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Microsatellite instability in thyroid tumours and tumour-like lesions

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Summary Fifty-one thyroid tumours and tumour-like lesions were analysed for instability at ten dinucleotide microsatellite loci and at two coding mononucleotide repeats within the transforming growth factor β (TGF-β) type II receptor (*TβRII*) and insulin-like growth factor II (IGF-II) receptor (*IGFIIR*) genes respectively. Microsatellite instability (MI) was detected in 11 out of 51 cases (21.5%), including six (11.7%) with MI at one or two loci and five (9.8%) with MI at three or more loci (RER$^+$ phenotype). No mutations in the *TβRII* and *IGFIIR* repeats were observed. The overall frequency of MI did not significantly vary in relation to age, gender, benign versus malignant status and tumour size. However, widespread MI was significantly more frequent in follicular adenomas and carcinomas than in papillary and Hürthle cell tumours: three out of nine tumours of follicular type (33.3%) resulted in replication error positive (RER$^+$), versus 1 out of 29 papillary carcinomas (3.4%, $P = 0.01$), and zero out of eight Hürthle cell neoplasms. Regional lymph node metastases were present in five MI-negative primary cancers and resulted in MI-positive in two cases.

Keywords: thyroid tumour: microsatellite instability; RER$^+$ phenotype; lymph node metastasis; transforming growth factor β type II receptor gene; insulin-like growth factor II receptor gene

Thyroid tumours are the most frequent neoplasms of the endocrine system. The diverse spectrum of tumours and tumour-like lesions originating from thyroid follicular cells provides an useful model for the study of alternative pathways of epithelial tumorigenesis at the molecular level (Hedigar et al, 1988; Farid et al, 1994). Mutations activating the *RAS* proto-oncogenes represent an early step in the pathway of follicular thyroid tumorigenesis (Lemoine et al, 1989), whereas translocations involving the *RET* and *TRK* oncogenes, with consequent expression of aberrant fusion proteins as well as amplification and overexpression of the *MET* oncogene, have been implicated in papillary thyroid carcinogenesis (Bongarzone et al, 1989; Di Renzo et al, 1992). A role for tumour-suppressor genes has also been evidenced: anaplastic carcinomas have a high frequency of point mutations of the *p53* gene, and deletions of chromosome 3p22 have been found in follicular carcinomas (Fagin et al, 1993; Hermann et al, 1996). Very recently, involvement of the *PTEN*/*MMAC1* tumour-suppressor gene has been linked to the 10q22–23 loss of heterozygosity (LOH) observed in thyroid tumours of follicular histotype associated with Cowden's syndrome (Liaw et al, 1997).

Microsatellites are simple and abundantly distributed polymorphic sequence repeats that represent 'hotspots' for base-pairing errors during DNA replication (Karran, 1996). A very high incidence of tumour-associated microsatellite mutations, consisting of contractions or expansions in the number of repetitive unit sequences, was initially reported in tumours of patients with hereditary non-polyposis colorectal cancer (HNPCC) and in a subset of sporadic colorectal cancers (Peltomäki et al, 1993; Thibodeau et al, 1993). HNPCC families present also extra-colorectal malignancies, notably including endometrial, gastric and ovarian carcinomas, but not thyroid tumours (Lynch et al, 1997). In HNPCC-associated tumours, widespread microsatellite instability (MI) is correlated with double-hit inactivation of one of at least four different genes, *hMLH1*, *hMSH2*, *hPMS1* and *hPMS2*, encoding mismatch recognition and repair (MMR) proteins (Hemminki et al, 1994; Liu et al, 1994; Nicolaides et al, 1994). Various degrees of MI have also been reported in colorectal and extracolorectal cancer cell lines (Boyer et al, 1995), and in several forms of sporadic extracolorectal tumours (Eshleman and Markowitz, 1995; Karran, 1996). At present, the genetic base(s) of MI in sporadic cancers are poorly defined because MMR gene mutations have been identified only in a minority of the cases (Konishi et al, 1996).

