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Original Paper

Benign and Malignant Thyroid Lesions Show Instability at Microsatellite Loci

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Forty-six benign and malignant tumours and tumour-like lesions of the thyroid were analysed for microsatellite instability (MI) at eight loci, mapping to four different chromosomes, 7 lesions (15%) displayed MI at one or more loci, including 2/13 nodular goitres, 2/15 follicular adenomas, 2/12 papillary carcinomas and 1/4 follicular carcinomas. Two benign and one malignant lesion among the seven unstable cases exhibited this phenotype at three or more loci. We found no mutations in the mismatch repair gene, *hMSH2*, in the seven affected cases, after screening all the exons by CDGE mutation analysis. At variance with the data on record, these results indicate that, despite being relatively infrequent, MI does occur not only in thyroid carcinomas but also in benign lesions (goitres and follicular adenomas of the thyroid). © **1997 Elsevier Science Ltd. All rights reserved.**

Key words: thyroid, nodular goitre, follicular adenoma, papillary carcinoma, microsatellite instability

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INTRODUCTION

MICROSATELLITE INSTABILITY (MI) is a genetic phenomenon manifested by shifts in the electrophoretic mobility of microsatellite repeat fragments. Mutations (generally small deletions or insertions) occurring during replication remain unrepaired and result in novel alleles. MI was first reported in sporadic and hereditary non-polyposis colorectal carcinoma (HNPCC) [1–3] and thereafter associated with inactivating mutations in the human DNA mismatch repair genes *hMSH2*, *hMLH1*, *hPMS1* and *hPMS2* [4–8]. It was further shown that cells harbouring such mutations were mismatch repair deficient and hypermutable [9, 10].

The prevalence of MI varies greatly in HNPCC-related sporadic carcinomas (colon, stomach, pancreas, ovary and endometrium) [1–3, 11–17], as well as in other types of tumours such as breast, lung and prostate carcinomas [12, 14, 18–22]. There also appears to be large variation in the prevalence of MI in precancerous lesions of different organs [23–26], thus leading to divergent assumptions on the timing of occurrence of this type of genomic instability in tumorigenesis [24, 26, 27].

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A limited number of thyroid carcinomas have been analysed for MI. Nine tumours reported by Vermiglio and associates did not exhibit MI, whereas thyroid carcinomas from 3 patients with multiple primary cancers did show this phenotype [29]. In addition, MI has not been reported, to the best of our knowledge, in any other benign or malignant endocrine tumour. In order to evaluate further this phenomenon in the thyroid, 46 lesions were analysed for instability at eight microsatellite loci.

MATERIALS AND METHODS

Tumour samples (n = 46)

In each case, fresh tissue samples from thyroid nodules were immediately snap frozen in liquid nitrogen and stored at −70°C until use. When available, DNA from normal parenchyma adjacent to the lesions ($n = 18$) was used as a constitutional control, otherwise, we used corresponding peripheral blood DNA ($n = 28$). High-molecular-weight DNA was isolated using standard methods [30]. Classification of the lesions was made according to Hedinger and associates [31] and Rosai and associates [32] into nodular goitre ($n = 13$), follicular adenoma ($n = 15$), papillary carcinoma ($n = 12$), follicular carcinoma ($n = 4$) and poorly differentiated carcinoma ($n = 2$). There was no

Table 1. *Thyroid lesions with microsatellite instability*

Cases	Lesion	Microsatellite markers							Number*
		D1S196	D1S103	D5S346	D2S102	CTLA4	D5S82	D16S265	
1	NG	–	–	–	–	–	MI	–	1/8
2	NG	MI	–	–	MI	–	MI	–	3/8
3	FA	MI	MI	MI	MI	–	–	–	4/8
4	FA	–	–	–	–	–	MI	–	1/8
5	PC	–	–	–	–	–	MI	–	1/8
6	PC	MI	MI	MI	ND	MI	MI	MI	6/7
7	FC	–	–	–	–	–	MI	–	1/8

*Loci with microsatellite instability/loci analysed.
NG, nodular goitre; FA, follicular adenoma; PC, papillary carcinoma; FC, follicular carcinoma; MI, microsatellite instability; ND, not determined; –, normal homozygote or heterozygote and unchanged in tumours.

history of familial cancer syndromes or multiple primary cancers in any of the 46 patients.

