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ORIGINAL ARTICLE

Loss of heterozygosity in 73 human thyroid tumors

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Abstract

OBJECTIVES: The aim of the study was to establish the LOH frequency of selected polymorphic markers in different histological types of thyroid tumors: 18 colloid goiters (CG), five follicular adenomas (FA), nine follicular carcinomas (FTC), 40 papillary carcinomas (PTC), and one anaplastic carcinoma (ATC). For PTC, tumors negative for RET/PTC rearrangements were preferred.
METHODS: LOH studies were performed using 14 highly polymorphic markers previously described as frequently lost in thyroid tumors.
RESULTS: In 20 cases (27%) the loss of at least one marker was found. No difference between the frequency of the LOH in FTC and PTC tumors was revealed (33% v. 33%). No differences between histopathological subtypes of PTC in LOH were found. Papillary thyroid carcinomas showed a tendency to higher LOH frequency from patients older than 45 years of age compared to younger ones (9/23 v. 4/17) although it was not statistically significant.
CONCLUSIONS: We conclude that papillary thyroid cancers, particularly those diagnosed in patients older than 45 years of age, do exhibit LOH at least with the same frequency as follicular cancers. This increased number of LOH events may contribute to the clinical aggressiveness of cancer in older patients.

Abbreviations

AC	– anaplastic carcinoma
BRAF	– V-RAF murine sarcoma viral oncogene homolog B1
CG	– colloidal goiter
CGH	– comparative genomic hybrydization
FA	– follicular adenoma
FTC	– follicular thyroid carcinoma
LOH	– loss of heterozygosity
METC	– hepatocyte growth factor receptor
PTC	– papillary thyroid carcinoma
PCR	– polimerase chain reaction
RET	– tyrosine kinase (rearranged during transection)
RT-PCR	– reverse transcriptase polimerase chain reaction
TSG	– tumor supressor gene
TRK1	– tyrosine receptor kinase 1

Introduction

Various studies have been performed to identify chromosomal regions harboring tumor supressor gene (TSG) involved in thyroid tumorigenesis. Most authors used loss of heterozygosity (LOH) technique to detect allelic deletion. There was not much known about LOH in colloidal goiters. Several chromosomal regions, such as 2p, 2q, 3p, 7q, 10p, 10q 11q, 13q, 17p and 17q, have been reported as frequently lost in thyroid adenomas and carcinomas [1–8]. Some data have proven that

LOH on 7q21.1 and 7q31.1 were characteristic events in FA/FTC but did not occur in PTC [5]. Most authors indicated that LOH was more frequently observed in FTC than in PTC [4]. Also, correlation between histopathological subtypes as well as clinical data and LOH changes in thyroid neoplasm were noticed but the data were contradictory [2, 5, 8].

RET and *TRK1* protooncogenes encoded for tyrosine kinase receptors are frequently involved in cytogenetic rearragements leading to the formation of chimeric oncogenes [9]. The presence of *RET* rearrangements has ranged between a few and more than 80% of PTC cases, with the highest estimates in populations of patients with known radiation history [10]. Both types of rearrangements may alternatively constitute an initiating step in PTC development, while the nature of initiating event in *RET/PTC* and *TRK1* negative PTC has still not been elucidated.

The aim of our study was to analyze the occurrence of LOH at different loci in human thyroid tumors and to estimate possible relationship between LOH and basic parameters such as histological type, stage and differentiation.

Material and methods

Tissue samples. The study group consisted of 73 thyroid tumors: 18 CG, 5 FA, 9 FTC, 40 PTC and one AC, diagnosed and classified according to WHO criteria [11]. The group of patients included 60 women and 13 men, ranging in age from 14 to 77 years (median 52±14), treated by thyroidectomy and, when indicated, neck modified lymphadenectomy.

For DNA extraction tumor fragments as well as normal tissue or blood were obtained from the same patients. Genomic DNA was prepared using standard procedure based on ionic detergent lysis and proteinase K digestion, phenol-chloroform extraction and isopropanol precipitation. The DNA concentration was quantitated by spectrophotometry (OD260/OD280nm).