A limited number of studies have examined the occurrence and the role of MI in thyroid tumours. The small series of nine cases reported by Vermiglio et al (1995) did not exhibit any MI, although a limited incidence of MI was observed in the larger series of tumours and tumour-like lesions analysed by Soares et al (1997). To explore the role of MI in thyroid carcinogenesis, we analysed the status of ten dinucleotide repeat microsatellite loci in a panel of 51 tumours and tumour-like lesions. We also tested the occurrence of mutations at two coding mononucleotide repeats within the transforming growth factor β (TGF-β) type II (*TβRII*) and insulin-like growth factor II (IGF-II) (*IGFIIR*) receptor genes

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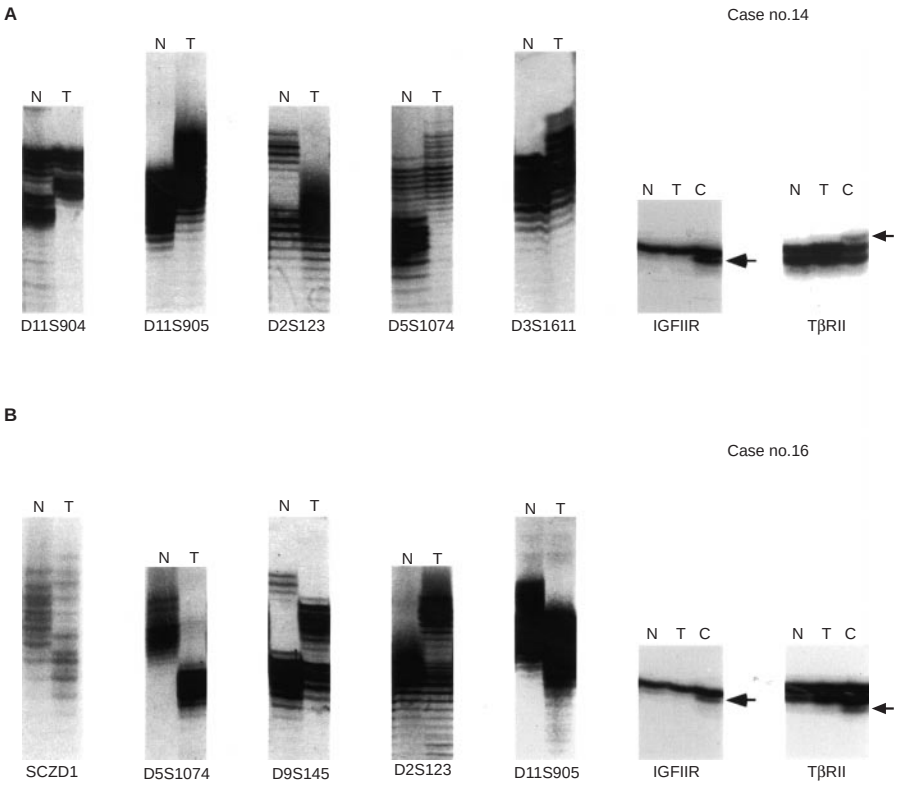

Figure 1 Microsatellite alterations and status of the *TβRII* poly-A_{10} and *IGFIIR* poly-G_8 mononucleotide repeats in paired normal thyroid (N) and tumour (T) DNAs from papillary carcinoma case no. 14 (**A**) and anaplastic carcinoma case no. 16 (**B**). **A** and **B**, corresponding to each microsatellite marker, depict a paired genotyping that presented novel tumour-associated alleles originating from compressions or expansions of repeats. Compressions of repeats are exemplified at D2S123 in case no. 14 and, in case no. 16, at SCZD1 and D9S145, in which instability involves both alleles, and at D11S905 and D5S1074, in which instability is associated to loss of one allele or co-migration of both alleles. Expansions of repeats are exemplified in case no. 14 at D11S904, D11S905, D5S1074 and D3S1611, and, in case no. 16, at D2S123. The banding pattern of the *TβRII* poly-A_{10} and *IGFIIR* poly-G_8 sequences is normal in both thyroid tumours (T), as compared with the respective normal thyroid controls (N) and to a mutated gastric cancer control (C)

respectively. Mutations at these repeats result in the inactivation of the *TβRII* and/or *IGFIIR* gene products and represent frequent events in MI-positive gastrointestinal malignancies (Markowitz et al, 1995; Myeroff et al, 1995; Parson et al, 1995; Souza et al, 1996; Ottini et al, 1998). In the present study, widespread MI tended to be associated with lesions of the follicular type (LiVolsi, 1990). There were no mutations at the mononucleotide repeats within the *TβRII* and *IGFIIR* genes. The analysis of lymph node metastases revealed that MI may occur during thyroid tumour progression.

