Exonic Deletions of Mismatch Repair Genes MLH1 and MSH2 Correlate with Prognosis and Protein Expression Levels in Malignant Melanomas

MONIKA KORABIOWSKA[1], ULRICH BRINCK[1], JERZY STACHURA[2], JACEK JAWIEN[3],
FRANK MICHAEL HASSE[4], CARLOS CORDON-CARDO[5] and GÖSTA FISCHER[1]

[1]Institute of Pathology, Reinhard Nieter Hospital, Academic Hospital of the University of Göttingen,
Friedrich Paffrath Str.100, 26389 Wilhelmshaven, Germany; [2]Department of Pathology and
[3]Department of Pharmacology, Jagiellonian University Krakow, Grzegorzecka 16, 31531 Krakow, Poland;
[4]Department of Surgery, Herzogin Elisabeth Heim, Hochstr.11, 38102 Braunschweig, Germany;
[5]Division of Molecular Pathology, Memorial Sloan Kettering Cancer Center,
1275 York Avenue, New York 10021, U.S.A.

Abstract. *The mutations of MLH1 and MSH2 have been reported to be responsible for malignant transformation and tumour progression in several sporadic tumours. Eighty-six primary malignant melanomas with known follow-up were investigated. Point mutations of DNA mismatch repair MLH1 and MSH2 in malignant melanomas were not found. Exon 12 (MSH2) was not present in 26 out of the 86 melanomas and exon 13 (MSH2) was lost in 25 of the tumours. The loss of exon 15 (MLH1) was observed in 22 out of the 86 tumours and the loss of exon 16 (MLH1) in 24 melanomas. The loss of exons correlated strongly with the loss of MLH1 and MSH2 protein expression. In multivariate analysis, including all 4 exons and expressions of MLH1 and MSH2, prognostic significance was found only for loss of exon 12 (MSH2) and loss of exon 15 (MLH1).*

DNA mismatch repair plays an important role in the preservation of genetic integrity from bacteria to mammals. For the first time, the defects of the repair genes MLH1 and MSH2 have been demonstrated in hereditary nonpolyposis colon cancer(HNPCC) (1-5). Subsequently, it was shown that HNPCC is caused by germ-line mutations in the human homologues of the bacterial and yeast MutS and MutL mismatch repair genes, including hMSH2 on chromosome

Correspondence to: PD Dr. Monika Korabiowska, Institute of Pathology, Friedrich Paffrath Str.100, 26389 Wilhelmshaven, Germany. Tel: 00494421891566, Fax: 00494421892771, e-mail: korabmon@yahoo.de

2p16 and hMLH1 on 3p21(6-9). Recently, the defects of the DNA mismatch repair system were reported to be responsible for malignant transformation and tumour progression in several sporadic tumours, including colorectal cancer, pancreatic and gastric carcinoma, endometrial, prostatic and breast cancer (10-12). In malignant melanomas, the DNA mismatch repair system has not yet been investigated in detail. The main goal of this study was to examine the presence of MSH2 and MLH1 mutations and their eventual prognostic outcome in a representative collection of malignant melanomas with known follow-up.

Materials and Methods

Patients. The material investigated consisted primarily of 106 malignant melanomas. It was possible to isolate DNA from 86 cases (pTis-12, pT1-4, pT2-18, pT3-23 and pT4-29) obtained from 39 females and 47 males. The melanomas were located as follows: head and neck 17, trunk 38, upper extremity 22, lower extremity 9. Twenty-two cases were classified as superficially spreading melanomas and 64 were nodular.