PCR and microsatellite analysis. LOH was detected by PCR-based 14 polymorphic microsatellite markers (*Table 1*). Primer sequences, cytogenetic localization and heterozygosity rate of markers were obtained from NCBI (http://www.ncbi.nlm.nih.gov). For the PCR amplification the forward primer was modified with 6-FAM, TET or HEX molecule (IDT Integrated DNA Technologies Inc.Coralville, IA, USA). Standard PCR reactions were carried out in a final volume of 15µl containing 50 ng of genomic DNA and the following concentrations: 0.33µM of each primer, 0.25nM dNTPs (Promega Corp. Madison, WI, USA), 2.5mM magnesium chloride and 0.04U/µl of Taq DNA Polymerase (MBI Fermentas). All PCR products were amplified using a PTC-200 Peltier Thermal Cycler (MJ Research, Watretown, MA, USA) under standard conditions. PCR products were prepared for capillary electrophoresis, with GenScan-500 TAMRA used as a size standard marker, according to the Applied Biosystem protocols (Applied Biosystem, Foster City, CA, USA). After denaturation, samples were analyzed in Genetic Analyzer ABI310 using ABI PRISM 310 Data Collection Software v 1.0.2. and ABI PRISM GeneScan Analysis Program. (Applied Biosystem, Foster City, CA, USA).

Determination of LOH. Definition of the allelic deletion was limited to informative (heterozygous) cases. Allelic loss was calculated using a normalized allele ratio (LOH ratio) equitation: LOH=(T1)(N2)/(T2)(N1) where N1 and N2 are peaks from normal DNA and T1 and T2 are peaks from tumor DNA. Marker in which the LOH ratio was below 0.6 was scored as lost.

Determination of RET/PTC rearrangements. Data for *RET/PTC* rearrangements were available for 31 PTC tumors. For *RET* rearrangements study, mRNA specimens were extracted from frozen tissues using Dynabeads mRNA DIRECT™ Kit (Dynal, Oslo, Norway) and then reverse-transcribed (cDNA Cycle Kit; Invitrogen, CA, USA). 2 µl of cDNA were amplified during PCR I to check whether any *RET* rearrangement is present according to the method described by Klugbauer et al. [12]. The PCR II was performed to amplify specific transcripts for 7 *RET/PTC* types (PTC1-7). PCR products were electrophoresed in 5% PAGE gels and stained with ethidium bromide.

Statistical analysis. For statistical analysis the Fisher exact test was used.

Results

The complete list of markers used in the study and the LOH results for each polymorphic marker are shown in *Table 1*.

The percentage of informative loci varied from 50–100% of all cases analyzed. In 20 cases (27%) the loss of at least one marker was found. Marker D10S607 (10q22.2) was the most frequently lost in the whole group of thyroid tumors (6/49–13%). The second most often deleted marker was D7S486 (7q31.1–*METC* gene locus) (5/58–9%). Five of 40 PTC cases (13%) and the anaplastic carcinoma sample presented LOH in multiple sites in different chromosomes. One case of 18 (6%) CG showed LOH in marker localized on 10q22.2 (D10S607). In two of five FA (40%) losses were found in two markers: D7S660 (7q21.1–1/5) and D10S607 (1/3). In follicular carcinoma LOH was present in three cases (3/9–33%), in markers D7S660 (1/8), D10S607 (1/7) and D17S520 (17p12–1/7). In the one analyzed case of anaplastic cancer, loss of two markers was found (D3S1435 and D7S660).

13 of 40 papillary carcinomas (33%) revealed LOH in at least one locus. In PTC we observed that losses were more frequently present in group of patients older than 45 years of age compared with younger ones (9/23 v. 4/17) but no statistical significance was found. Detailed results obtained in the above two groups are presented in *Table 2*. The differences in deleted loci in both groups were noticed. In the older group of patients the marker most frequently lost was D7S486 (22%–4/18). In the group of younger patients marker D17S520 (17p12) was lost in two out of 13 tumors.

For 31 PTC tumors the corresponding mRNA was available and the analysis of *RET/PTC* rearrangements

Table 1: Localization of the chromosome markers used in this study of 73 human thyroid tumors and summary of LOH versus informative cases in each locus studied.

