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Altered Expression and New Mutations in DNA Mismatch Repair Genes MLH1 and MSH2 in Melanoma Brain Metastases

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Abstract. *Brain metastases, including those of malignant melanoma (known for its high genomic instability), are the most common intracranial tumors. The main objective of this study was to investigate expression and mutation in the DNA mismatch repair system in melanoma brain metastases. Expression of MLH1, MSH2, PMS1 and PMS2 was investigated immunohistochemically in 31 melanoma metastatic tumors. Mutational analysis of MLH1 and MSH2 was performed in 17 melanoma brain metastases. Loss of MLH1 and MSH2 expression was found in 10/31 and 12/31 tumors. PMS1 (27/31) and PMS2 (28/31) expression was preserved in the majority of lesions. Potential missense mutation was found in MSH2 (exon 13) in 2/17 melanomas. Mutation in the intron sequence between exon 14 and 15 of MLH1 (exon 15) was observed in 4/17 cases. Our results indicate that the two major DNA mismatch repair genes, MLH1 and MSH2, are more frequently affected by alterations in the DNA mismatch repair system than the helper genes PMS1 and PMS2. The presence of mutations of MSH2 and MLH1 in melanoma brain metastases, which has not been found in primary melanomas, indicates the high genomic instability of melanoma brain metastases.*

Brain metastases are the most common intracranial tumors and are up to 10 times more frequent than primary brain tumors. Neurologically, they cause encephalopathy and a range of other dysfunctions. Brain metastases develop when tumor cells enter the nervous system and secondarily colonize the brain. Intracranial metastases may involve the brain tissue, the cranial nerves and the blood vessels and sinuses. The most common location is in the brain tissue itself. Among adults, the most common sources of brain metastases are the lung, breast, unknown primary sites and the skin including malignant melanoma. Metastasizing lesions from colon, breast and renal cell carcinoma are often single, while malignant melanoma and lung cancer tend to form multiple lesions, sometimes resulting in a much more severe clinical picture (1-3).

Much progress has been made in investigating the various types of tumor-associated genes over the past 10 years and recently attention has been focused on the group of genes responsible for DNA mismatch repair. DNA mismatch repair plays an important role in the preservation of genetic integrity between bacteria and mammals (4-6). A mismatched base-pair is recognized by MSH2, which starts the repair process together with MSH6. MLH1 at the same time cooperates with different enzymes (nuclease, polymerase and others), also playing an active part in the repair process. In humans, MLH1 has two additional helper proteins: PMS1 and PMS2.

For the first time, defects in the repair genes MLH1 and MSH2 have been demonstrated in hereditary nonpolyposis colorectal cancer (HNPCC) (7-9). 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 2p16 and hMLH1 on 3p21 (10-12).

Recently, defects in the DNA mismatch repair system were reported to be responsible for malignant transformation and tumor progression in several sporadic tumors including colorectal, pancreatic, gastric, endometrial, prostatic and breast cancer (13-17).

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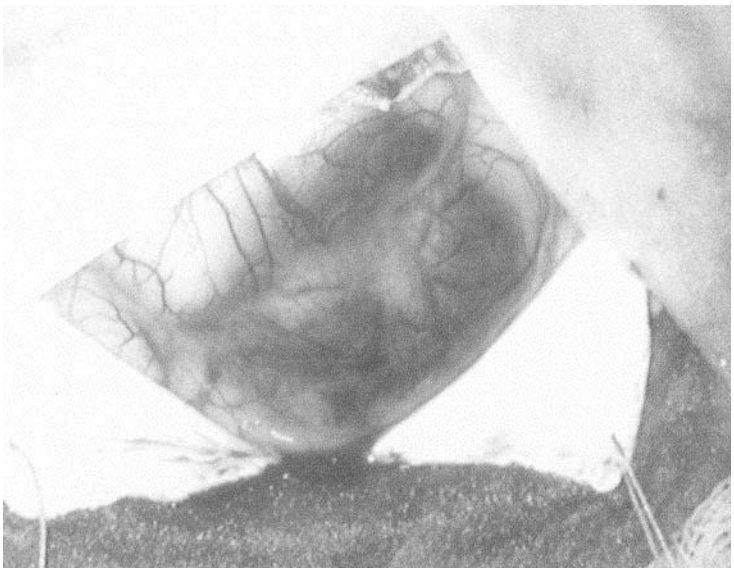

Figure 1. *Macroscopic image of a melanoma metastatic lesion during an operation and before extraction in a 27-year-old male. Note the contrast and black colour of the edges of the tumor.*

In malignant melanoma, we showed that not mutations but loss of gene expression might be responsible for defects in DNA repair. We have so far concentrated on lentigo maligna, primary melanoma and melanoma metastases in the lymph nodes (18-22). The main objective of this study was to investigate DNA mismatch repair genes in a representative sample of melanoma metastases in the brain.

