Clinico-pathological significance of cell-type-specific loss of heterozygosity on chromosome 7q21: analysis of 318 microdissected thyroid lesions

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Abstract

A careful pathological examination often reveals the presence of different lesions at various stages of tumor progression and invasion, even in those thyroid glands presenting with solitary nodules. Each thyroid lesion is composed of many different cell types, reflecting the marked heterogeneity of normal thyroid tissue. Among the different chromosome regions altered in thyroid tumors, 7q21 appears to be specifically involved in malignant tumors, especially of the follicular type. This study was conducted to analyze the loss of heterozygosity (LOH) pattern at 7q21 in pure populations of cells from each single lesion harbored in surgically removed thyroid glands, and to evaluate its clinical significance. One hundred and forty-two thyroid glands were examined, all showing, as a common trait, a goitrous appearance associated with one single lesion in 114 cases and with more than one in the remaining 28 cases. A total number of 318 lesions was analyzed, consisting of 142 goiters (TG), 48 hyperplasias (TH), 80 adenomas (TA) and 48 carcinomas (TC). Five different types of cells were isolated by laser capture microdissection from each lesion. DNA was analyzed by PCR and polyacrylamide gel electrophoresis in search of LOH affecting five microsatellite markers, D7S660, D7S630, D7S492, D7S657, and D7S689. We detected LOH at 7q21 not only in thyroid malignant tumors but also in benign lesions. Allelic loss occurred exclusively in dark nucleus and eosinophilic cytoplasm cells, commonly observed in the follicular type of lesions. In these types of lesions allelic loss frequency increases along with neoplastic transformation (9\% in TG, 41\% in TH, 68\% in TA and 100\% in TC), and is directly correlated with thyroid gland volume as well as with the presence of multiple lesions. The highest LOH rate was observed for D7S492, indicating that the recurrent region of deletion was localized at the corresponding genetic locus at 7q21.2, in the same position where the common fragile site FRA7E was previously mapped. LOH at this locus represents an early event in the development of follicular TC and is associated with intense growth of thyroid glands.

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Introduction

Thyroid tissue is characterized by the rather frequent occurrence of co-existing lesions in the context of the same gland. In this regard, it has been observed that only one third of the apparently solitary thyroid nodules proves to be truly unique at ultrasound examination (Brander et al. 1992). In addition, it has been demonstrated that the number of nodules, detected by both clinical and sonographical evaluation, can be increased after a careful pathological examination in one third of surgically removed thyroid glands (Rosai et al. 1992b). Finally, many lesions are diagnosed in thyroid tissue in which no nodules are visible. In this regard, many thyroid nodules, initially classified as solitary, are observed in the context of multinodular glands, hyperplasias, toxic goiters and thyroiditis (Baloch & LiVolsi 2002). In the same way, small foci of papillary thyroid carcinomas (PTC) are observed in thyroid glands affected by many different alterations, and a frequent association of follicular thyroid carcinomas (FTC) with nodular hyperplasias has also been described (Thompson et al. 2001). All these observations indicate that the cohabitation of many different lesions in the same thyroid gland is common, even though the meaning of this association still remains unexplained. The development of each different type of lesion is sustained by the involvement of cell-specific pathogenetic mechanisms responsible for the various phenotypes observed. Loss of heterozygosity (LOH) analysis has been extensively used to detect the loss of genetic material in neoplastic tissues. LOH at a specific chromosomal locus may be the result of a localized or diffuse genomic instability, and it is useful in the localization of putative tumor suppressor genes (TSG), especially when it occurs at the same specific physical chromosomal location where fragile sites have been mapped (Sutherland et al. 1998). LOH represents a rather frequent molecular alteration in thyroid tumors, mostly detected in follicular neoplastic lesions such as follicular adenomas (FA) and FTC (Fagin 1992, 2002). It has been suggested that LOH at 11q13 may be involved in the early steps of neoplastic transformation of thyroid follicular cells (Matsuo et al. 1991), while LOH at 3p and 7q appears to be associated with later steps, and in particular with the progression of the FA towards FTC (Grebe et al. 1997, Trovato et al. 1999). So far, ten studies have investigated LOH on chromosome 7q in thyroid tumors, and all of them have focused their attention on two specific regions, namely 7q21 and 7q31 (Kubo et al. 1991, Zedenius et al. 1995, Califano et al. 1996, Tung et al. 1997, Segev et al. 1998, Zhang et al. 1998, Trovato et al. 1999, Kitamura et al. 2000, 2001, Oriola et al. 2001). A meta-analysis of the results of these studies showed that LOH at 7q21 is undetectable in normal as well as in hyperplastic thyroid lesions. It is rare in PTC (8%) and in oncocytic adenomas (OA) (10%); it is more frequent in FA (27%) and in FTC (45%), and it is constantly observed in anaplastic thyroid carcinomas (ATC) (100%). According to these data, the chromosomal locus 7q21 appears to be relevant in thyroid tumor development and progression, and this observation was also confirmed by a recent paper even though the analysis was conducted without any preliminary microdissection of the thyroid surgical specimens (Trovato et al. 1999). However, the clinical and pathological significance of the occurrence of LOH at this locus in thyroid tumors still needs to be elucidated. In addition, the precise mapping of the smallest common deleted region is not available yet. The present study was performed to elucidate the significance of this genetic alteration by performing a detailed analysis of pure populations of cells from each single lesion harbored in surgically removed thyroid glands, and to correlate LOH data with clinicopathological features.

