in the surrounding area was involved in the development of PTC. To our knowledge, it is the first report concerning the presence of LOH/MSI exactly at the *THRB locus* in the Polish population with diagnosed sporadic PTC.

Material and methods

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

We used tumour tissue specimens (50-100 mg) obtained from 23 patients with primary PTC, who had undergone surgery (total thyroidectomy) at the Department of Oncological Surgery, Centre of Oncology – the MSC Memorial Institute, Gliwice, Poland, and at the Department of General and Colorectal Surgery, the University of Lodz, Poland, during the years 2002-2006.

In each case, PTC was cytologically diagnosed before surgery (fine-needle aspiration biopsy – FNAB evaluation) and verified by histopathological postoperative examination. PTC variants were as follows: PTC, classic variant – 11 cases, PTC, follicular variant – 9 cases, and PTC, tall-cell variant – 3 cases.

The mean age of all the studied patients (16 females and 7 males) was 45.05±16.12 years (x±SD), the range between 16 and 74 years. For each PTC case, matching pairs of macroscopically unchanged thyroid tissue were used as a wide-type control. After surgery, the tissue fragments were immediately snap-frozen in dry ice and stored at –70°C until further processing.

DNA isolation from tissue

Extraction of genomic DNA was carried out from frozen neoplastic and macroscopically matching unchanged tissue samples, using a commercially available kit [Genomic Midi AX, (A&A Biotechnology, Gdynia, Poland)]. Briefly, 50-100 mg of tissue was mechanically homogenized and DNA isolation was performed, according to the manufacturer's protocol. DNA concentration and purity were assessed spectrophotometrically by measuring the absorbance at 260 and 280 nm (Ultrospec 2000 UV/Visible Spectrophotometer, Pharmacia Biotech, Sweden) and DNA concentration was estimated within the range of 0.1-1.0 µg/µL.

PCR amplification with polymorphic markers

The nucleotide sequences of polymorphic *cleaved amplified polymorphic sequence* (CAPS) and microsatellite markers (TIB-MOLBIOL, Gdynia, Poland) and the sizes of PCR products are shown in Table I.

PCR reactions with polymorphic CAPS markers were performed in a TRIO-Thermoblock thermocycler (Biometra, Göttingen, Germany) in a total volume of 50 µL, containing: 40 ng of DNA, commercial HotStarTaq DNA Polymerase Kit: [10×PCR buffer: Tris-Cl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$, pH 8.7], 5×Q-solution, dNTP mix (10mM – of each), 5 U/µL of HotStarTaq DNA Polymerase (QIAGEN, Hilden, Germany) and 25 pmol of each (i.e. sense and antisense) primers and nuclease-free water. PCR reactions for microsatellite markers were performed in a Mastercycler personal (Eppendorf, Hamburg, Germany) in a total volume of 25 µL, under the same conditions as mentioned above. In PCR amplifications, DyeAmidite 667 (Indodicarbocyanine dye amidite; Pharmacia Biotech) 5' labelled sense primer of each microsatellite marker and 1000 ng DNA were used. Amplification reactions were carried out for 36 cycles. Each cycle consisted of initial denaturation at 95°C for 15 min (1 cycle), denaturation at 94°C for 1 min (1 cycle), annealing at a specific temperature for each pair of primers:

Table I. Nucleotide sequences of polymorphic markers and the size (bp) of PCR products

