Acta Derm Venereol 2001; 81: 242–245

INVESTIGATIVE REPORT

Microsatellite Instability in Malignant Melanomas

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Microsatellite instability (MSI) is caused by deficient DNA mismatch repair, and results in a "mutator" phenotype. Recent studies have produced contradictory results about the frequency and significance of MSI in malignant melanomas. In this study, we therefore determined the time of onset and relative frequency of MSI during the progression of melanocytic tumours, including benign melanocytic naevi. We examined 7 different microsatellite loci in 9 melanocytic naevi, 25 primary malignant melanomas and 8 melanoma metastases. None of the melanocytic naevi showed MSI. In contrast, moderate frequency of MSI in 1/12 (8%) was detected in thin melanomas of < 0.75 mm vertical thickness and in 1/8 (12%) of those with a thickness > 0.75 mm and < 1.5 mm. The rate of MSI was increased in tumours thicker than 1.5 mm (2/5) and in melanoma metastases, with over 25% (2/8) of the lesions investigated. We conclude that MSI occurs in a considerable subset of malignant melanomas and that there is a pattern consistent with increasing frequency of MSI with progression of melanocytic tumours. ***Key words: malignant melanoma; microsatellite instability.***

(Accepted July 2, 2001.)

Acta Derm Venereol 2001; 81: 242–245.

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Replication errors due to mismatch repair deficiency have been recognized as a fundamental principle of carcinogenesis (1). Mismatch repair deficiency leads to genetic instability of both non-coding DNA, most notably microsatellite repeats, and coding DNA (2). Subsequent mutations in tumour-suppressor genes and proto-oncogenes can result in increased proliferation and thereby counteract apoptosis (3). The occurrence of microsatellite instability (MSI) secondary to mismatch repair deficiency is very well documented as an important event in the pathogenesis of hereditary non-polyposis colorectal carcinoma (4–9). Whereas data about intestinal tumours are abundant and definite conclusions can be drawn, the evaluation of skin cancer is still controversial. In 1994, Honchel et al. (10) reported MSI in a subset of skin lesions in patients with Muir-Torre syndrome which was characterized by the presence of at least one sebaceous tumour and at least one visceral malignancy. In addition to the findings of Honchel, Langenbach et al. (11) have shown that MSI plays an insignificant part in the induction of sporadic keratoacanthomas in contrast to keratoacanthomas associated with Muir-Torre syndrome. A larger study on MSI in sporadic skin cancer by Quinn et al. (12) revealed a rate of 5% of MSI in malignant melanomas. In contrast, in the studies of Peris et al. (13), Haley et al. (14), Tomlinson et al. (15) and Talwalkar et al. (16), a MSI phenotype was found in up to 25% of the malignant melanomas analysed. Rübben et al. (17) observed MSI phenotypes in metastatic subclones of one single case of melanoma. In addition, cases of MSI in benign melanocytic naevi and Spitz naevi are reported (14, 18). The variation in the absolute frequency of MSI in the aforementioned studies could be a result of different sampling strategies of cases and microsatellite loci. The current consensus is that a considerable subset of malignant melanomas shows the MSI phenomenon. However, the question remains whether the appearance of MSI secondary to mismatch repair deficiency is correlated with various phenotypes of melanomas and whether the frequency of MSI events increases during tumour progression. In this study, we therefore determined the time of onset and relative frequency of MSI during the progression of melanocytic tumours, including benign melanocytic naevi. We found that none of the melanocytic naevi showed MSI. In contrast, moderate frequency of MSI was detected in thin melanomas. The rate of MSI was increased in tumours thicker than 1.5 mm and melanoma metastases, with about 25% of all lesions investigated. Our data confirm previous estimates of almost a quarter of all sporadic malignant melanomas being MSI in advanced stages. The presence of a MSI status appears to be linked to the malignant phenotype of melanocytic tumours. As the rate of MSI cases is increased in advanced stages, our results support a progression-dependent accumulation of somatic mutations of microsatellites and counter MSI as an early causative event in melanomas.