PCR analysis of MLH1 and MSH2 exons. The histological material was cut into sterile Eppendorf microfuge tubes. After washing once with xylene and twice with ethanol (96%) to remove xylene rests, the cells and cellular debris were obtained by centrifugation at 500 rpm at 4˚C. The DNA was isolated from the samples with a QIAamp DNA Mini Kit (Qiagen, Germany). Five μl of extracted DNA were used for PCR performed on a thermal cycler model 480 (Perkin Elmer, Weiterstadt, Germany). Each PCR was performed on a 50-μl mixture, in a thin-walled reaction tube, consisting of 1 unit Tfl DNA polymerase, polymerase buffer (BIOzym, Hessisch Oldendorf, Germany) and 20 μM of each dNTP. The mixture was overlaid with mineral oil (Sigma, Munich, Germany). The PCR primers were synthesised by MWG (Ebersberg, Germany) and

added at a final concentration of 40 pmol/l per assay. Standard precautions against cross-contamination were taken (13).

The presence of non-degraded DNA in the samples was tested by a semi-nested PCR specific to the human β-globin gene. β-globin DNA was amplified using the primer pairs 5'-ATGGTGCACCT GACTCCTGAGG-3' (sense 1)/5'-GCCATCACTAAAGGCACC GAGC-3' (anti-sense) and 5'-CTGTGGGGCA AGGTGAACG-3' (sense 2)/ anti-sense. The sense 2/anti-sense primers yield a 290-bp product. For amplification, the test tubes were heated to 95°C for 7 min, followed by 40 cycles at 95°C for 45 sec, 60°C for 20 sec and 72°C for 1 min and a final extension at 72°C for 7 min. For the second PCR run, 1-2 μl of the first run PCR product was used. The PCR conditions in runs 1 and 2 were identical.

Only β-globin-positive cases were used for the nested PCR analysis of the MLH1 and MSH2 exons. Exon 12 of MSH2 was amplified with the help of the following primers: 5'-TTTC TGTTTTTATTTTTTTACAGG-3' (forward primer), 5'-AAACG TTACCCCCACAAAG-3' (reverse primer). For the analysis of exon 13 of MSH2, the following primers were applied: 5'-CTAA CAATCCATTTATTAGTAGC-3' (forward primer) and 5'-CATTT CTATCTTCAAGGGACTAGGA-3' (reverse primer). Exon 15 of MLH1 was investigated applying the primers: 5'-ATTTGTCCC AACTGGTTGTATCTC-3'(forward primer) and 5'-ACTATACAA TACAGCAACTATCCT-3'(reverse primer). Exon 16 of MLH1 was analysed using the primers: 5'-GCTTGCTCCTTCATGTT CTTG-3'(forward primer) and 5'-CACCCGGCTGGAAATTT TAT-3'(reverse primer). For the amplification of MLH1 exons 15 and 16 and MSH2 exons 12 and 13, the test tubes were heated to 95°C for 7 min, followed by 40 cycles at 95°C for 45 sec, 60°C for 20 sec and 72°C for 1 min and a final extension at 72°C for 7 min.

The aliquots of the final reaction product were analysed by electrophoresis in 3% agarose gels containing ethidium bromide and visualised under ultraviolet light. The amplified fluorescent PCR products were mixed with TAMRA 350-internal size standard (Perkin-Elmer, Weiterstadt, Germany) and analysed by GENESCAN analysis software 672 on the automatic DNA analyzer (ABI 310, Weiterstadt, Germany).

Protein expression of MLH1 and MSH2. The following antibodies were applied to demonstrate DNA mismatch repair gene expression: a) N-20, rabbit polyclonal antibody against the epitope corresponding to an amino acid sequence mapping at the amino terminus of hMSH2 of human origin (Santa Cruz Biotechnology Inc., Heidelberg, Germany); b) C-20, rabbit polyclonal antibody against the epitope corresponding to an amino acid sequence mapping at the carboxy terminus of hMLH1 of human origin (Santa Cruz Biotechnology, Inc.).