MATERIALS AND METHODS

Pathological samples and clinical data

Forty-eight cases of sporadic thyroid neoplasms from unrelated patients were diagnosed, classified and staged according to the UICC criteria (Hermanek and Sobin, 1987) by one of the authors (FN). Forty-one fresh paired normal thyroid and tumour samples (cases no. 1–41) were obtained from partial or total thyroidectomy specimens surgically removed during the last 3 years at the University 'La Sapienza', Rome, Italy. To minimize the possibility of an inadvertent inclusion of normal tissue in the tumour samples and vice versa, samples were selected from microscopically controlled areas. The samples were promptly frozen and stored at –80°C until DNA extraction. Seven additional cases of sporadic thyroid tumours were retrieved from surgical pathology files (cases AR1–AR7). Moreover, three papillary thyroid carcinomas occurring in distinct familial adenomatous polyposis (FAP) patients from a single FAP kindred (Civitelli et al, 1996) were also analysed. Altogether, the 51 cases analysed comprised three follicular nodules, considered as putative follicular preneoplastic lesions (LiVolsi, 1990), seven follicular adenomas, including two microfollicular and one trabecular variants, six Hürthle cell adenomas, two follicular carcinomas, two Hürthle cell carcinomas, two anaplastic carcinomas and 29 papillary carcinomas, two of which contained areas with follicular differentiation. Regional lymph node metastases were analysed in five cases, including a Hürthle cell carcinoma (AR7) with 14 distinct metastases and four papillary carcinomas: case AR2, with three metastases; cases AR5 and no. 11, with two metastases; and case no. 29, with a single metastasis.

DNA extraction

Extraction of normal tissue and tumour DNA from fresh samples was performed using standard methods (Lazzereschi et al, 1997). For formalin-fixed, paraffin-embedded samples, sections, 5–10 μm in thickness, were collected on glass slides and stained with haematoxylin. After pathological review, areas of normal tissue and tumour were marked and microdissected; where the areas of interest could not be clearly separated from surrounding tissue, selective ultraviolet radiation fractionation (Shibata et al, 1996)

Table 1 Clinicopathological characteristics of the primary and metastatic thyroid tumours and tumour-like lesions with evidence of microsatellite instability (MI)

Code no.	Gender/ age	Type	TNM	Clinical stage	Size (cm)	Origin of sample[a]	MI
37	M/49[b]	Follicular nodule	NA[c]	NA	1.0	T	D2S123
2	M/24	Follicular adenoma	NA	NA	3.5	T	635/636, D2S174, D5S1074, D11S904
9	W/37[b]	Follicular adenoma	NA	NA	3.0	T	635/636, D9S145, SCZD1
AR6	W/35	Follicular carcinoma	T4NxMx	I	3.5	T	D2S123, D2S174, D3S1611, D5S1074, D9S145, SCZD1, D11S905
AR7	M/74	Hürthle cell carcinoma	T1N2Mx	II	ND[d]	T	–
					< 1	M1	D11S905
					2	M2	D3S1611
					1.8	M3	D9S145
					0.7	M4	D9S145
					1.2	M5	D2S174, D17S250
1	W/26	Papillary carcinoma	T4N0Mx	III	3.0	T	635/636
14	M/32	Papillary carcinoma	T1N0Mx	I	< 1	T	D2S123, D3S1611, D5S1074, D11S904, D11S905
25	W/32	Papillary carcinoma	T1NxMx	I	0.8	T	635/636
27	M/70	Papillary carcinoma	T2NxMx	II	1.7	T	D11S904
AR1	W/ND	Papillary carcinoma	T2N1Mx	ND	1.2	T	D9S145, D11S905
AR4	W/32	Papillary carcinoma	T2NxMx	I	1.2	T	D11S904
AR5	M/40	Papillary carcinoma	T2N1Mx	I	ND	T	–
					1.2	M1	D9S145
16	W/72	Anaplastic carcinoma	T4NxMx	IV	ND	T	D2S123, D5S1074, D9S145, SCZD1, D11S905