MATERIALS AND METHODS

Tissue samples and DNA extraction

Formalin-fixed paraffin-embedded as well as cryo-preserved tissue specimens of 9 benign melanocytic naevi, 25 sporadic primary cutaneous melanomas and 8 metastases of malignant melanomas were collected at the Departments of Dermatology and Pathology, University of Regensburg, Germany. Diagnoses were confirmed by two independent histopathologists. Clark levels of primary malignant melanomas ranged from II to IV. Metastases included 7 cutaneous metastases and one lymph node metastasis. A 10-μm section was taken from each tissue block and stained with H&E in order to localize the tumour area within the sample. Subsequently, multiple sections were dissected under microscopic control. First, the paraffin-embedded sections were deparaffinized and rehydrated. Contaminating parts of the epidermis, dermis and subcutaneous fat were removed and collected into cell lysis buffer. The remaining tumour fragments were collected separately into cell lysis buffer. DNAs from the tumour region and from the corresponding normal tissue were then prepared by a standard phenol extraction method. To eliminate RNA entirely, extracted DNA samples were treated with RNase (Boehringer Mannheim, Germany).

Microsatellite polymerase chain reaction (PCR)

For assessment of MSI, a set of 7 microsatellite loci was analysed. PCRs were performed using the following primers: *D5S346 sense:*

 ISSN 0001-5555

ACT CAC TCT AGT GAT AAA TCG, *antisense:* AGC AGA TAA GAC AGT ATT ACT AGT T; *D10S89 sense:* AAC ACT AGT GAC ATT ATT TTC, *antisense:* AGC TAG GCC TGA AGG CTT CT; *D17S520 sense:* AGG GAT ACT ATT CAG CCC GAG GTG, *antisense:* ACT GCC ACT CCT TGC CCC ATT C; *D10S197 sense:* ACC ACT GCA CTT CAG GTG AC, *antisense:* GTG ATA CTG TCC TCA GGT CTC C; *D11S904 sense:* ATG ACA AGC AAT CCT TGA GC, *antisense:* CTG TGT TAT ATC CCT AAA GTG GTG A; *D13S175 sense:* TAT TGG ATA CTT GAA TCT GCT G, *antisense:* TGC ATC ACC TCA CAT AGG TTA; *D9S171 sense:* AGC TAA GTG AAC CTC ATC TCT GTC T, *antisense:* ACC CTA GCA CTG ATG GTA TAG TCT.

For each pair of tumours and normal DNA, separate reactions with the above-mentioned set of primers were performed. For PCR 50 ng of each template DNA were added to a mixture of 1 × Taq buffer with 1.5 mM $MgCl_2$ (Boehringer, Mannheim, Germany), 200 mM dNTPs, 0.5 µM of each primer, and 5U/Rx Taq-Polymerase (Boehringer, Mannheim, Germany) in a volume of 50 µl. PCR conditions were 1 min at 95°C, 1 min at 55–60°C (depending on the primers) and 1 min 30 s at 72°C. The PCR reaction products were then electrophoresed on 6% denaturing polyacrylamide gels and stained in accordance with a previously developed silver-staining protocol (19). MSI was assumed if at least one out of 7 of the tested loci showed somatic variations in microsatellite length. Loss of heterozygosity (LOH) was defined if in an informative microsatellite locus (showing both alleles) one allele was lost in the tumour sample and a shift of one allele to the length of the other allele could be excluded because of the equal intensity of the band.

RESULTS

The analysis of 9 benign melanocytic naevi, 24 sporadic primary cutaneous melanomas, and 8 metastases of malignant melanomas matched with normal tissue samples of each patient revealed MSI exclusively in malignant lesions (Fig. 1). MSI was not observed in benign melanocytic naevi. The primary malignant melanomas presented MSI in 4/25 cases, i.e. we observed an average of 16% MSI in malignant melanomas (Table I). MSI was observed most frequently at the D10S89 and the D17S520 locus. In one tumour with Breslow thickness of 6.2 mm, MSI was seen at 2 loci; D10S89 and D5S346, respectively. Metastases showed the highest incidence of MSI (2/8). MSI was found in 1/12 of the tumours of < 0.75 mm vertical thickness and in 3/13 of those > 0.75 mm thick.

In addition to MSI, we observed LOH at chromosome 10 for both of the microsatellite markers tested. D10S89 showed LOH in 1/8 (12%) and D10S197 in 1/7 (14%) of the informative cases.