Primer sequences	Amplicon size (bp)
CAPS marker THRB (Hind III)	
Sense: 5'-TCATTCGAG TTAGTGCAAAG-3'	432
Antisense: 5'-ACGTTAGTGGCTCATATGAG-3'	
CAPS marker THRB (MspI)	
Sense: 5'-AACGTTGGACCTCAAGCCCAT-3'	683
Antisense: 5'-CAGGGTTCCTTCTATAAACATGG-3'	
Microsatellite marker: THRB	
Sense: 5'-GATCACAAGGATGCTAGAGT-3'	197
Antisense: 5'-TCAAAGGAGTCAGGCTGTAG-3'	
Microsatellite marker: D3S1435	
Sense: 5'-TGGATACATTAGTATACTGAATT-3'	154
Antisense: 5'-TAAGACGGAAGCAAGGAAGG-3'	
Microsatellite marker: D3S1304	
Sense: 5'-TTCGCTCTTTGATAGGC-3'	253-269
Antisense: 5'- ATTTCATTTGTAATTTACTAGCAG-3'	
Microsatellite marker: D3S1597	
Sense: 5'-AGTACAAATACACACAAATGTCTC-3'	162-180
Antisense: 5'-GCAAATCGTTCATTGCT-3'	

55°C (CAPS markers), 56°C (D3S1604), 61°C (THRB, D3S1435), 65°C (D3S1597) for 1 min (35 cycles), elongation at 72°C for 1 min (35 cycles), and final elongation at 72°C for 10 min. Several water blank controls for PCR contaminations were included in each amplification reaction.

Cleaved Amplified Polymorphic Sequence (CAPS) analysis

PCR products with CAPS polymorphic markers were digested with selected restriction enzymes (i.e. Hind III and Msp I) as follows: 10 µL of PCR products were incubated overnight in a volume of 20 µL with 20 units of proper restriction enzyme in a salt-adjusted 1×NEBuffer (10 mM Tris-HCl, 50 mM NaCl, 10 µM $MgCl_2$, 1mM Dithiothreitol, pH 7.9) (BioLabs, New England).

Digested PCR products were identified using 8% polyacrylamide-TBE [Tris-borate-ethylenediamine tetraacetate (EDTA)] gels, visualized by ethidium bromide staining (0.5 mg/mL) and photographed (Sony UPP-11HD film).

After digestion, the allelotype was determined for each pair of samples (PTC tissue – T vs. corresponding macroscopically unchanged tissue – N) and heterozygosity was estimated. Only those cases which demonstrated heterozygosity in macroscopically unchanged tissue were considered informative.

PCR-based LOH analysis with microsatellite markers

All the results obtained by the *Cleaved Amplified Polymorphic Sequence* method were reviewed using the ALFexpress™ II DNA analysis system (Amersham Biosciences, Uppsala, Sweden). Primer sequence and cytogenetic localization for the microsatellite marker were obtained from NCBI (http:/www.ncbi.nlm.nih.gov/genome/sts/sts).

PCR products (after denaturation in 95°C, 5 min) were run on denaturing ReproGel high resolution gel (Amersham Biosciences, Uppsala, Sweden) using ALFexpress™ DNA Sequencer. The results were analyzed using Allelelinks™ v. 1.00 software (Pharmacia Biotech) with an internal ALFexpress Sizer® 300 and an external ALFexpress™ Sizer® 50-500 (Pharmacia Biotech). Allelic loss was calculated using the normalized LOH ratio (R) by the equation R=(A1)(N2)/(A2)(N1), in which A1 and A2 are the values of fluorescence (RQ units) of the alleles from tumour tissue and N1 and N2 are the allele fluorescence values from the matching macroscopically unchanged thyroid tissue. The cases in which the LOH ratio (R) was either ≤0.8 or ≥1.25 were scored as LOH, taking into consideration that the LOH ratio was calculated by R or by 1/R if R was >1. Microsatellite instability (MSI) was scored when the additional bands that were not present in the corresponding macroscopically unchanged tissue were observed in

DNA from PTC tissue. Additionally, PCR products with each of the microsatellite markers D3S1435, THRB, D3S1597 and D3S1304 were analyzed for LOH/MSI presence using ion-pair reversed-phase high performance liquid chromatography (IP-RP-HPLC) on a Transgenomic WAVE System (Omaha, NE, USA). The assessment of DNA fragments (separation PCR product, sizing and typing of alleles) was compared to the WAVE optimized sizing standard – WAVE DNA sizing control (Transgenomic Inc.) and MSI/LOH results were analyzed using Navigator™ software v. 2.6.1. The alleles were sized in their native (i.e. double stranded) state.