[a]T, primary thyroid tumour or tumour-like lesion; M1–5, different lymph node metastases. [b]M, man; W, woman. [c]NA, not applicable. [d]ND, not determined.

was performed. Genomic DNA from microdissected paraffin-embedded samples was extracted as previously described (Ottini et al, 1995, 1997). One microlitre (1 µl) of DNA was used to set up 10-ml polymerase chain reactions (PCR).

Microsatellite analysis

For microsatellite typings, a two-step protocol was used consisting of a non-radioactive external PCR, followed by a radioactive internal PCR (nested PCR) and utilizing a $1:10^4$ dilution of primary PCR products as template. The following ten microsatellite dinucleotide repeats were analysed: *D2S123*, *D2S174*, *D3S1611*, *SCZD1*, *D5S1074*, *D9S145*, *D11S904*, *D11S905*, *635–636* and *D17S250*. Primers, PCR mixture, cycling conditions, electrophoretic separation and autoradiography were as previously described (Ottini et al, 1995). The 73-bp region encompassing the poly-A_{10} repeat of the *TβRII* gene at nucleotides 709–718 and the 110-bp region including the poly-G_8 tract of the *IGFIIR* gene at nucleotides 4089–4096 were amplified by using previously reported primer sets (Parson et al, 1995; Souza et al, 1996). The presence of mutations was tested using mutated MI-positive gastric cancers as positive controls (Ottini et al, 1998). PCR mixture, cycling conditions, electrophoretic separation and autoradiography were as previously used for MI analysis (Ottini et al, 1995).

Statistical analyses for the correlation of microsatellite alterations with clinicopathological characteristics were performed using the χ^2 test. The *P*-values were computed after combining the fraction of cases with MI at three or more loci, versus the fraction of cases with no evidence of MI and with MI limited to one or two loci. Statistical significance was considered at $P \leq 0.05$.

RESULTS

A series of thyroid tumours and tumour-like lesions, paired to their associated normal tissues, was tested for MI at ten dinucleotide repeat microsatellite loci. The same samples were also analysed for the presence of coding repeat mutations at a poly-A_{10} tract within the *TβRII* gene (nucleotides 709–718) and at a poly-G_8 tract within the *IGFIIR* gene (nucleotides 4030–4140). All MI-positive typings were confirmed in duplicate or triplicate experiments, using samples from independent DNA extractions. Allelic imbalances were not considered in the present study.

Out of the 51 primary thyroid lesions analysed, 11 (21.5%) were positive for MI, as identified by the presence of tumour-associated electrophoretic mobility shifts of microsatellite alleles at one or more loci. MI-positive samples included one out of three follicular nodules, two out of seven follicular adenomas, 6 out of 29 papillary carcinomas, one out of two follicular carcinomas and one out of two anaplastic carcinomas. Interestingly, the three FAP-associated papillary carcinomas were MI negative.

No abnormally migrating bands indicative of mutations in the *TβRII* poly-A_{10} and *IGFIIR* poly-G_8 sequences were detected in the 51 cases examined, including the five tumours with MI at three or more loci. In spite of the absence of mutations at the *TβRII* poly-A_{10} repeat, it is noteworthy that 7 out of 11 MI-positive cases reported in this study, including three out of five cases with MI at three or more loci, showed reduced *TβRII* expression at the RNA and/or protein level, as demonstrated in a previous work (Lazzereschi et al, 1997). Figure 1 shows paired normal tissue and tumour microsatellite genotypings from two cases, papillary carcinoma no. 14 and anaplastic carcinoma no. 16, that manifested MI at five out of ten loci but had a normal banding pattern of the *TβRII* poly-A_{10} and *IGFIIR* poly-G_8 sequences compared with mutated gastric cancer controls. Notably, anaplastic carcinoma no. 16 previously demonstrated reduced *TβRII* expression (Lazzereschi et al, 1997).