Materials and Methods

The material investigated was taken from 31 patients with melanoma brain metastases: 16 women and 15 men aged between 38 and 71 years. The patients were treated in the Neurosurgical Departments of the Universities of Göttingen and of the Saarland (Figure 1), Germany and in Memorial Sloan Kettering Cancer Center in New York, USA. The sites of the metastases were; frontal lobe – 17; temporal lobe – 4; vertebral canal – 7; frontal and temporal lobe – 3.

For 12 patients primary melanoma tumors were available and they were located in the head and neck area, 4 tumors, trunk, 3, lower, 1 and upper extremities, 4. Five of the primary tumors represented pT3 stage and 7 of them were classified as pT4.

PCR analysis of MLH1 and MSH2 mutations. The histological material was cut into sterile Eppendorf microfuge tubes. After washing once with xylene and twice with 96% ethanol to remove the xylene residue, cells and cellular debris were obtained by centrifugation at 500 rpm and 4˚C. DNA was isolated from the samples using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Extracted DNA (200 ng) was used for PCR performed on a thermal cycler model 480 (Perkin Elmer, Weiterstadt, Germany). Each PCR was performed in a thin-walled test tube on 50 μl of a mixture consisting of 1U Tfl DNA polymerase, polymerase buffer (Biozym, Hessisch Oldendorf, Germany) and 20 μmol of each dNTP. The mixture was overlaid with mineral oil (Sigma, Munich, Germany). The PCR primers were synthesized by MWG (Ebersberg, Germany) and added at a final concentration of 40 pmol/l per assay. Standard precautions against cross-contamination were taken.

3'(anti-sense) and 5'-CTGTGGGGCAAGGTGAACG-3'(sense2/antisense). For amplification, 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, 72 ˚C for 1 min and a final extension at 72˚C for 7 min. For the second PCR, 1-2 μl of the first-run PCR product was used. PCR conditions in run 1 and 2 were identical.

Only β-globin-positive cases were used for nested PCR analysis of MLH1 and MSH2 exons. Exon 12 of MSH2 was analysed using the primers: 5'-TTTCTGTTTTTATTTTTTACAGG-3'(forward) and 5'-AAACGTTACCCCCACAAAG-3'(reverse). Exon 13 of MSH2 was analyzed using the primers 5'-CTAACAATCCATTTA TTAGTAGC-3'(forward) and 5'-CATTTCTATCTTCAAGGGA CTAGGA-3'(reverse). Exon 15 of MLH1 was analysed using the primers: 5'-ATTTGTCCCAACTGGTTGTATCTC-3'(forward) and 5'-ACTATACAATACAGCAACTATCCT-3'(reverse). Exon 16 of MLH1 was analysed using the primers: 5'-GCTTGCTCCTT CATGTTCTTG-3' (forward) and 5'-CACCCGGCTGGGAAA TTTTAT-3'(reverse). For amplification of the exons, 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, 72˚C for 1 min and final extension at 72˚C for 7 min.

For visualization, 5 μl of each PCR product was separated on a 3% (w/v) agarose gel containing 0.5 μg ethidium bromide per ml. The gel was recorded using a CCD camera (Biometra, Göttingen, Germany). The MSH2 and MLH1 amplification products were purified using a QIAquick PCR Purification Kit (Qiagen).

A total of 200 ng isolated DNA was labeled with the PRISM Ready Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany) according to the manufacturer's instructions and analyzed in an Applied Biosystems DNA sequencer (model 310). Oligonucleotides previously used for amplification of fragments served as sequencing primers.