		LOH / informative cases (%)					
Locus symbol	**Band location**	**CG n=18**	**FA n=5**	**FTC n=9**	**PTC n=40**	**AC n=1**	**total n=73**
MYCL	1p32	0/14	0/5	0/7	1/26 (4)	0/1	1/53 (1.9)
D2S123	2p16	0/15	0/3	0/7	2/25 (8)	0/1	2/51 (3.9)
D2S1328	2q14.3	0/11	0/2	0/6	3/26 (12)	nd	3/45 (6.7)
D3S1101	3p12	0/16	0/5	0/9	1/32 (3)	0/1	1/63 (1.6)
D3S1481	3p14.2	0/13	0/5	0/9	1/32 (3)	0/1	1/60 (1.7)
D3S1435	3p24.2	0/14	0/4	0/7	0/26	1/1 (100)	1/52 (1.9)
D7S665	7p15	0/13	0/3	0/5	1/29 (3)	0/1	1/51 (2)
D7S660	7q21.1	0/10	1/5 (20)	1/7 (14)	0/22	1/1 (100)	3/45 (6.7)
D7S492	7q21.1	0/16	0/3	0/7	1/24 (4)	0/1	1/51 (2)
D7S486	7q31.1	0/16	0/5	0/8	5/28 (18)	0/1	5/58 (8.6)
D10S607	10q22.2	1/13 (8)	1/3 (33)	1/6 (17)	3/26 (12)	nd	6/48 (12.5)
D15S97	15q11.2	0/13	0/2	0/4	1/28 (4)	0/1	1/48 (2.1)
D17S520	17p12	0/16	0/4	1/7 (14)	2/33 (6)	nd	3/60 (5)
TP53	17p13.1	0/10	0/4	0/5	0/22	0/1	0/47
Total		1/18 (5.6)	2/5 (40)	3/9 (33)	13/40 (33)	1/1 (100)	20/73 (27)

CG – colloidal goiter, FA – follicular adenoma, FTC – follicular carcinoma, PTC – papillary carcinoma, AC – anaplastic carcinoma; LOH – loss of heterozygosity, nd – no data.

Table 2: The comparison of LOH frequency between patients of papillary carcinoma diagnosed under and over 45 years of age

	Number of cases with LOH in			
	one locus	**two loci**	**three or more loci**	**total (%)**
PTC (n=40)				**P=0.298**
under 45 yrs (n=17)	2	2	0	4 (23)
over 45 yrs (n=23)	6	2	1	9 (39)
RET/PTC negative (n=31)				**P=0.1061**
under 45 yrs (n=16)	2	2	0	4 (25)
over 45 yrs (n=15)	5	2	1	8 (53)

was performed. Their absence was revealed by a RT-PCR study. Among confirmed *RET/PTC* negative PTC cases the frequency of LOH-positive tumors was 39% (12/31), with 4/16 cases diagnosed in patients under 45 years of age (25%) and 8/15 cases observed in older PTC patients (53%).

Discussion

Loss of genetic material in thyroid neoplasm has been studied using cytogenetic methods as well as LOH techniques. Different chromosomal regions have been suggested to be involved in the development of thyroid cancer, but the findings are still controversial. We used LOH technique to analyze markers which were suggested by other authors to be specifically lost in different histological types of thyroid tumors.

In our studies only one case of colloidal goiter showed LOH in marker localized in 10q22.2 (D10S607). To our knowledge this is the first case of CG which reveals any LOH. Interestingly, marker D10S607 was most frequently lost in different subtypes of thyroid tumors analyzed (6/48–13%), which may suggest the early role of the TSG localized at this loci.

Several authors found that LOH at 10q22–24 was more frequently observed in FA than in FTC, suggesting that there might be two different pathways in developing thyroid follicular tumors [1,6]. Our results displayed the same phenomenon although low number of informative cases prevented us from performing statistical analysis.

In studies performed by Trovato et al. [5] four markers (D7S660, D7S492 from 7q21.1 and D7S486, D7S655 from 7q31.1) were lost in all FTC studied and in 10–20% of FA cases, whereas no losses in PTC were observed. It was suggested that the above-mentioned markers could be used for distinguishing FA/FTC from PTC. These authors also found the 100% prevalence of LOH of 7q markers in ATC cases, which could prove that FTC and ATC shared genetic abnormalities and that there were two subsequent stages of the same pathological pathway [5]. Our results as well as other data did not confirm the observation that 7q markers were selectively lost in FTC but not in PTC. [7,8,13]. In both FA and FTC we found LOH (2/12–17%) only in marker D7S660 (7q21.1), while in PTC allelic losses were found in two markers – D7S486 (5/28–18%) and D7S492 (1/24–4%). These results suggest that these markers are not characteristically lost in FA/FTC subtypes. Since consistent losses of markers localized in chromosome 7q were described by many authors it was suggested that at least one TSG is localized in 7q in the vicinity of the *METC* locus [5,7,8].