Results

In our analysis with CAPS marker (Hind III and Msp I), 74% of the studied patients (17/23 cases) were informative, being heterozygous for at least one of the studied markers. We did not notice any significant differences in the frequency of heterozygosity in DNA derived from the PTC sample, compared with the matching, macroscopically unchanged tissue for Hind III marker (48% vs. 57%), while in the case of Msp I marker, there was even the same rate of heterozygosity in PTC and macroscopically unchanged thyroid tissue (52%).

Interestingly enough, we observed allelic deletion in two cases of informative patients at the Hind III marker. Both of them (one case PTC – classic variant, $pT_{2a}N_0M_0$, and the other one PTC – follicular variant, $pT_{4a}N_{1a}M_0$) showed homozygous deletion at the Hind III marker in PTC tissue, although both alleles were present in the macroscopically unchanged tissue. Only one of them (PTC – classic variant, $pT_{2a}N_0M_0$) was taken into consideration in our LOH/MSI study because of remaining heterozygous also for the other studied marker (Msp I). Anyway, because the detected DNA alteration in PTC tissue was observed only for one informative CAPS marker, we supposed that the result could just be a single polymorphism only (Figure 1; line 4). Moreover, we did not detect any allelic losses in PTC at another – Msp I marker.

In order to check if allelic loss at the Hind III marker observed in one patient (PTC – classic variant, $pT_{2a}N_0M_0$) presented only a single polymorphism, harbouring the site of the Hind III marker, we performed an LOH/MSI study at the *THRB locus*, using the ALFexpress II DNA analysis system with microsatellite THRB marker. Our experiment did not prove any presence of LOH/MIS in the studied case, thereby confirming the single polymorphism limited to the Hind III marker.

The summary of LOH/MSI results for all the markers used in our study is shown in Table II.

The percentage of informative *loci* varied from 65 to 83% of all the analyzed cases. Examination of all the studied patients for LOH/MSI presence by the WAVE System (using D3S1435, THRB, D3S1597, D3S1304 markers) revealed LOH only at the *THRB locus* (THRB marker) in one (1) among 18 informative cases (1/18) of PTC tall-cell variant, ($pT_3N_xM_x$) (Figure 2, picture 3).

Additionally, in the ion-pair-reversed-phase HPLC analysis, different chromatogram patterns were observed in cancer tissue, as compared with DNA from the macroscopically unchanged tissue. A significant increase in the size of one of the homoduplexes was distinct, as visualized by an elevated detector signal at the *THRB locus* (THRB marker) in two (2) out of 18 informative (2/18) cases of PTC: one in the PTC follicular variant ($pT_{2b}N_{1a}M_0$) and the other one in the PTC tall-cell variant ($pT_{4b}N_0M_0$) (Figure 2, picture 4). Despite the observed chromatogram pattern differences, we did not recognize those DNA alterations to be MSI. In our study, using the remaining microsatellite markers, we did not detect any presence of LOH/MSI in PTC tissue (Table II). None of the macroscopically unchanged thyroid tissue had LOH/MSI at the *THRB locus* or in the bordering region (3p24.2~3pter).