The immunohistochemical reactions in the paraffin-embedded tumour tissue were carried out using the Stravigen Multilink kit (Biogenex Laboratories, Hamburg, Germany). The histological sections were mounted on uncoated slides and were deparaffinated by xylol and then transferred to a descending alcohol series and rinsed with distilled water. Before incubation with the primary antibodies, the sections were heated for 10 min on a hot-plate (85°C) in citrate buffer (pH=6). Afterwards, incubation with the primary antibodies was carried out overnight at 4°C at an antibody concentration of 1:50. The histological specimens were then rinsed with Tris buffer solution and incubated at room temperature with the link (Stravigen Multilink, Biogenex Laboratories) for 45 min. After the detection reaction had been performed using a label
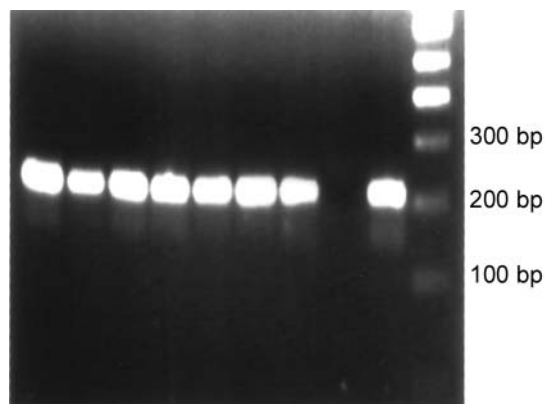

Figure 1. *Positive PCR product for exon 13 of MSH2 in malignant melanomas. Exon 13 presence was found in cases 1-7 and 9 and lost in case 8.*

(Stravigen Multilink, Biogenex Laboratories) in combination with chromogen fast red (Biogenex Laboratories, Hamburg, Germany), the nuclei were counterstained with hematoxylin. In the control reactions, the primary antibodies were omitted. The sections were evaluated by the CAS200 image analysis (Becton-Dickinson, Hamburg, Germany) system and the results were expressed as percentages of immunolabelled cell-indices.

Statistics. The data were analysed using the statistical analysis system (SPSS, Version 7.5) on an IBM-compatible PC under Windows NT 4.0. The data were first scanned into the spread-sheet (Microsoft Excel 97), where they were made available to the statistics program *via* an ODBC driver (open database connectivity).

The Kaplan-Meier method was employed to calculate the survival rates (14). The significance of differences in the survival curves was calculated with log-rank tests. Cox regression was the multivariate method used for predicting the survival rate based on several parameters (15). This method estimates the regression coefficients that make it possible to form a prediction equation. The Cox regression was used repeatedly to explore the set of data. Parameters were selected and the dataset was modelled using the methods offered by SPSS forward selection and backward selection. The first method adds parameters to a survival prediction model until the exclusion criterion is reached. By contrast, backward selection eliminates parameters from the model until the inclusion criterion is reached. The terminal criteria (for inclusion or exclusion) was the likelihood ratio based on partial likelihood estimators. In general, the Cox regression helps provide a formula for predicting the survival and testing the significance of individual predictors.

Results

Exonic deletions of MSH2 and MLH1. Exon 12 of MSH2 was present in 60 cases investigated and lost in 26 melanomas. Exon 13 of MSH2 was positive in 61 tumours and negative in 25 melanomas (Figure 1). Exon 15 of MLH1 was found to be present in 64 melanomas, but it was not possible to
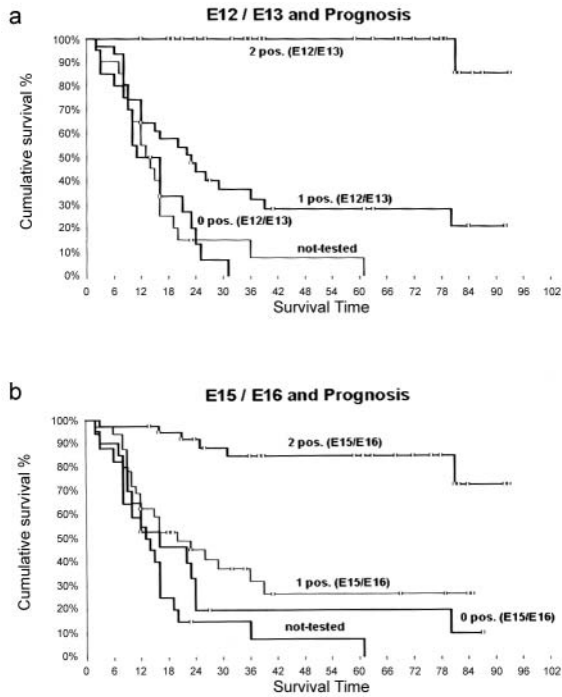
Figure 2. *a. Kaplan-Meier survival curves of exon 12 and exon 13 (MSH2)-positive (E=1) and -negative (E=0) melanomas. Survival time expressed in months. b. Kaplan-Meier survival curves of exon 15 and exon 16 (MLH1)-positive (E=1) and -negative (E=0) melanomas. Survival time expressed in months.*