It has been reported that allelic losses in FA and FTC (33% and 60% cases) were significantly more frequent than in PTC (23%) [4]. Other studies seemed to prove the above results [1, 2, 3, 8]. In our studies the difference between the frequency of the LOH in FTC and PTC was not found (33% vs. 33%). In order to analyze the possible reasons of the higher frequency of LOH observed by us, we inspected the influence of age at PTC diagnosis and of the *RET* mutations occurrence.

RET rearrangements are found in 6–84% cases of PTC, depending on the population investigated, radiation exposure and method of estimation. In average, 25–30% PTC are *RET*-positive, with the higher incidence in younger PTC patients. In our experience the frequency of *RET* mutations (mainly intrachromosomal inversions) in the Polish population of PTC approaches 50% in patients younger than 30 years of age at diagnosis while it is several times lower (under 10%) in patients diagnosed with PTC after the age of 50 [14]. The age-dependence of *RET* mutations has been reported by others [15,16]. On the other hand, others relate the occurrence of *RET/PTC* mutations only to radiation-induced PTC [12]. Molecular events leading to the development of PTC in the absence of *RET* or *TRK* rearrangements are largely unknown. Recent reports indicate on the high frequency of BRAF mutations which may lead to the conclusion that the activation of the alternate steps of the tyrosine kinase receptors-RAS-MAPK pathway may result in development of papillary thyroid cancer [17,18]. However, the impact of other, still unrecognized genes is also discussed and our observation, that *RET/PTC* negative tumors exhibit a nearly 40% frequency of LOH, may help to localize the potential TSG involved. This mechanism may preferentially operate in older PTC patients, in whom the clinical symptoms of malignancy are more distinctly expressed and more often lead to a fatal outcome [19,20]. As *RET* rearrangements were excluded in majority of PTC cases in this study, other molecular events leading to the development of PTC could be easier detected. We did not find any information about the occurrence of *RET* rearrangements in other studies of LOH in PTC.

Using CGH analysis, some authors displayed an increasing number of alterations accompanied by increased aggressiveness of thyroid cancer, but this relationship was present only in papillary not follicular tumors [21,22]. In another report, LOH events were observed more frequently in cases deceased of papillary carcinoma [23]. We did not correlate our results with the clinical course of the disease, as the number of patients with very advanced PTC was rather small. Instead, we performed a comparison of the LOH occurrence in two age groups of PTC (under and above 45 years of age). In the group of older PTC patients allelic deletions were present more frequently (39% vs. 23%) but it was not statistically significant. In relative terms, the last difference was even more distinct, when proven RET/PTC-negative tumors were considered – a two-fold increase was seen in older patients. It is known, that the prognosis is much poorer in PTC patients older than 40–50 years of age [19,20]. Thus, the increased number of LOH events may contribute to the clinical aggressiveness of cancer in older patients, especially in those papillary thyroid tumors which are not initiated by a RET/PTC rearrangement. In conclusion, our study shows that papillary thyroid cancers, particularly those diagnosed in patients older than 45 years of age, do exhibit LOH at least with the same frequency as follicular cancers.

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REFERENCES

1 Zedenius J, Wallin G, Svensson A, Bovee J, Hoog A, Backdahl M, et al. Deletions of the long arm of chromosome 10 in progression of follicular thyroid tumors. Hum Genet 1996; **97**:299–303.
2 Grebe SK, McIver B, Hay ID, Wu PS, Maciel LM, Drabkin HA, et al. Frequent loss of heterozygosity on chromosomes 3p and 17p without VHL or p53 mutations suggests involvement of unidentified tumor suppressor genes in follicular thyroid carcinoma. J Clin Endocrinol Metab 1997; **82**:3684–91.
3 Tung WS, Shevlin DW, Kaleem Z, Tribune DJ, Wells SA, Goodfellow PJ. Allelotype of follicular thyroid carcinomas reveals genetic instability consistent with frequent nondisjunctional chromosomal loss. Genes Chromosome Cancer 1997; **19**:43–51.
4 Ward LS, Brenta G, Medvedovic M, Fagin JA. Studies of allelic loss in thyroid tumors reveal major differences in chromosomal instability between papillary and follicular carcinomas. J Clin Endocrinol Metab 1998; **83**:525–30.
5 Trovato M, Fraggetta F, Villari D, Batolo D, Mackey K, Trimarchi F, et al. Loss of heterozygosity of the long arm of chromosome 7 in follicular and anaplastic thyroid cancer, but not in papillary thyroid cancer. J Clin Endocrinol Metab 1999; **84**:3235–40.
6 Yeh JJ, Marsh DJ, Zedenius J, Dwight T, Delbridge L, Robinson BG, et al. Fine-structure deletion mapping of 10q22-24 identifies regions of loss of heterozygosity and suggests that sporadic follicular thyroid adenomas and follicular thyroid carcinomas