Materials and methods

Study group

A total of 142 nodular thyroid glands, surgically removed and collected at the Dept. of Human Pathology, University of Messina was examined. Patients’ ages at surgery ranged from 18 to 80 years, with a mean value of 46.1 years. The maximum diameter of multi-nodular thyroid glands at macroscopic examination ranged from 7 to 19 cm, with a mean size of 9.7 cm. The volume of each lobe was calculated according to the formula: 

\[ V = 0.479 \times (d) \times (w) \times (l), \]

where d is the depth, w the width and l the length of each lobe. Thyroid volume is calculated by adding the volumes of the two lobes without including the volume of the isthmus, and is measured in ml.

The common histological trait of these glands was the nodular goiter, either colloid or parenchymatous. In 114 thyroids, the goiter was associated with another hyperplastic, adenomatous or carcinomatous lesion, while in 28 glands the goiter made up the background for more than one lesion. We have analyzed a total of 318 different lesions encountered in the context of our study group. The size of the lesions ranged between 1 and 8 cm with a mean value of 3.9 cm. According to standard criteria of the Armed Forces Institute of Pathology (Rosai et al. 1992a), and considering the histological growth pattern, thyroid lesions were classified as goiters (142 cases), hyperplasias (48 cases), adenomas (80 cases) and carcinomas (48 cases), including 22 FTC, 21 PTC, 1 oncocytic carcinoma (OC), 1 clear cell carcinoma and 3 ATC variants. Thyroid glands with a histological aspect of Hashimoto’s thyroiditis were not included in this study. Each lesion sample was treated
Thyroid follicular cells were microdissected out of 5-Laser capture microdissection (LCM) with GGN-EC were seen exclusively in 21 PTC. showing CN-EC were recognized in 9 hyperplasias. Cells characterized by DN-CC were observed in 3 seen in 13 hyperplasias, in 18 OA and in 1 carcinoma. Cells showing CN-EC were recognized in 9 hyperplasias. Cells with GGN-EC were seen exclusively in 21 PTC. from each lesion, showing the same cytological features, was captured in each cap. For each slide, at least two separate caps were obtained including one cap with thyroid follicular cells derived from inside the lesion and another cap containing cells retrieved from the surrounding goiter. In the case of multiple lesions in the same gland, cells obtained from each single lesion were processed separately. An example of microdissection of histological slides for various lesions encountered in the same thyroid gland is shown (see Fig. 1). All caps were handled with gloves to reduce contamination and kept separately from the others during all the following procedures. Thyroid lesions that proved positive for LOH were examined and microdissected two additional times, and two independent experiments were performed to confirm the initial results.