Discussion

The molecular background of PTC has already been characterized in a number of reports [21, 22]. At the same time, it is a rather odd fact that not all genetic factors involved in PTC carcinogenesis have yet been investigated. It has been documented that DNA alterations – related to LOH/MSI presence – occur in the initiation phase as well as in the progression phase of thyroid carcinogenesis [6, 7, 16]. MSI/LOH has been proved to be helpful in understanding the genetic bases of the multistep process of thyroid cancer development. In our study, we examined whether LOH/MSI alterations in the

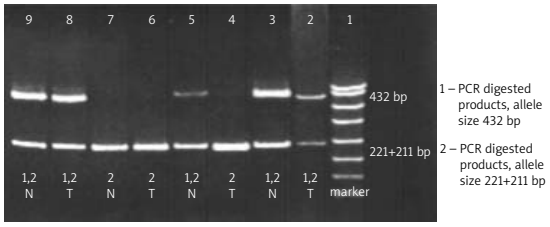

Figure 1. Example of ethidium bromide, stained with 8% PAA gel, containing Hind III digested PCR products of *THRB* polymorphic region (CAPS method). Allelic deletion in case of PTC – classic variant ($pT_{2a}N_0M_0$), referring to the absence of one (1) of the two alleles (1 and 2) in tumour DNA (T) (lane 4), compared with the presence of both alleles (1 and 2) in DNA derived from matching, macroscopically unchanged tissue (N) (lane 5). The sizes of the two alleles are indicated next to the DNA weight marker (lane 1) and the allelotype for each sample is denoted beneath each line. For other abbreviations, see the legend for Figure 1

Table II. Chromosome location of the microsatellite and CAPS markers used in the study, and the summary of LOH/MSI results in all the informative cases

Marker and *locus* map position	LOH/informative cases	MSI/informative cases
D3S1597 3p24.2~3pter	0/19	0/19
D3S1304 3p24.2~3pter	0/15	0/15
D3S1435 3p24.2	0/17	0/17
THRB 3p24.3	1/18	0/18
CAPS marker THRB (Hind III) 3p24.3	0/13	0/13
CAPS marker THRB (Msp I) 3p24.3	0/12	0/12