develop along distinct neoplastic pathways. Genes Chromosome Cancer 1999; **26**:322–8.
7 Kitamura Y, Shimizu K, Ito K, Tanaka S, Emi M. Allelotyping of follicular losses in chromosome arms 7q, 11p and 22q. J Clin Endocrinol Metab 2001; **86**:4268–72.
8 Oriola J, Halperin I, Mallofre C, Muntane J, Angel M, Rivera-Fillat F. Screening of selected genomic areas potentially involved in thyroid neoplasms. Eur J Cancer 2001; **37**:2470–4.
9 Pierotti MA. Chromosomal rearrangements in thyroid carcinomas: a recombination or death dilemma. Cancer Lett 2001; **166**:1–7.
10 Gimm O. Thyroid cancer. Mini review. Cancer Letters 2001; **163**:143–56.
11 Hedinger C, Williams E., Sobin L. The WHO histological classification of tumors: a commentary on the second edition. Cancer 1989; **63**:908–11.
12 Klugbauer S, Lengfelder E, Demidchik EP, Rabes HM. High prevalence of *RET* rearrangement in thyroid tumors of children from Belarus after Chernobyl reactor accident. Oncogene 1995; **11**:2459–67.
13 Zhang JS, Nelson M, McIver B, Hay ID, Goellner JR, Grant CS, et al. Differential loss of heterozygosity at 7q31.2 in follicular and papillary thyroid tumors. Oncogene 1998; **17**:789–93.
14 Wiench M, Wloch J, Oczko M, Gubala E, Jarzab B. Rearrangement of the RET gene in papillary thyroid carcinoma. Wiad Lek 2001; **54**:64–71 (in Polish with English abstract.)
15 Bongarzone I, Fugazzola L, Vigneri P, Mariani L, Mondellini P, Pacini F, et al. Age-related activation of the tyrosine kinase receptor protooncogenes *RET* and *NTRK1* in papillary thyroid carcinoma. J Clin Endocrinol Metab 1996; **81**:2006–9.
16 Bongarzone I, Vigneri P, Mariani L, Collini P, Pilotti S, Pierotti MA. *RET/NTRK1* rearrangements in thyroid gland tumors of the papillary carcinoma family: correlation with clinicopathological features. Clin Cancer Res 1998; **4**:223–8.
17 Cohen Y, Xing M, Mambo E, Guo Z, Wu G, Trink B, et al. BRAF mutation in papillary thyroid carcinoma. J Natl Cancer Inst 2003; **95**:625–27.
18 Namba H, Nakashima M, Hayashi T, Hayashida N, Maeda S, Rogounovitch TI, et al. Clinical implications of hot spot BRAF mutation, V599E, in papillar thyroid cancers. J Clin Endocrinol Metab 2003; **88**:4393–7.
19 Schlumberger MJ. Diagnostic follow-up of well-differentiated thyroid carcinoma: historical perspective and current status. J Endocrinol Invest 1999; **22**(11 Suppl):3–7.
20 Mazzaferri EL, Kloos RT. Clinical review 128: Current approaches to primary therapy for papillary and follicular thyroid cancer. J Clin Endocrinol Metab 2000; **86**:1447–63
21 Frisk T, Kytola S, Wallin G, Zedenius J, Larsson C. Low frequency of numerical chromosomal aberrations in follicular thyroid tumors detected by comparative genomic hybridization. Genes Chromosomes Cancer 1999; **25**:349–53.
22 Kjellman P, Lagercrantz S, Hoog A, Wallin G, Larsson C, Zedenius J. Gain of 1q and loss of 9q21.3-q32 are associated with a less favorable prognosis in papillary thyroid carcinoma. Genes Chromosomes Cancer 2001; **32**:43–9.
23 Kitamura Y, Shimizu K, Tanaka S, Ito K, Emi M. Association of allelic loss on 1q, 4p, 7q, 9p, 9q and 16q with postoperative death in papillary carcinoma. Clin Cancer Res 2000; **6**:1819–25.