**Cell selection and cytological features**

All thyroid lesions were reviewed by the same pathologist and carefully evaluated for a detailed classification and selection of cells to be microdissected and analyzed. The above-mentioned 318 pathological lesions were further subdivided according to the morphological features of nucleus and cytoplasm at light microscopy. Cells have been classified into the following five different types: (1) dark nucleus and eosinophilic cytoplasm (DN-EC), (2) dark nucleus and oncocytic cytoplasm (DN-OC), (3) dark nucleus and clear cytoplasm (DN-CC), (4) clear nucleus and eosinophilic cytoplasm (CN-EC), and (5) ground glass nucleus and eosinophilic cytoplasm (GGN-EC). The DN-EC cells were the major cell type present in all 142 goiters, in 23 hyperplasias, in 57 FA and in 25 carcinomas (22 FTC and 3 ATC). Cells characterized by DN-OC were seen in 13 hyperplasias, in 18 OA and in 1 carcinoma. Cells characterized by DN-CC were observed in 3 hyperplasias, 5 adenomas, and 1 carcinoma. Cells showing CN-EC were recognized in 9 hyperplasias. Cells with GGN-EC were seen exclusively in 21 PTC.

**Laser capture microdissection (LCM)**

Thyroid follicular cells were microdissected out of 5-μm sections, stained with hematoxylin and eosin, under direct microscopic visualization using the Pix Cell II Laser Capture microscope (Arcturus Engineering, Mountain View, CA, USA). Cells were retrieved by means of the activation of a thermoplastic polymer film mounted on a dedicated cap, using a laser beam (7.5, 15 or 30 μm diameter) focused on the selected tissue area. A mean number of 500 cells from each lesion, showing the same cytological features, was captured in each cap. For each slide, at least two separate caps were obtained including one cap with thyroid follicular cells derived from inside the lesion and another cap containing cells retrieved from the surrounding goiter. In the case of multiple lesions in the same gland, cells obtained from each single lesion were processed separately. An example of microdissection of histological slides for various lesions encountered in the same thyroid gland is shown (see Fig. 1). All caps were handled with gloves to reduce contamination and kept separately from the others during all the following procedures. Thyroid lesions that proved positive for LOH were examined and microdissected two additional times, and two independent experiments were performed to confirm the initial results.

**Cell lysis and DNA extraction**

After microdissection each cap was removed by using a dedicated cap holder and was placed in the aperture of a 0.5 ml microfuge tube containing 70 μl freshly prepared lysis buffer (0.04% Proteinase K, 10 mM Tris–HCl, pH 8, 1 mM EDTA, and 1% Tween-20). The tubes were then placed upside down to allow the buffer to cover the cells and were incubated overnight at 42°C. The tubes were then centrifuged for 3 min at 3000 r.p.m. After the centrifugation step, the tubes were heated at 100°C for 10 min to inactivate the Proteinase K. An aliquot of this reaction was used directly as template for PCR amplification.

**PCR-based LOH analysis**

DNA extracted from microdissected cells was amplified by PCR in the presence of specific primers flanking the sequences of five different microsatellite markers. These markers are located on the long arm of chromosome 7 in a region spanning approximately 10.33 cM. The microsatellite markers analyzed were: D7S660, located at 7q21.1, D7S630 and D7S492, located at 7q21.2, and D7S657 and D7S689 located at 7q21.3. They were chosen according to the Radiation Hybrid, Genebridge4, Stanford G3 and Genethon indications and for maximal heterozygosity (Kong et al. 2002). Name, cytogenetic localization, length of the sequence, genetic size recognized by recent master map deCode, and primer information of the chosen microsatellites are listed in Table 1. To increase specificity and yield of the reaction, a second PCR was performed in the presence of one of the two primers used for the first reaction and a nested one, internal to the other primer used for the first PCR. All the oligonucleotide primers used in the study were synthesized and purchased from MWG Biotech (Ebersberg, Germany). PCR amplifications of DNA obtained from microdissected cells were performed by means of the hot start technique in a 20 μl reaction mixture containing 200 μM dNTP mix, 0.6 μM primers, and 1 × PCR reaction buffer (containing 1.25 mM MgCl2). PCR amplifications were run in the Thermal Cycler PCR GeneAmp System 2400 (Perkin Elmer Corp., Norwalk, CT, USA). The reactions consisted of an initial denaturation step at 94°C for 5 min, at the end of which 1 unit of Taq polymerase (Perkin Elmer Corp.) was added, followed by 35–40 cycles. Each cycle was composed of a denaturation step at 94°C for 30 s, an annealing step at the requested temperature for 30 s, and an extension step at 72°C for 30 s. The last cycle was followed by a final 5-min incubation step at 72°C. Annealing temperature was determined empirically after an initial estimate based on primer length and composition.
LOH was detected by polyacrylamide gel electrophoresis (PAGE) analysis. Amplified DNA was mixed with an equal volume of formamide loading dye (95% formamide, 0.05% bromophenol blue and 0.05% xylen cyanol) and loaded onto a gel consisting of 10–12% acrylamide (19:1 acrylamide:bisacrylamide), 0.089 M Tris (pH 8.3), 0.089 M borate, and 0.002 M EDTA. Samples were electrophoresed at 150 volts for 2–4 h and analyzed by silver staining using the Silver Stain kit (Bio-Rad Lab, Hercules, CA, USA). LOH results were visualized and evaluated in an independent and blind fashion and with the assistance of an optical densitometry scanner. For informative cases, the criteria for LOH were complete or near complete loss of one or both alleles of the DNA band, corresponding to the microsatellite sequence. In particular, LOH was defined according to the following formula:

\[
\text{LOH index} = \frac{T_2}{T_1} = \frac{G_2}{G_1},
\]

where \(T\) was the tumor sample, \(G\) was the matching goiter sample, and 1 and 2 were the intensities of smaller and larger alleles respectively. LOH was considered positive in the presence of values of LOH index \(< 0.6\) or \(> 1.7\). The efficiency in LOH detection was greatly improved by the concomitant analysis of at least two different cellular populations derived from the same thyroid gland. Reproducibility of each loss was confirmed by at least two independent experiments. Constitutional homozygosity was considered as non-informative. Experiments in which allelic imbalances were detected were repeated an additional two times and the results were reproducible in all the tests performed. The fractional allele loss (FAL) index, expressed as the presence of LOH in all chromosomal loci investigated, was calculated for each individual sample as follows: FAL index = (total number of chromosomal loci with LOH)/(total number of informative loci examined).

### Statistical analysis

The differences in size and volume of glands in both LOH-positive and -negative samples were examined by the Student’s \(t\)-test. The pattern of LOH for each microsatellite marker as well as the FAL index were correlated with the volume of the glands, the number of lesions and the cell type.

### Results

In our study, the informative rate of each microsatellite marker is never lower than 92%. In particular, among 318 lesions the 5 microsatellite markers analyzed are informative in a total of 292 cases for D7S689, 295 for D7S660, 305 for D7S630 and D7S657, and 306 for D7S492. Informative rate and frequency of positive cases for each microsatellite are summarized in Table 2. Two illustrative cases of multi-nodular thyroid glands with the LOH pattern for each microsatellite analyzed are shown in Fig. 1.

### Table 1

Characteristics of the microsatellite markers used in this study.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Primer name</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>D7S660</td>
<td>AFM277xd5</td>
<td>Forward: TATGCAAGGCACCTGTGGAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Forward-Internal: TAGGCCAACACTGGGGAGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: AGCTTGATAGTGGAATCATTT</td>
</tr>
<tr>
<td>D7S657</td>
<td>AFM263yd9</td>
<td>Forward: TCTCTCTTTGGACCTATCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Forward-Internal: GTCAAGTGAGATGGACATTCC</td>
</tr>
</tbody>
</table>

### Table 2

Informative rate and frequency of LOH-positive cases for each microsatellite marker.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Informative lesions</th>
<th>LOH-positive lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(% total)</td>
<td>(n)</td>
</tr>
<tr>
<td>D7S660</td>
<td>295 (93)</td>
<td>19</td>
</tr>
<tr>
<td>D7S630</td>
<td>305 (96)</td>
<td>11</td>
</tr>
<tr>
<td>D7S492</td>
<td>306 (96)</td>
<td>49</td>
</tr>
<tr>
<td>D7S689</td>
<td>292 (92)</td>
<td>28</td>
</tr>
<tr>
<td>D7S657</td>
<td>305 (96)</td>
<td>25</td>
</tr>
</tbody>
</table>
Allelic loss at 7q21 is more frequent in enlarged thyroid glands containing multiple lesions