DISCUSSION

Two lines of evidence can be drawn from our data: 1) Our data confirm previous estimates of almost a quarter of all sporadic malignant melanomas being MSI in advanced stages. These findings require a more detailed evaluation of a possible prognostic consequence of a mismatched repair (MMR) deficit, since MSI status may have consequences for the response to therapy (3). In addition, there is preliminary evidence that aggressive chemotherapy makes it even more likely that MSI subclones, obviously resistant to chemotherapy, are selected (20, 21). Cell lines that were selected for resistance to chemotherapeutic agents such as etoposide and fotemustine exhibited a 2.0- to 2.5-fold increase in MSI rates, suggesting a link between MMR deficiency and drug resistance. This implies speculation that detection of a mismatch repair deficiency status in melanoma metastases may be of prognostic importance indicating an elevated risk for non-response to chemotherapeutic regimes. 2) The part that MSI/mismatch repair deficiency plays in the causal pathogenesis of sporadic melanocytic tumours is probably different from the intestinal system as far as hereditary non-polyposis colorectal carcinomas are concerned and different from the conditions in Muir-Torre syndrome as well. In contrast to those conditions, in melanomas we have no evidence of an early onset of mismatch repair deficiency, for instance already in benign melanocytic naevi, comparable to actinic keratoses in Muir-Torre syndrome. Instead, the presence of a MSI status appears to be linked to the malignant phenotype and is more frequent for advanced stages, i.e. thick melanomas and metastases. Our results therefore support a progression-dependent accumulation of somatic mutations of microsatellites rather than MSI as an early causative event in melanomas.

Accordingly, the molecular genetics of melanoma MMR deficiency are different from the findings in hereditary non-polyposis colorectal carcinoma. Genes that are frequently mutated in colon cancer seem to retain integrity in malignant melanomas, even when a mutator phenotype can be observed. Variable levels of the mismatch repair gene hMSH2 were found to be expressed in melanoma cell lines, primary malignant melanomas, and melanoma metastases (22). Furthermore, the control-function of the p53 tumour suppressor protein on the hMSH2 expression turned out to be functional in malignant melanomas. Consequently, no mutations of hMSH2 or hMLH1 could be observed in MSI

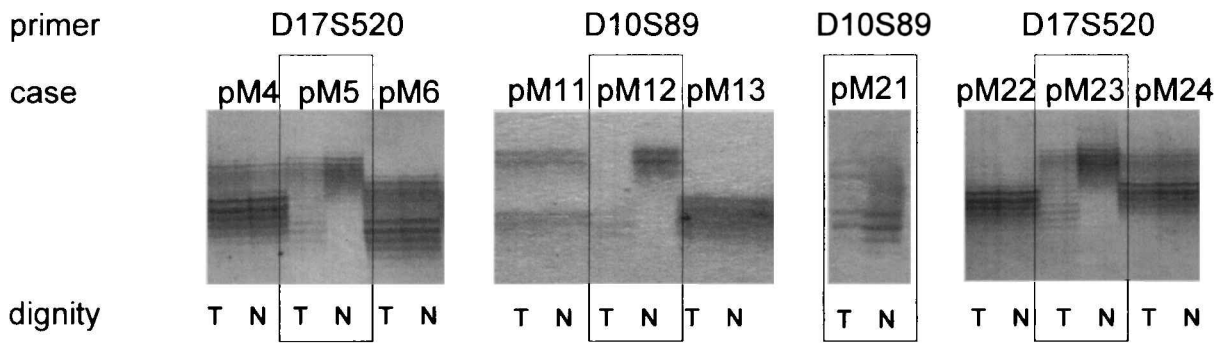

Fig. 1. Microsatellite instabilities of the D17S520 locus (pM5, pM23) and of the D10S89 locus (pM12, pM21) of 4 malignant melanomas are shown. Normal tissue of the same patients serves as control.