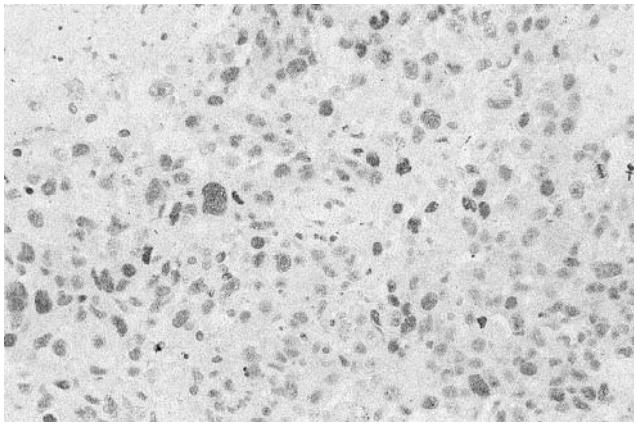

Figure 2. *Nuclear staining for MLH1 in melanoma brain metastasis.*

Table I. *Expression of DNA mismatch repair genes in melanoma brain metastases.*

Marker	Rate of positive cases	Percentage of positive cells		
		Minimum	Median	Maximum
MLH1	21/31	0.00	24.00	65.00
MSH2	19/31	0.00	19.00	72.00
PMS1	27/31	0.00	26.00	52.00
PMS2	28/31	0.00	36.00	58.00

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 epitope corresponding to an amino acid sequence mapping at the amino terminus of hMSH2 of human origin.
b) C-20, rabbit polyclonal antibody against epitope corresponding to an amino acid sequence mapping at the carboxy terminus of hMLH1 of human origin.
c) K-20, rabbit polyclonal antibody against epitope corresponding to an amino acid sequence mapping at the amino terminus of hPMS1 of human origin.
d) C-20, rabbit polyclonal antibody against epitope corresponding to an amino acid sequence mapping at the amino terminus of hPMS2 of human origin.

All antibodies were supplied by Santa Cruz Biotechnology Inc., Heidelberg, Germany.

The immunohistochemical reactions in the paraffin-embedded tumor tissue were carried out using the Stravigen Multilink kit (Biogenex Laboratories, Hamburg, Germany). The histological sections were mounted on uncoated slides. They were deparaffinized using xylol and then transferred to a descending alcohol series and rinsed with distilled water. Before incubation with primary antibodies, the sections were heated for 10 min (85˚C) in citrate buffer (pH=6). They were then incubated overnight with the primary antibodies 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 link (Stravigen Multilink, Biogenex Laboratories, Hamburg, Germany) for 45 min. After detection reaction using a label (Stravigen Multilink, Biogenex Laboratories) in combination with chromogen fast red (Biogenex Laboratories), the nuclei were counterstained with hematoxylin. Primary antibodies were omitted from control reactions. Sections were evaluated by CAS200 image analysis (Becton-Dickinson, Hamburg, Germany) and results were expressed as percentages of immunolabeled cell indices.

Results

Expression of DNA mismatch repair genes in melanoma brain metastases. MLH1 expression was totally lost in 10 out of 31 cases. The MLH1 index (percentage of MLH1-positive cells) ranged between 0 and 65%, with a median of 24% (Figure 2). MSH2 was totally lost in 12 cases. The MSH2 index ranged between 0 and 72%, with a median of 19%.

PMS1 (27/31) and PMS2 expression (28/31) were preserved in most lesions. The PMS1 index did not exceed 52% and the median was 26%. The PMS2 index was similar with a maximum of 58% (Table I).

Expression of DNA mismatch repair genes in primary melanomas. MLH1 expression was found in 7 out of 12 primary melanomas investigated and its index ranged between 0 and 82% with a median 31%. MSH2 was found in 10 out of 12 melanomas investigated with maximal expression of 43%. PMS1 and PMS2 were expressed in all primary melanomas and their expression peaked at 61% and 53%, respectively. Comparison of the expression of DNA mismatch repair genes in primary melanomas and their brain metastases did not reveal any significant differences.