Only 121 (85%) out of the total number of 142 thyroid glands are informative, while the remaining 21 glands (15%) are non-informative (NI) in at least one microsatellite. The results of LOH analysis are referred to the total number of informative cases in each group. LOH in at least one microsatellite marker is detected in 52 (43%) out of 121 multi-nodular glands. A positive correlation is found between the maximum diameter of the gland and the presence of LOH, so that only multi-nodular glands with a maximum diameter equal to or larger than 10 cm show LOH at 7q21. The volume of LOH-positive multi-nodular glands ranges from 62.76 to 1807.488 ml (486.383 ± 380.329, mean ± S.D.). The volume of the thyroid glands negative at LOH analysis is lower, ranging from 32.426 to 903.744 ml (213.601 ± 165.013, mean ± S.D.). The difference between the mean values of the volumes of LOH-positive and LOH-negative glands is statistically significant (P < 0.000003). When thyroid glands are grouped according to their volume, a linear correlation is observed between thyroid gland volume and the presence of LOH at 7q21 (r = 0.9954) (Fig. 2). The mean LOH frequency is significantly different between the 99 multi-nodular glands harboring two lesions (37.4%) and the 22 glands containing more than two lesions (68.2%). The mean value of the FAL index, calculated in each LOH-positive lesion, does not differ in these two groups. Thyroid glands with more than two lesions show a mean FAL index of 0.314 compared with 0.36 in glands with only two lesions. Considering the total number of 52 LOH-positive multi-nodular thyroid glands, a simultaneous occurrence of LOH affecting at least two microsatellites is observed in 36 cases. Simultaneous LOH is more frequent (73.3%) in multi-nodular glands harboring more than two lesions compared with glands with only two lesions (67.6%). In 12 multi-nodular glands (8 with more than two lesions and 4 with two lesions) a simultaneous LOH for the same microsatellite marker in two different lesions is detected. Simultaneous LOH affects more frequently the D7S492 polymorphic microsatellite marker (66.7%). Two illustrative cases of LOH in thyroid glands with two different lesions as well as glands with more than two lesions are shown in Fig. 1.

Allelic loss at 7q21 is detected only in DN-EC thyroid follicular cells

All the different kinds of thyroid lesions composed of DN-EC cells are possibly affected by LOH at 7q21.
The correlation between this type of cell and LOH at this locus is so strong that we do not observe allelic loss in any lesion composed of the other types of cells (Fig. 3A). In fact, all the other types of cells, namely DN-OC, DN-CC, CN-EC and GGN-EC cells, obtained from many different types of lesions, always retain heterozygosity at 7q21. However, the frequency of LOH in DN-EC cells is significantly correlated with the various stages of neoplastic transformation and progression of the thyroid gland (Fig. 3B).