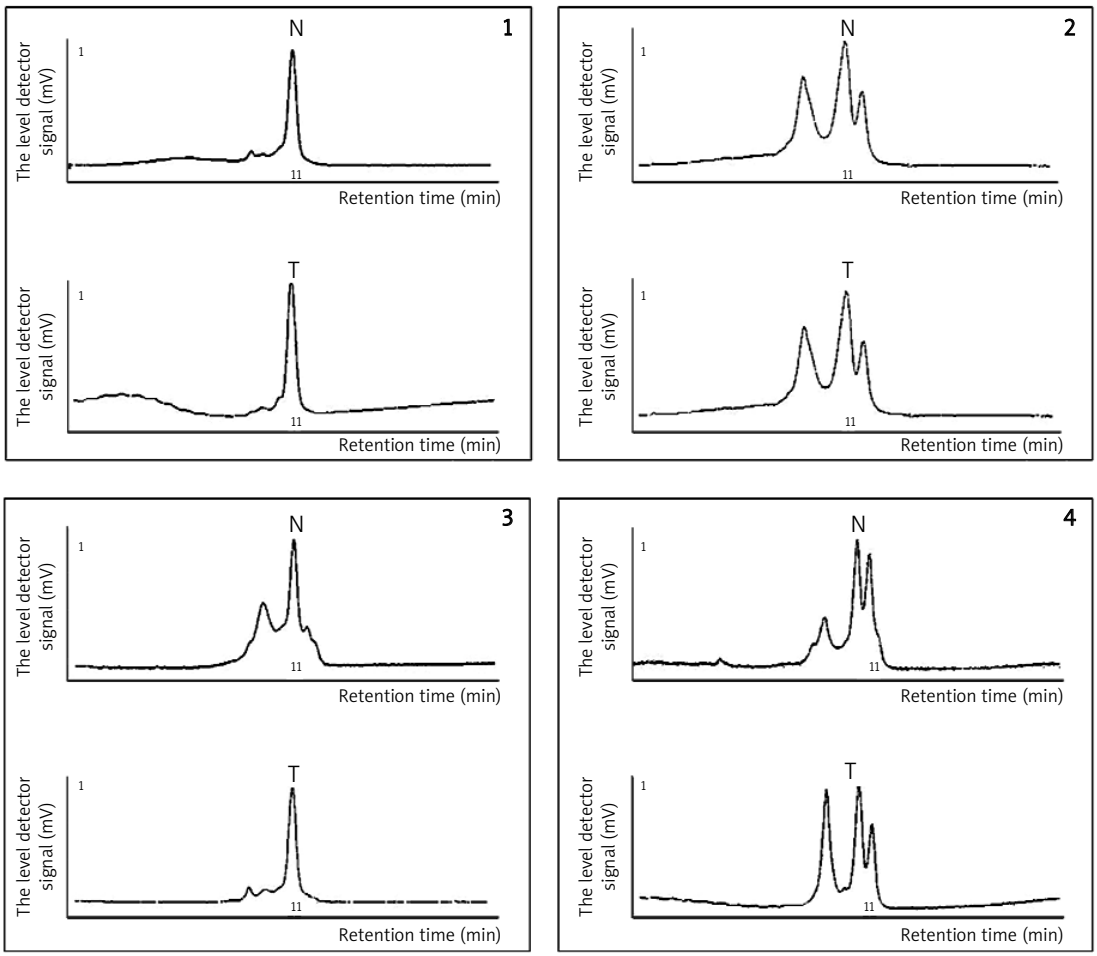

Figure 2. Results of the LOH/MSI method, using the WAVE DNA fragment analysis. The chromatograms show peak profiles for *THRB* microsatellite marker, derived from tumour DNA (T) and DNA from matching, macroscopically unchanged tissue (N); 1 – an example of constitutionally homozygous DNA (PTC classic variant). The chromatogram shows a single peak, i.e. one allelic homoduplex; 2 – an example of heterozygous DNA (PTC follicular variant); the chromatogram shows two allelic homoduplexes, one with shorter retention time; 3 – an example of LOH, with the presence of only one main peak, i.e. one allelic homoduplex in the tumour DNA (PTC tall-cell variant, $pT_3N_xM_x$); 4 – an example of allelic homoduplex, different in height, was observed in the tumour DNA (PTC – follicular variant, $pT_{2b}N_{1a}M_0$)

3p24.2~3pter region were involved in PTC carcinogenesis. The low incidence of LOH (1 out of 18) in PTC tissue on the 3p chromosome obtained in our study is consistent with the results of other authors [6, 7, 11]. The majority of reports [6-8, 11, 12], as well as our own results, have proved that LOH on the 3p chromosome is not a common feature in PTC. On the other hand, the low incidence of LOH obtained for the Polish population in the present study, precisely in the 3p24.2~3pter region, did not confirm the significance of LOH (9-30%) in the 3p25.3-3p24.2 region in PTC carcinogenesis, as estimated by Rodrigues-Serpa et al. [16]. In our present study, LOH was observed only at one *locus* (*THRB gene*) in the case of the tall-cell variant of PTC ($pT_3N_xM_x$), whereas others observed LOH mainly in classic and follicular variants of PTC [11, 16]. In our results, the presence of LOH in the tall-cell variant of PTC (more aggressive than other variants, e.g. PTC classic or follicular variants) [21] may confirm the prevalence of allelic loss in more aggressive phenotypes, reported for thyroid tumours [7, 9]. Because our study is initial and the examined group is small, further studies in larger groups of patients are needed in order to confirm our observations.

So far, only a few studies on MSI in thyroid tumours have been published. Therefore, we decided to estimate MSI as a characteristic genetic event in thyroid carcinogenesis and establish the prognostic value of this DNA alternation. In the present study, MSI was not detected while examining four dinucleotide microsatellite markers in any of the studied *loci*.