Analysis of MLH1 and MSH2 mutations. All primary melanomas were screened for mutations of MLH1 and MSH2 without any positive results. It was possible to isolate DNA from 17 melanoma brain metastases. The quality of the DNA was assessed on β-globin positivity. Sequencing analysis revealed the potential missense mutation in exon 13 of MSH2 causing a CGA-GGA conversion, which may replace the amino acid Arg by Gly in 2 out of 17 tumors investigated. The corresponding electropherogram shows 2 peaks at the same nucleotide position. The other positions show optimal signals and are inapparent. It is possible that the PCR reaction indicates mutant and non-mutant MSH2 alleles in the sample.

In 4 out of 17 tumors sequencing analysis indicated a mutation in the intron sequence between exon 14 and 15 of the MLH1 gene. The A-G mutation is located at position

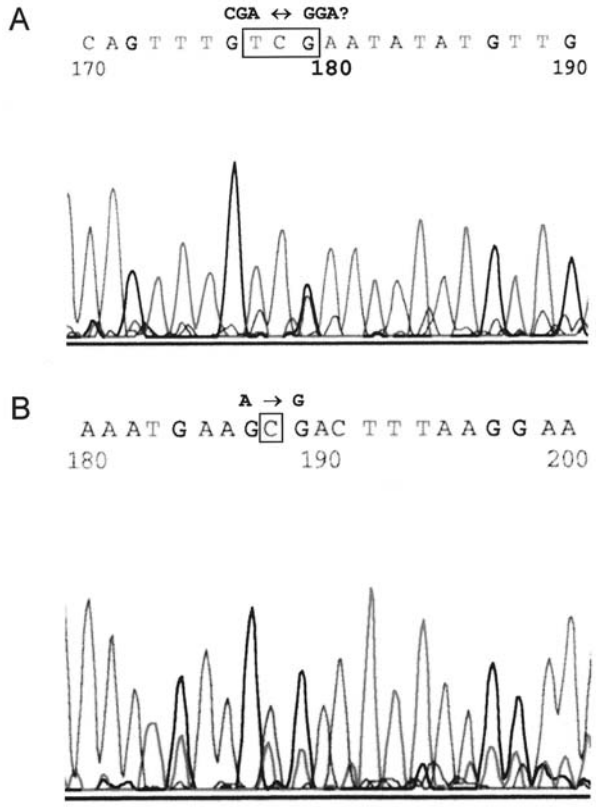

Figure 3. *Sequence analysis of PCR products amplified from MSH2 and MLH1 DNA. Reverse primers were used for automatic sequencing. Upper sequence - A potential mutation was found in MSH2 exon 13 causing a CGA – GGA conversion which may replace the amino acid Arg by Gly. Note that the corresponding electropherogram shows double peaks at the same nucleotide position. The other positions show optimal signals and are inapparent. Therefore, it is possible that the PCR reaction indicates mutant and non-mutant MSH2 alleles in the sample. Sequence below - The analysis indicates a mutation in the intron sequence between exon 14 and 15 of the MLH1 gene. The A-G mutation is located at position -19 near the splice acceptor site of this intron. It is unclear whether the alteration causes exon skipping and formation of alternative MLH1 splicing transcripts.*

19 near the splice acceptor site of this intron. It is unclear whether the alteration causes exon skipping and formation of alternative MLH1 splicing transcripts (Figure 3) (Table II).

Discussion

The relatively new discovery of defects in melanoma brain metastases and in endometrial, breast, prostate, bladder and gastric cancers indicate that defects of DNA mismatch repair are a more widespread phenomenon than first assumed. Considerable evidence exists that the MI-positive phenotype in HNPCC tumors is due to inactivation of DNA mismatch repair, probably due to inherited mutations of mismatch repair genes.

Table II. *Mutational analysis of MLH1 and MSH2.*

Gene	Exon	Number of cases with mutation	
MLH1	15	0/17	4/17
MLH1	16	0/17	0/17
MSH2	12	0/17	0/17
MSH2	13	2/17	0/17

Our study analysed comparatively the expression of DNA mismatch repair genes in primary melanomas and their brain metastases. Significant differences in the gene expression at the protein level were not found. In contrast, some of the metastatic lesions demonstrated mutations and polymorphic variants of MLH1 and MSH2, which were not found in primary melanomas.