Microsatellite marker analysis

Paired samples of tumour and normal control DNA were amplified by PCR (polymerase chain reaction) at the following eight loci containing dinucleotide repeat sequences (chromosomal location in parentheses): D1S103 (1q), D1S196 (1q), D2S102 (2q), CTLA4 (2q), D5S346 (5q), D5S82 (5q), D16S265 (16q) and D16S301 (16q). PCR was performed in 25 μl volumes of a mixture containing 10 mM Tris-HCl (pH 8.0), 50 mM of KCl, 1.5 mM $MgCl_2$, 100 μM of each deoxynucleotide triphosphate except dCTP, 10 μM of dCTP, 1 μCi of [α-^{32}P]dCTP, 0.4 μM of each primer, 0.75 units of *Taq* DNA polymerase, and 30–50 ng of genomic DNA. Thirty cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.75 min were performed, with an initial denaturation step of 94°C for 5 min and a final extension step of 72°C for 10 min using a Perkin-Elmer 9600 GeneAmp PCR System. PCR reaction products were diluted 1:1 with a loading buffer (98% formamide, 0.1% xylene cyanol FF, 0.1% bromophenol blue, and 10 mM EDTA [pH 8.0]) and then denatured for 5 min at 95°C. Subsequently, 2 μl of this solution were electrophoresed in 6% polyacrylamide gels containing 7 M urea and 32.5% formamide for 2–3 h at 60 W. After electrophoresis, gels were fixed in 10% acetic acid, washed, dried and exposed to X-ray field for 12–24 h. All scorings were made independently by two observers. According to Thibodeau and associates [3], genetic alterations involving the gain or loss of two or more repeat units are classified as Type I and alterations of one repeat unit as Type II. Eight loci were analysed per case and, for the sake of simplicity, a tumour was considered positive (MI) when at least one locus displayed a different mobility band. This option does not mean we belittle the existence of aetiopathogenic differences between cases with single-repeat slippage at just one locus and cases with slippage at several loci. Such differences are taken into account in the comparison of the different groups (see Results and Table 1). All RER$^+$ loci were confirmed by a new PCR and electrophoretic run.

hMSH2 *mutation analysis*

All 16 exons of *hMSH2* were screened for mutations using constant denaturant gel electrophoresis (CDGE) [33]. The separation principle of CDGE is based on the unique melting behaviour of each DNA fragment. The detailed procedure for such analysis of *hMSH2* has been recently published [34]. Briefly, theoretical melting profiles were calculated for all exons, followed by primer design using MacMelt 1.0 (MedProbe A/S) and OLIGO (National Biosciences) computer programs. Template DNAs were amplified by PCR, and the products separated by CDGE (D-GENE system, BioRad). If a mutation is indicated by CDGE, sequencing of the affected exon will identify the exact position of the changed base(s). By using the conditions described by Børresen and associates [34], it was estimated that approximately 65% of all mutations will be detected within the analysed fragments.

RESULTS

The results are summarised in Tables 1 and 2. Seven of the 46 thyroid lesions displayed MI at one or more loci (Table 1). Among these, there were two nodular goitres and two follicular adenomas, thus leading to an overall MI prevalence of 14.3% in the 28 benign lesions (Table 2). Two papillary carcinomas and a follicular carcinoma displayed MI, thus leading to a MI prevalence of 16.7% in the 18 carcinomas.

Three out of the seven lesions displayed MI at three or more loci (a nodular goitre (Figure 1)), a follicular adenoma and a papillary carcinoma (Figure 2), whereas the remaining four lesions showed MI at a single locus (Table 1).

Cases 1, 4 and 7, with MI at a single locus, displayed Type II alterations, whereas case 5, also with one altered locus, displayed a Type I alteration (see Materials and Methods). Cases 2 and 3, with three and four unstable loci respectively, displayed both Type I and Type II alterations. Case 6 displayed Type I alterations at all affected loci.

Both papillary carcinomas with MI displayed discrete to moderate lymphoid infiltration which could also be observed in most of the papillary carcinomas without MI. Comparison of the histological features of other lesions with MI with those of their non-unstable counterparts did not disclose any notable difference.

Among the seven microsatellite unstable tumours, no aberrantly migrating bands were observed by CDGE of the 16 *hMSH2* exons, and thereby no mutations were indicated.

Table 2. *Frequency of microsatellite instability in thyroid lesions*

Diagnosis (*n*)	MI ⩾ 1 locus	MI ⩾ 3 loci
Nodular goitre (13)	2 (15.4%)	1 (7.6%)
Follicular adenoma (15)	2 (13.3%)	1 (6.6%)
Papillary carcinoma (12)	2 (16.7%)	1 (8.3%)
Follicular carcinoma (4)	1 (25.0%)	0 (0%)
Poorly differentiated carcinoma (2)	0 (0%)	0 (0%)

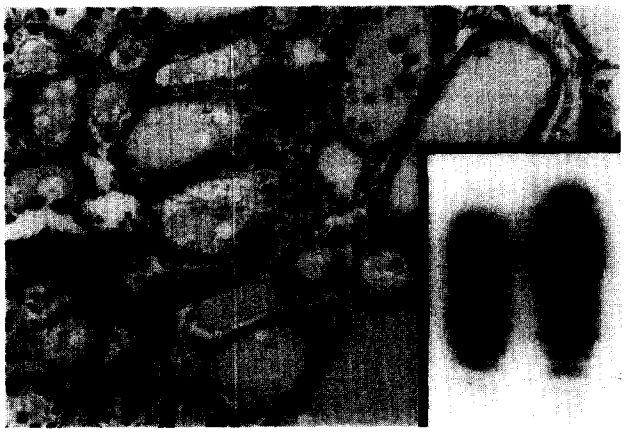

Figure 1. Case 2: This nodular goitre displayed MI at three loci. Haematoxylin and eosin. Inset: PCR amplification of locus D5S82 showing Type II alteration (left lane, normal thyroid; right lane, goitre). Magnification ×125.