MSI is believed to be associated with mutations in the DNA mismatch repair (MMR) system. In our study, we observed different chromatogram patterns in cancer tissue, as compared with DNA from the macroscopically unchanged tissue – i.e. significantly increased height of one of the homoduplexes that might indicate the occurrence of MSI. However, because both peaks were present in both samples (cancer and macroscopically unchanged tissues) and additional peaks were not visible in other nearby *loci*, we could not regard that DNA alternation as MSI. The results obtained by others [14-16] indicate the low frequency of MSI and confirm the limited role of DNA mismatch repair (MMR) system defects in thyroid carcinogenesis. Regarding the relationship between MSI and benign or malignant status of thyroid lesions, tumour size or such features as patient's age at diagnosis, or gender, very controversial results were found [1, 8, 14]. Similarly, according to our results, the prognostic value of MSI or correlation with the histopathological variant of PTC or tumour size seems to be doubtful. Regarding the prognostic aspects, Onda et al. [1] have confirmed the correlation between MSI in tumour tissue and better long-term survival. Also, a strong association between MSI/LOH presence and iodine deficiency as well as tumour stage and metastases was demonstrated [23]. Because of the small group of PTC cases analyzed in our study, it was not possible to calculate correlations between LOH and the histopathological variant of PTC or to determine the assignment of patients to a particular stage in the clinical staging system [24]. Additionally, our patient group comes from an area (Poland) where the problem of iodine deficiency is no longer observed – normalization of iodine supply has been achieved. Similarly, no correlations were analysed between LOH and such parameters as patient's age at diagnosis or gender. In conclusion, the low incidence of LOH and lack of MSI in PTC in our study, juxtaposed with many controversial results of others, seems to be associated with the high heterogeneity of thyroid cancer at the molecular level, as well as with different criteria in screening of the MSI/LOH alterations.

All the genetic events leading to sporadic PTC are found with variable frequency, depending on the study population and screened *loci* (particularly in the case of the LOH/MSI study). It is also known that activation of the signalling pathway of some of protooncogenes (i.e. *RAS, MET, RET, NTRK1, BRAF*) is a dominant event in thyroid carcinogenesis [21]. The low frequency of LOH and lack of MSI in our study group, simultaneously with the relatively high frequency of *RET* and *NTRK1* protooncogene rearrangements in PTC in the Polish population [25], may confirm the above view. Moreover, in our study, no LOH/MSI presence was observed in patients with earlier confirmed *RET*/PTC and *Trk* rearrangements [25].

The rare incidence of LOH and lack of MSI at the 3p24.3 *locus* observed in our study confirmed Rocha et al.'s [26] findings suggesting that mutations in the *THRB* gene (in a hot-spot region) do not play a role as a relevant mechanism for human thyroid carcinogenesis. On the other hand, the aberrant expression of the *THRB* gene and the high frequency of somatic mutations of this gene in PTC tissues (93.75%), as observed by others [27], need some attention as well. All these discrepancies in point mutation screening and/or LOH/MSI evaluation may be explained by different methodologies, different numbers of studied cases or different microsatellite *loci*, investigated in various reports. In view of the importance of LOH/MSI analysis (prognostic value), these molecular applications require some more updated improvement. The WAVE System, used in our study, was the most advantageous, fully automated and optimal high-throughput assay for the LOH/MSI analysis. IP-RP-HPLC, using a computer-

controlled system, has been widely used in many recent studies [28, 29]. Here we report that the LOH/MSI analysis, using the WAVE System under correct conditions, can clearly be established more efficiently and far more quickly than by conventional approaches.

In many cases, the results obtained from LOH/MSI analysis, using cancer cells, are difficult to evaluate because of no uniform pattern of DNA instability or LOH status in cancer tissue. Many distinct subtypes of microsatellite alterations were found in human cancers, including those observed in thyroid tumours [7, 30].

Conclusions

Our present results do not *speak for* the possible role of the *THRB* gene in the development of PTC. On the other hand, although we have also noticed that the allelic losses on chromosome 3p (3p24.2~3pter) are rather rare features of PTC, the significance of those DNA lesions in PTC requires further studies.

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