Defects of DNA mismatch repair have often been reported for brain tumors. Microsatellite instability is associated with the development of primary gliomas rather than with the recurrence or progression of these tumors. Astonishingly, the occurrence of microsatellite instability in gliomas was not connected with structural alterations in the MLH1 and MSH2 genes. Comparison of the microsatellite patterns in primary and secondary gliomas revealed different modalities of clonal evolution, including clonal identity, clonal deletion, clonal progression and different clonality, suggesting that intensive clonal selection may play a central role in the recurrence of gliomas (23).

Another research group reported that microsatellite instability and mutations of DNA mismatch repair genes are present in a subset of young glioma patients and that these patients and their families are at risk of developing HNPCC-related tumors. These results have practical relevance for the genetic testing and management of young patients with gliomas and their families (24).

DNA mismatch repair system defects do not seem to be of relevance in human medulloblastomas, where the repair system is not commonly deficient (25). A low level of microsatellite instability has also been reported for oligodendrogliomas (17.4%), pituitary adenomas (3.2%), meningiomas (2.4%) and astrocytomas (3%). Gangliogliomas and schwannomas do not show instable loci (26,27).

So far, there have been no reports of defective DNA repair systems in melanoma brain metastases in the literature. An important role of defective DNA mismatch repair genes in the progression of malignant melanoma has been confirmed by different research groups. MSH2 may be of importance in genetic stability, tumorigenesis and

progression of malignant melanoma. Increased genomic instability in malignant melanoma is associated with an elevated protein level of this DNA repair enzyme, and MSH2 expression may also be regulated by the p53 protein (28,29). Prognostic significance has also been reported for MSH2. Elevated levels of MSH2 expression have been shown to correlate with recurrence or death from the disease (30).

Reports of a connection between defective DNA mismatch repair systems and the presence of microsatellite instability in malignant melanoma are controversial. Hussein *et al.* (31) reported that correlations between microsatellite instability and the expression and function of DNA mismatch repair genes were not present in melanocytic dysplastic naevi and in cutaneous malignant melanoma. Another research group demonstrated an association between high frequency microsatellite instability and defective DNA mismatch repair in human melanoma. Tumors with highly reduced or non-existent MLH1 and PMS2 expression displayed a high frequency of microsatellite instability. The absence of unstable loci on the mononucleotide level indicates that, in malignant melanoma, microsatellite instability is not associated with mutator-phenotype repair-deficient tumors characteristic of mismatch. Microsatellite instability at dinucleotide repeats increases with melanoma progression and indicates that expansions of triplet repeats may occur in melanocytic tumors (31-35).

Generally, defects in DNA repair systems can have practical relevance in chemotherapy. The increased repair of oxidative DNA damage might mediate increased chemoresistance through improved repair of drug-induced DNA damage. In contrast, reduced DNA mismatch repair might confer resistance by preventing futile degradation of newly synthesized DNA-opposite alkylation damage, or by apoptosis (36).

In this study, we demonstrated altered expression of DNA mismatch repair genes in melanoma metastases, more frequently connected with the two major DNA mismatch repair genes, MLH1 and MSH2, than with the helper genes PMS1 and PMS2. Sequencing analysis revealed potential missense mutation in exon 13 of MSH2 causing a CGA – GGA conversion which may replace the amino acid Arg by Gly. The corresponding electropherogram showed double peaks at the same nucleotide position. We speculate that PCR reaction indicated mutant and non-mutant MSH2 alleles in the tumor sample.

The second mutation was found in the intron sequence between exon 14 and 15 of the MLH1 gene. This A-G mutation was located at position -19 near the splice acceptor site of this intron. This alteration can potentially cause exon skipping and formation of alternative MLH1 splicing transcripts (37-39). Neither alteration has been reported in the literature for malignant melanoma.

The presence of mutations of DNA mismatch repair genes in melanoma brain metastases confirmed the high genomic instability of these lesions. Such alterations were not found in the earlier stages of progression of malignant melanoma (lentigos and primary cutaneous tumors) (22). Our results support the existence of a mutator phenotype that develops at an early stage in the formation of human tumors and results in an intrinsic genetic instability. Brain melanoma metastases represent the last stages of the malignant spread of a tumor. A high rate of genetic instability, mutations and a reduced level of mismatch gene expression also support Vogelstein's hypothesis that mutations are the primary cause of human tumors and that these can result from errors in DNA sequencing or DNA damage (40). The results of this study agree with these theories.

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