Loss of heterozygosity was found at D1S103 and D1S196 in a follicular adenoma and a follicular carcinoma, respectively.

DISCUSSION

Two of the three microsatellite unstable carcinomas were only affected at one out of eight loci, implying that these two cases could have been scored as negative if fewer loci had been analysed. Therefore, the absence of MI in thyroid carcinomas reported by Vermiglio and associates [28] probably reflects the limited number of analysed markers and the small size of their series.

The three thyroid carcinomas with MI reported by Horii and associates [29] were observed in a series of patients with multiple primary cancers, and are thereby not directly comparable to those of our series. The tumours of the present study were from patients with no history of familial cancer nor evidence of other primary cancers. Taking our results together with those of Vermiglio and associates [28], one may conclude that the prevalence of MI in thyroid carcinomas, outside the context of multiple primary cancers, lies below the values reported for HNPCC-related sporadic carcinomas [1–3, 11, 12, 14, 16, 17].

The discrepant prevalences of MI in thyroid carcinomas, prostatic carcinomas [18, 19], and breast carcinomas [20, 21], rules out the possibility of establishing a close relationship between hormone-dependent tumours in general and prevalence of MI.

Figure 2. Case 6: This papillary carcinoma displayed MI at six loci. Haematoxylin and eosin. Inset: PCR amplification of locus CTLA4 showing Type I alteration (left lane, normal thyroid; right lane, papillary carcinoma). Magnification ×125.

MI has been detected in lesions of ulcerative colitis, regardless of the presence of dysplasia [35]; Brentnall and associates proposed that MI in this setting could result from saturation of the DNA repair mechanisms due to the stress of chronic inflammation. It is unlikely that such a mechanism is operative in the unstable tumours of the present series, namely in the goitre cases, since none of them displayed signs of inflammation. The occurrence of MI at one or few loci may also represent a random event [36]. This possibility cannot be definitely ruled out especially in those cases exhibiting single locus alterations. The same does not hold true, however, for thyroid lesions with instability at multiple loci—as many as six out of seven unstable loci were observed in one of our cases. We do not know if the use of tri- and tetranucleotide markers would lead to a higher number of altered loci in thyroid tumours, since it is known that different patterns of alterations of short tandem repeats are found in different types of tumours [20, 37–39].

The finding of a subset of benign thyroid tumours and tumour-like lesions displaying MI at several loci suggests that MI can be an early event in thyroid tumorigenesis, thus fitting well with Loeb's hypothesis [40] and with the observation of MI in some precancerous dysplasias of the stomach [24], adenomas and/or dysplasias of the colon [25–27], and Barrett's metaplasia of the oesophagus [23]. Moreover, the occurrence of MI at several loci in a nodular goitre fits with the concept that at least some of the nodules of the so-called adenomatous goitres appear to be neoplastic rather than hyperplastic [41].

We found a similar prevalence of MI in benign lesions and carcinomas of the thyroid, both when the comparison concerns the cases with affected loci, regardless of their number, and when it is restricted to cases with several affected loci, but a larger series is necessary to confirm this finding, which contrasts with the usually much lower frequency of MI in benign lesions than in their malignant counterparts in several organs [23, 24, 26]. In colorectal tumours, the majority of unstable adenomas are affected at a single locus, whereas more than half of the unstable carcinomas show novel alleles at several loci [13, 26], suggesting that these changes accumulate during tumour growth.

We cannot rule out the putative involvement of DNA polymerases at least in the cases presenting alterations at a single locus, since it has been reported that mutations of DNA polymerases may interfere with poly(GT) tract stability [42], whereas the occurrence of MI at multiple loci suggests the existence of abnormalities of DNA mismatch repair genes [42, 43]. Six of seven tumours showed novel alleles at the D5S82 locus, suggesting that this locus is highly unstable.

The mismatch repair gene *hMSH2* is apparently not mutated in any of the microsatellite unstable cases of our series. This may reflect the fact that thyroid carcinoma is not part of HNPCC; the size of the series is, however, too small to allow any definitive conclusion on this issue and it is estimated that only approximately 65% of all mutations of *hMSH2* will be detected within the analysed fragments under the conditions we have used [34]. Additional tumours need to be collected for MI in order to study further *hMSH2* and to search for mutations in other DNA mismatch repair genes to see if we can find in thyroid pathology, as in other systems, a relationship between MI

and abnormalities of at least one mismatch repair gene [34, 44].

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