The MI status of regional lymph node metastases could be examined after microdissection of metastatic foci and compared with that of the corresponding primary carcinoma in five cases. As

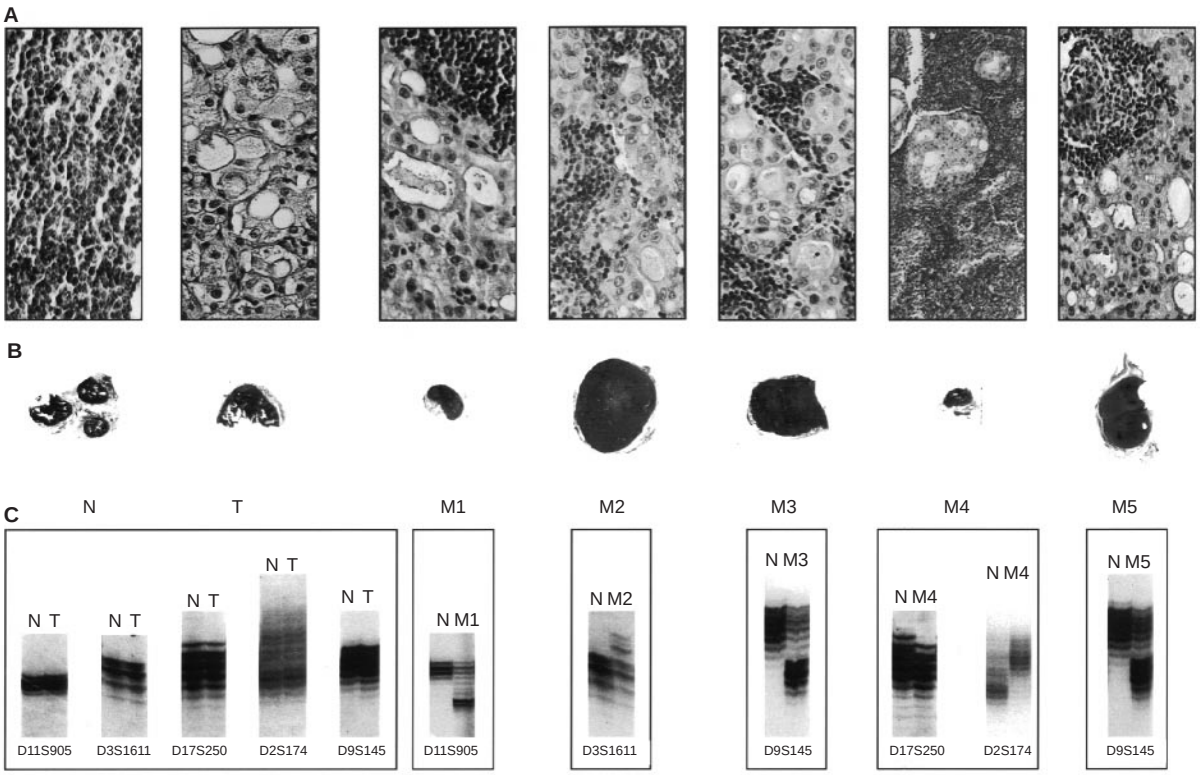

Figure 2 Microsatellite profiles of primary tumour (T), matched normal tissue (N) and individual regional lymph node metastases (M1, M2, M3, M4, M5) from Hürthle cell carcinoma AR7. **A** and **B** respectively show the histology and low magnification views of the samples that were individually analysed after microdissection, **C** shows the banding patterns of the microsatellite markers that demonstrated MI in lymph node metastases. The primary tumour does not show any microsatellite alteration, as compared with normal thyroid tissue. The five metastases depicted here exhibit heterogeneous MI patterns at D11S905 (M1), D3S1611 (M2), D9S145 (M3 and M5, both sharing the same pattern), D2S714 and D17S250 (M4)

reported in Table 1, metastases from two MI-negative primary cancers, a papillary and a Hürthle cell carcinoma, were MI positive. In particular, the unique lymph node metastasis of papillary carcinoma AR5 was MI positive at D9S145, whereas 5 out of 14 metastases from Hürthle cell carcinoma AR7 demonstrated heterogeneous MI patterns, indicative of clonal metastatic diversity. Figure 2 shows relevant microsatellite profiles from case AR7: metastases designated M1, M2, M3 and M5 demonstrated MI at one locus, corresponding to D11S905 (M1), D3S1611 (M2) and D9S145 (M3 and M5 both sharing the same MI pattern), metastasis M4 was MI positive at loci D2S174 and D17S250.