Figure 3. *Nuclear reaction product of MLH1 in malignant melanoma, x400.*

Figure 4. *a. Boxplot demonstrating the MSH2 expression in exon 12 and exon 13 (MSH2)-positive (E=1) and -negative (E=0) melanomas. Y-axis: percentage of MSH2-positive cells. b. Boxplot showing the MLH1 reactivity in exon 15 and exon 16 (MLH1)-positive (E=1) and -negative (E=0) melanomas. Y-axis: percentage of MLH1-positive cells.*

detect its presence in 22 of the tumours investigated. Exon 16 of MLH1 was observed in 62 tumours and lost in 24 melanomas.

Kaplan-Meier survival curves of melanomas, with and without exonic deletions. When comparing the Kaplan-Meier survival curves for exon 12 and exon 13 (MSH2)-positive and - negative melanomas the difference between them was significant ($p=0.013$) (Figure 2a). In contrast, the difference for exon 15 and exon 16 (MLH1)-positive and -negative melanomas was highly significant ($p=0.006$) (Figure 2b).

Protein expression of MSH2 and MLH1. MSH2 expression oscillated between 0 and 98%. Significant and highly significant relationships between MSH2 expression and the loss of exons 12 and 13 (MSH2) are demonstrated in Figure 4a. MLH1 expression ranged between 0 and 96% (Figure 3). The relationship between MLH1 expression and loss of exons 15 and 16 (MLH1) is shown in Figure 4b.