**Frequency of allelic loss at 7q21 increases along with neoplastic tranformation**

LOH at 7q21 is present in all the various types of lesions analyzed, but its frequency is different, and it progressively increases along with the neoplastic transformation (Fig. 3). In our study, 12 out of 121 informative goiters (10%), 7 out of 39 informative hyperplasias (18%), 34 out of 71 informative adenomas (48%), and 20 out of 39 informative carcinomas (51%) show LOH in this chromosomal region. The results of LOH for each microsatellite in the different thyroid lesions are shown in Fig. 4 where only LOH-positive samples are reported. The mean FAL value is similar in goiters and hyperplastic lesions (0.28 vs 0.25 respectively) and significantly increases along with neoplastic transformation, with a mean value ranging from 0.32 for FA up to 0.6 in the case of FTC and ATC. All 12 goiters and 7 hyperplasias that are positive at LOH analysis are associated with a coexisting neoplastic lesions (FA, FTC or ATC) located in another part of the same gland. The size of LOH-positive lesions ranges from 1 to 6 cm with a mean value of 3.9 cm. No difference between the mean diameter value of the lesions is observed in LOH-positive compared with -negative groups. The 76 colloid and 66 parenchymatous nodules composed exclusively of DN-EC cells and with overall informative rates of 85% and 86% respectively, show LOH in a total of 12 cases out of 121 (10%) informative nodules (Fig. 3A). LOH for at least one marker is more frequent in parenchymatous (16%) than in colloid (4.6%) goiter, while the co-existence of LOH at two markers in the same nodule is a rare event in goiters (Fig. 3A, B). Interestingly, LOH for the two markers telomeric to D7S492 is never seen in the parenchymatous nodules. The highest LOH rate is observed for D7S492 (6.6%). All hyperplastic nodules are composed of homogeneous populations of follicular cells. In half of the cases the cells are characterized by a DN-EC appearance. LOH is observed only in hyperplastic lesions composed of this type of cell (Fig. 3A, B). In particular, LOH is detected in 7 out of 17 informative cases. D7S492 is the marker most frequently involved, with a total of 4 cases out of 17 informative ones (23.5%). Interestingly, as already observed in goiters in hyperplastic nodules, LOH is always found in multi-nodular glands in which a coexisting LOH-positive FA and/or FTC is also present. LOH at 7q21 is detected at a variable frequency among the 71 informative cases out of the 80 TA, which encompass 3 different histological variants. No LOH can be detected in the 17 informative cases out of the 18 oncocytic adenomas, which consist of DN-OC follicular...
cells, and in all 4 informative cases out of the 5 clear cell adenomas, composed of DN-CC follicular cells. Among the 57 follicular adenomas, all composed of the same type of DN-EC cells, LOH is detected in 34 cases out of 50 informative cases (68%) (Fig. 3). However, frequency is different in the various histological types of follicular adenomas (Fig. 3C). All the 8 cases of solid follicular adenomas are positive for LOH in at least one marker, a picture similar to that observed in FTC. The number of cases showing simultaneous occurrence of LOH at different markers of the 7q21 region in follicular adenomas is increased compared with goitrous and hyperplastic lesions. In fact, the FAL index is increased in LOH-positive samples (Fig. 4). In thyroid adenomas (TA), D7S492 is the single marker most frequently affected by LOH (44% of the cases) as already observed in the other types of lesions. Loss of genetic material at 7q21 is found exclusively in two types of carcinomas, namely FTC and ATC. A total of 18 out of 22 informative FTC, and 2 out of 2 ATC are positive in at least one marker. The common trait of these two types of carcinomas relies on the fact that they are composed of DN-EC cells. In this regard, it is interesting to note that two FTC, characterized by a different cell population (clear cells and oncocytic cells), are negative to LOH analysis. No LOH is detected in any of the PTC examined. All of the 18 informative FTC out of 22 cases show LOH for at least one marker, and again D7S492 is the marker most frequently affected by allelic loss (80%). Simultaneous allelic loss for more than two markers is rather frequent with a FAL index even greater than that observed in adenomas. In one widely invasive tumor (case #15 in Fig. 4D; see also Fig. 1A) the simultaneous allelic loss for all five markers is observed. No specific combination of LOH simultaneously affecting two markers is observed in any carcinomatous lesions.

The smallest common deleted region is mapped at 7q21.2

Considering all thyroid lesions, without any distinction based on histological or cytological criteria, the microsatellite marker showing the highest LOH incidence is D7S492 (16.0%) (Fig. 5A). The LOH rate is reduced in

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**Figure 3** LOH frequency at 7q21 in follicular thyroid lesions, divided according to histology. (A) A summary of LOH results for each histotype, subdivided into the five different cytotypes, is shown. (B) LOH rates are shown in each different histological lesion examined, regardless of any cell selection (w/o cell selection; open bars) or in lesions exclusively composed of DN-EC cells (solid bars). (C) LOH rates in the various types of adenomas are shown. Thyroid lesions are considered positive when at least one microsatellite marker at 7q21 is affected by LOH.
**Figure 4** Detailed LOH analysis of five different microsatellite markers at 7q21 in four different types of thyroid lesions. Only thyroid lesions showing LOH in at least one microsatellite marker are shown. For each case the retention of heterozygosity (empty box), the loss of heterozygosity (half-filled box), the non-informative condition (NI) as well as the FAL index are reported. LOH-positive samples are grouped according to their histology in (A) goiter, (B) hyperplasia, (C) adenoma and (D) carcinoma. In (D) case #15, showing LOH in all microsatellites analyzed, and case #10, showing LOH in two different lesions of the same gland, are highlighted in boxes. PAGE showing LOH results of these two cases are shown in Fig. 1.
the two microsatellite markers D7S689 (9.5%) and D7S657 (8.1%), located at the telomeric border of the 7q21 region analyzed (Fig. 5A). An almost twofold reduction is observed comparing the LOH rate at D7S492 with these two markers, separated only by 2.5 cM and mapping in a different chromosomal locus (7q21.3). This finding suggests the possibility that the distal (telomeric) boundary of this 'hot spot' locus is located at the region included between D7S492 and D7S689. A lower LOH frequency is found at the two microsatellite markers located in a centromeric position with respect to D7S492. In particular, the most centromeric marker, i.e. D7S660, shows an LOH frequency of 6.4%. Remarkably, the lowest LOH rate (3.6%) is observed at D7S630, the nearest centromeric marker with respect to D7S492. A 4.4-fold decrease in LOH rate is observed by comparing the results at the two contiguous markers D7S492 and D7S630, separated by 1.04 cM and mapping in the same 7q21.2 chromosomal locus. This result indicates that the proximal (centromeric) boundary of the critical region at 7q21 is flanked by the microsatellite marker D7S630.