It is presently accepted that only widespread MI, defined by the presence of instability at 30% or more of the loci analysed and referred to as replication error-positive (RER^+) phenotype, is associated with a distinct clinicopathological profile (Eshleman and Markowitz, 1996; Karran, 1996). In the present study, 5 out of 51 (9.8%) cases of primary thyroid tumours and tumour-like lesions, comprising two follicular adenomas, one follicular carcinoma, one anaplastic carcinoma and one papillary carcinoma, manifested MI at three or more loci. The remaining six MI-positive cases (11.7%), including one solitary follicular nodule and five papillary carcinomas, manifested MI limited to one or two loci (Table 1). No preference for instability at any specific microsatellite locus was observed: in fact, seven out of the ten loci analysed were altered at the same frequency. Table 2 reports the fractions of primary tumours and tumour-like lesions with low (one or two loci) and high (three or more loci) MI according to a series of clinicopathological features. MI was not exclusive to malignancies because it was detected in a solitary follicular nodule and in two follicular adenomas. In particular, MI at three or more loci was observed in 2 out of 16 benign lesions (12.5%) and in 3 out of 35 carcinomas (8.6%). High-level MI (three or more loci) tended to be associated with lesions of follicular type: in fact, three out of nine (33.3%) tumours with follicular growth pattern, including two out of seven follicular adenomas and one out of two follicular carcinomas, manifested MI at three or more loci, versus 1 out of 29 (3.4%) papillary carcinomas ($P = 0.01$) and zero out of eight Hürthle cell tumours which included six adenomas and two carcinomas (Tables 1 and 2). Moreover, MI at three or more loci was detected in one out of two anaplastic carcinomas, usually considered poorly differentiated lesions of follicular lineage (Werner and Ingbar, 1991). With regard to clinical stage, 5 out of 29 carcinomas of stages I or II manifested MI, including three cases with MI at one or two loci and two cases with MI at three or more loci, compared with two out of five tumours of stages III or IV, including a papillary carcinoma with MI at one locus and an anaplastic carcinoma with MI at three or more loci. Differences in MI positivity in relation to patients' gender and age or tumour size were unremarkable.

DISCUSSION

In spite of their common origin from follicular epithelium, the great variety of histotypes and the differences in biological behaviour of thyroid tumours and tumour-like lesions suggest

Table 2 Fractions of cases with microsatellite instability (MI) at one or two and three or more loci according to selected clinicopathological features in the 51 primary thyroid tumours and tumour-like lesions examined

		Cases with MI	
	Men	**1 or 2 loci**	**≥ 3 loci**
Gender	Men	2/16	2/16
	Women	4/35	3/35
Age (years)	< 45	3/33	4/33
	≥ 45	2/13	1/13
	Unknown	1/5	0/5
Type of lesion	Benign	1/16	2/16
	Malignant	5/35	3/35
Tumour size	≤ 1 cm	2/12	1/12
	≤ 4 cm	4/32	3/32
	> 4 cm	0/4	0/4
	Unknown	0/3	1/3
Histological pattern	Nodule[a]	1/3	0/3
	Follicular[b]	0/9	3/9 *P* 0.01
	Hürthle cell[b]	0/8	0/8
	Papillary	5/29	1/29
	Anaplastic	0/2	1/2
Clinical stage	I	2/25	2/25
	II	1/4	0/4
	III	1/2	0/2
	IV	0/3	1/3
	Unknown	1/1	0/1

[a]Follicular nodule. [b]Adenomas and carcinomas combined.

different pathways of thyroid tumorigenesis (Hedigar et al, 1988; Werner and Ingbar, 1991). This is consistent with the existence of alternative genetic pathways for tumour development and progression, confirmed at the molecular level by distinct gene alterations in the papillary and in the follicular histotypes (Farid et al, 1994; Wynford-Thomas, 1997).