Multivariate analyses (Cox regression model forward and backward). In forward and backward selection, including the loss of all 4 exons and the expression of MSH2 and MLH1,
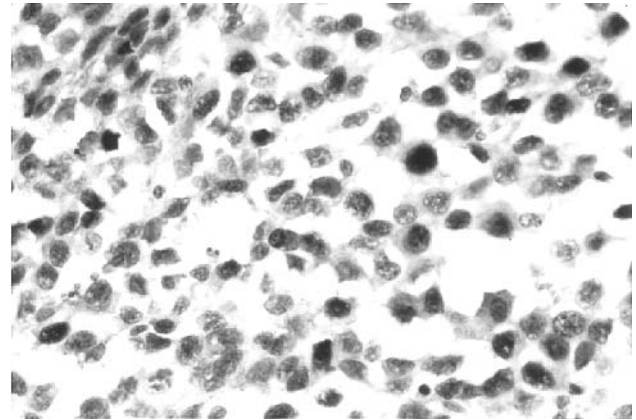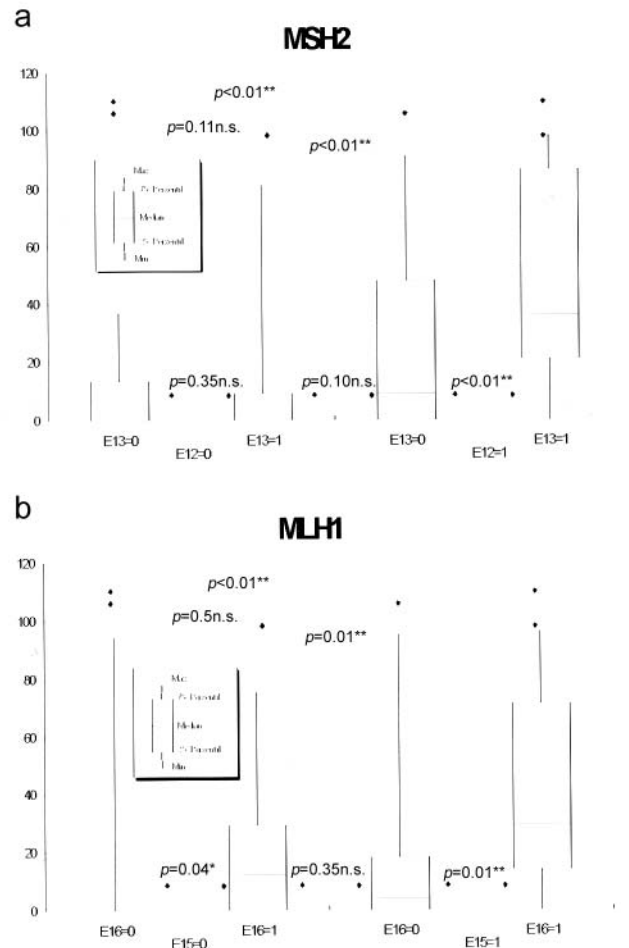
only the loss of exon 12 of MSH2 ($p<0.001$, coefficient of regression 0.8574, change of risk +136%, confidence limits +56% to +256%) and the loss of exon 15 of MLH1 ($p=0.024$, coefficient of regression 0.431, change of risk +54%, confidence limits +6% to +124%) had a significant effect on patient survival.

Point mutations of MLH1 and MSH2. No point mutations of the DNA mismatch repair genes, MLH1 and MSH2, were found in malignant melanomas.

Discussion

We analysed four of the most frequently mutated exons of MLH1 and MSH2 in 86 cases of malignant melanomas with known follow-up. Although there was a visible trend toward the loss of all 4 exons, prognostic significance was only defined for the loss of exon 12 of MSH2 and the loss of exon 15 of MLH1. Point mutations in MSH2 and MLH1 in malignant melanomas were not found.

Previous studies have suggested that the MSH2-MLH1-PMS1 pathway of mismatch repair primarily corrects insertions or deletions of one or two units of dinucleotide repeats (16). Given the importance of genomic instability in generating the multiple mutations necessary for multi-step tumorigenesis, mutator phenotype tumours might be expected to have accelerated progression. Indeed, HNPCC tumours often give the clinical impression of quicker progression. An increase in mutation rate might be expected to accelerate progression to the same end-point, because fewer divisions are needed to alter the usual genetic transformation barriers.

In malignant melanomas, not the mutation phenotype, but the phenomenon of exonic deletions seems to play a more important role concerning the prediction of patient prognosis. In our previous study, we demonstrated that the loss of the protein expression of repair genes, especially MLH1, MSH2, Ku70, Ku80 and APC, negatively influenced the prognosis in melanoma patients (17-24). The loss of gene expression was demonstrated to be functionally related to either the methylation of the promotor region or to mutational changes (25-26). Our study demonstrated some significant and, even partly, highly significant relationships between the loss of MLH1 and MSH2 protein expression and exonic deletions of these genes.

Multivariate analysis demonstrated that the loss of exon 12 of MSH2 and the loss of exon 15 of MLH1 have a marked prognostic significance for malignant melanomas. Our results correspond with cytogenetic anomalies of the chromosomes on which MLH1 and MSH2 are located. Chromosome 2 is often translocated, as reported for melanoma brain metastases (27), and chromosome 3 could be lost, as reported for uveal melanomas (28-30).

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