When the results of our LOH analysis are evaluated considering the different histology and cell composition of the lesions, the relevance of the previously identified chromosomal locus becomes even more evident. Our approach, in fact, enables us to identify the cell population specifically affected by LOH at this region in the various thyroid lesions. LOH frequency, reported for each microsatellite analyzed and subdivided into the traditional histological criteria, namely goiter, hyperplasia, adenoma and carcinoma, is shown in Fig. 5B. However, only lesions composed of DN-EC cells show LOH at 7q21, and results are expressed considering only these types of cells. Again D7S492, located at 7q21.2, is the microsatellite marker most frequently affected in all different types of lesions. LOH frequency for this marker is 80% in carcinomas, 44% in adenomas, 10% in hyperplasias and 6.6% in goiters. The other centromeric and telomeric markers show progressively lower LOH rates.

**Discussion**

The development of thyroid cancer is thought to be the result of a progressive accumulation of genetic events which are responsible for the various stages of thyroid proliferation from goiter and hyperplasia to neoplastic lesions such as adenoma and well or poorly differentiated carcinoma. According to the current model of carcinogenic progression through multisteps, the specific subset of germinal and/or somatic genetic alterations, accumulated by the thyroid follicular cell, is responsible for the acquisition of the various phenotypes observed at histological evaluation in the different thyroid tumors. The identification of these genetic alterations may be useful in early recognition of those cellular clones already committed to cancer transformation. Previous LOH studies have demonstrated that the two major variants of thyroid cancer, namely the PTC and the FTC, follow two separate tumorigenic pathways. Allelic loss appears to be a rather frequent event in FTC, while PTC seems not to be associated with this kind of genetic damage, at least not in the loci examined so far (Ward et al. 1998). LOH analysis has been used in many solid tumors to localize the presence of putative TSGs, whose loss may lead to alterations in the activity of critical genes involved in the regulation of cell proliferation and apoptosis (Thiagalingam et al. 2002). The occurrence of LOH is usually considered one of the earliest events in cell transformation and tumor development. In addition, a close correlation between cancer and the genetic alterations such as LOH, chromosomal breakage, gaps and rearrangements has
been demonstrated at specific genetic loci, named fragile sites (Yunis & Soreng 1984). To date, many of these chromosomal loci have been identified on the long arm of chromosome 7 (Huang et al. 1999, Zenklusen et al. 2000, Han et al. 2003), but no data are available regarding their involvement in thyroid tumor development. In the present study, we have focused our attention on a locus located at 7q21, and we have analyzed the presence of allelic loss in a large population of thyroid lesions. The LOH pattern of five different microsatellite markers, included in a region spanning approximately 10.33 cM inside 7q21, has been evaluated. Our results indicate that the polymorphic microsatellite marker D7S492, located at 7q21.2 and corresponding to the genetic size of 100.97 cM, maps inside the shortest most common deleted region. This region may be considered as a 'hot spot' for thyroid tumors, and it probably contains a yet to be identified putative TSG. In addition, this hot spot region co-localizes with a previously identified common fragile site, named FRA7E. The expression of FRA7E has been characterized in breast cancer, where it appears to be associated with later stages of development (Dhillon et al. 2003), but no association has been reported so far between FRA7E and thyroid cancer. We believe that characterization and analysis of this fragile site in thyroid tumors will lead to the identification of those genes that are likely to play a key role in thyroid cancer development.