Microsatellite instability is an indicator of decreased replication fidelity of genomic DNA, which, when at multiple loci, is believed to be associated with genetic defects that promote tumorigenesis (Eshleman and Markowitz, 1996; Karran, 1996). Few works have analysed the MI status of thyroid tumours and tumour-like lesions. A preliminary analysis based on a small series of cases, tested at a limited number of microsatellite markers, failed to evidence any MI (Vermiglio et al, 1995), whereas a second report was suggestive of a limited role of MI in both benign and malignant tumours and even in non-neoplastic lesions such as goitres (Soares et al, 1997). To further investigate the frequency and role of MI in thyroid carcinogenesis, a panel of cases encompassing all the most common tumours and tumour-like lesions has been examined for MI at ten dinucleotide repeats. Overall, MI at one or more loci was found in 21.5% of thyroid tumours, a frequency comparable to those reported for tumours of other origins (Thibodeau et al, 1993; Eshleman and Markowitz, 1995; Karran, 1996). Widespread MI, i.e. at 30% or more of the loci (RER^+ phenotype), was found in 9.8% of the thyroid tumours, corresponding to 45% of all MI-positive cases.

It is noteworthy that cases with MI at multiple loci were significantly more frequent in follicular type tumours and tumour-like lesions relative to papillary and Hürthle cell-type tumours. Interestingly, three FAP-associated thyroid papillary carcinomas, occurring in carriers of a germline mutation at codon 1061 of the adenomatous polyposis coli (APC) gene (Civitelli et al, 1996), resulted MI negative. The occurrence of widespread MI in benign thyroid lesions and its low frequency in papillary carcinomas were in agreement with the results of Soares et al (1997). There were no associations between MI status and patients' age and gender or tumour size.

The comparison of the microsatellite profiles of lymph node metastases with those of primary thyroid tumours and matched normal tissues revealed that metastases with heterogeneous MI-positive patterns may originate from primary cancers with no evidence of MI. As in gastrointestinal cancer and melanoma (Shibata et al, 1996; Richetta et al, 1997), this indicates either dominant metastatic expansion of tumour cell clones underrepresented in the primary lesion, or diversification of microsatellite alleles and clonal evolution during population doublings after metastasization. The occurrence of MI in metastases from MI-negative primary tumours and the presence of widespread MI in two out of five high-stage primary carcinomas raises the possibility that genomic instability at sequence repeats might be associated with tumour progression in some thyroid cancers. Further investigations of larger series of cases are required to verify this hypothesis.

TGF-β is the most important growth-inhibitory factor in thyroid cells (Colletta et al, 1989), and resistance or escape from TGF-β control could contribute to the neoplastic transformation of follicular epithelium (Blaydes and Wynford-Thomas, 1996; Brattain et al, 1996; Coppa et al, 1997). *TβRII* mutations could have been responsible for the loss of TGF-β responsiveness and for the reduced levels of *TβRII* mRNA and protein that we previously found in thyroid malignancies, including cases investigated in this study (Lazzereschi et al, 1997). Nevertheless, none of the presently analysed 51 primary tumours and tumour-like lesions, including the 11 MI-positive cases, revealed mutations in the *TβRII* and *IGFIIR* coding repeats analysed, which represent frequent sites of inactivating mutations in MI-positive gastrointestinal cancer (Markowitz et al, 1995; Myeroff et al, 1995; Parson et al, 1995; Souza et al, 1996; Ottini et al, 1998). This suggests that in thyroid tumorigenesis loss or down-regulation of *TβRII* expression occurs via MI-independent mechanisms.

In conclusion, our results suggest that MI may influence the biology of follicular thyroid tumours and tumour-like lesions and the molecular mechanisms of progression of thyroid cancer in general. This warrants further research on the genetic base(s) and functional consequences of genomic instability in thyroid carcinogenesis.

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