Table I. *Overview of all specimens of primary malignant melanomas (pM) investigated and the occurrence of microsatellite instability (MSI)*

Primary melanomas	Sex	Age (years)	Type	Clark's level	Breslow's index (mm)	Growth phase	Localization	MSI
pM1	m	52	SSM	III	0.8	H	trunk	–
pM2	m	40	SSM	IV	1.4	V	back	–
pM3	m	68	SSM	II	0.5	H	back	–
pM4	m	36	SSM	III	0.6	H	back	–
pM5	f	47	SSM	III	0.8	H	lower leg	D17S520
pM6	m	65	SSM	IV	1.8	V	trunk	–
pM7	m	75	SSM	IV	1.4	V	head	–
pM8	f	74	SSM	II	0.3	H	face	–
pM9	m	62	SSM	III	0.6	H	back	–
pM10	f	71	SSM	IV	0.9	V	shoulder	–
pM11	m	79	SSM	III	0.4	H	neck	–
pM12	f	61	SSM	IV	2.0	V	trunk	D10S89
pM13	m	43	SSM	III	0.8	H	trunk	–
pM14	m	73	SSM	III	0.6	H	back	–
pM15	m	54	SSM	III	1.1	H	shoulder	–
pM16	f	78	SSM	IV	1.9	V	back	–
pM17	m	58	SSM	II	0.5	H	forearm	–
pM18	m	47	SSM	III	0.6	H	trunk	–
pM19	f	42	SSM	II	0.2	H	forearm	–
pM20	f	66	SSM	II	0.4	H	forearm	–
pM21	f	57	NMM	IV	6.2	V	upper arm	D5S346 D10S89
pM22	m	68	ALM	II	0.2	H	praeputium	–
pM23	f	52	LMM	II	0.2	H	face	D17S520
pM24	f	35	SSM	IV	1.4	V	trunk	–
pM25	f	35	SSM	III	1.8	V	upper arm	–

H = horizontal, V = vertical.
SSM = superficial spreading malignant melanoma; NMM = nodular malignant melanoma; LMM = lentigo maligna melanoma, Clark's level, Breslow's index, vertical or horizontal growth and localization are summarized. ALM = acral lentiginous melanoma.

melanomas (15). How the statistically significant increase of MSI from a spontaneous rate of around 10^{-4} per locus and cell division (23) to the level observed in this study can be explained remains to be elucidated.

At the moment, there is no consensus about the significance of an MSI phenotype in any of the skin tumours nor about the rate at which this can be expected. In investigating a possible reason for MMR deficiency in patients with and without Torre-Muir syndrome, Quinn et al. (12) found MSI in less than 5% of all tumours studied. They concluded that the MSI phenomenon was uncommon in patients with skin cancer but that it could play a part in some individuals. In contrast, Peris et al. (13) found MSI phenotypes in 8 of 40 (20%) melanomas at one of 10 loci examined. In later studies, this rate of MSI phenotypes in melanomas has been reproduced close to that in our study. Three groups, Healy et al. (14), Tomlinson et al. (15) and Talwalkar et al. (16) described MSI phenotypes in 17% (7/42 cases), 18% (6/32 cases) and 25% (5/20 cases), respectively, of the melanomas analysed. The variations observed in the aforementioned studies may be due to sampling of cases, as no tumour-thickness stratified analysis of the data has been performed, i.e. low rates may indicate sampling of early lesions and high rates advanced lesions. Therefore, we tried to sample tumours that represented the full spectrum, including benign melanocytic tumours, primaries of variable thickness, and metastases. On the other hand, MSI events may not be equally distributed over the genome and sampling of microsatellite loci could be a critical factor too.

Further reason for turning to a larger scale of microsatellite analysis in melanomas comes from very recent studies indicating links between genome stabilization by administering non-steroidal antiphlogistic drugs. Rüschoff et al. (3) investigated the effects of non-steroidal antiphlogistic drugs in mismatch repair deficient colorectal cancer cell lines and found that non-steroidal antiphlogistic drugs, such as aspirin or sulindac, induce a genetic selection for microsatellite stability in mismatch repair deficient cells. The use of non-steroidal antiphlogistic drugs may therefore provide an effective prophylactic therapy for hereditary non-polyposis colorectal cancer kindreds. One could speculate that non-steroidal antiphlogistic drugs might also be beneficial in keeping the genome stable in MMR-deficient malignant melanomas during aggressive chemotherapy. If using drugs like etoposide or fotemustine, non-steroidal antiphlogistic drugs could even help to avoid selection of MSI clones with resistance to the therapy applied.

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