In a previous study, we analyzed LOH at 7q on thyroid glands in which only one solitary nodule was analyzed and the results were compared with those obtained with the normal matching tissue (Trovato et al. 1999). In the present study, a more detailed analysis of this chromosomal region was conducted, including some microsatellite markers already evaluated in the previous report such as D7S492. Moreover, thyroid glands examined in the present study were characterized by the presence of at least two different lesions, one of which was invariably represented by the goiter. In our study, surgically removed thyroid tissue showed a rather enlarged appearance with a median volume higher than normal and with some cases exceptionally large (8 cases with a volume greater than 1000 ml). This finding may be related to the relative iodine deficiency of the geographical area (Messina and Sicily) from which the patients came. The mean size of LOH glands (11.6 cm) led us to suspect that this type of genetic alteration arises in glands subjected to intense growth stimuli. It is possible that the progressive development of thyroid nodules, caused by unknown factors, creates a growth background favorable to the occurrence of genetic mutations such as ras mutations and the deregulation of the activators of the MAP kinase cascade. A significant increase in LOH rate for all markers analyzed is observed in thyroid glands with more than two lesions (68.2%) as compared with those with only two lesions (37.4%). This study confirms our previous observations concerning LOH at 7q21 in glands containing FA, FTC and ATC (Trovato et al. 1999). However, our careful analysis of every single lesion present in each thyroid gland allowed us to detect LOH also in goiters and hyperplastic lesions that were present in the thyroid tissue surrounding other solitary nodular lesions. Interestingly, we detect LOH in goiters and/or hyperplasias only in those glands showing LOH for other coexisting neoplastic lesions. The presence of LOH in goiters and hyperplasias led us to suppose that LOH at 7q21 may represent a very early event in thyroid tumorigenesis. In fact, not all LOH-positive cells are believed to form a specific neoplastic lesion, but our data indicate that most neoplastic lesions and all carcinomatous lesions composed of DN-EC cells will develop and progress in those cells affected by allelic loss at 7q21. On the other hand, it is also possible that previous studies have failed to detect any LOH at 7q21 in the matching normal tissues of thyroid follicular neoplastic lesions because the analysis was performed on cells without any microdissection procedure. For this reason, the possible presence of LOH in a specific type of cell could have been undetected because of contamination with other types of cells characterized by the retention of heterozygosity at 7q21.

The loss of genetic material may occur as a result of chromosomal instability and other defects in the DNA replication (Hay et al. 1984, Oller et al. 1989). However, it is also possible that this genetic alteration does not necessarily represent a primary event in tumorigenesis but may be a late secondary phenomenon, as frequently observed in growing tissue (Glick et al. 1999). According to this interpretation, genetic mutations promoting growth occur more frequently in cells with a high intrinsic replication rate (Saaavedra et al. 2000). In this regard, it has been hypothesized that nodular lesions arise from a small fraction of cells characterized by having an innately shorter than average cell cycle that allows them to generate progenies tending to overgrow their neighboring cells, evolving through time into the generation of nodules (Studer & Derwahl 1995). The consensus of clinical and ultrasound studies indicate that the thyroid might become nodular in the course of a lifetime even in the absence of a chronic stimulus from thyrotropin (Mortensen et al. 1955, Studer 1988). However, the appearance of LOH in middle age (45 years) excludes this alteration as a mutation of old age and is in agreement with the rare occurrence of a spontaneous mutation acquired during the natural life span (LiVolsi 1990).

In this study we have analyzed a large number of thyroid neoplastic and non-neoplastic lesions, directly at
the single cell level recruited by LCM. This approach enabled us to study the LOH pattern on pure populations of follicular cells, subdivided according to their nuclear and cytoplasmic features. We find a strict correlation between cytological appearance and allelic loss at 7q21. In fact, LOH is exclusively detected in lesions that, regardless of the architectural and prognostic differences, show a similar cytological appearance consisting of DN-EC cells. All carcinomas, most adenomas, less than half of hyperplasias and few goiters with these kinds of cells show LOH. The rest of the cellular types (DN-OC, DN-CC, CN-EC and GGN-EC), independently from the lesions in which they are detected, retain heterozygosity at this locus. These results support the true importance of a cellular distinction in the evaluation of the molecular alterations affecting thyroid tumors for a clear assessment of the phenotype/genotype correlation.

Up to 80% of DN-EC lesions are diagnosed by fine-needle aspiration (FNA) as follicular neoplasms, without any possible distinction between benign or malignant lesions (Fonseca & Sabrinho-Simoes 1995; Baloch et al. 2001). On the basis of our observations, it could be hypothesized that LOH analysis at 7q21 may be applied to cytological specimens obtained by FNA for a better characterization of these thyroid lesions. Further experiments are needed to prove the usefulness and accuracy of the absence of allelic loss at this locus as a marker of benignity in thyroid follicular lesions.

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