Immunohistochemistry of DNA Mismatch Repair Enzyme MSH2 Is Not Correlated with Prognostic Data from Endometrial Carcinomas

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Abstract. Background: The human Mut-S-homolog-2 (MSH2) is part of the DNA mismatch repair system (MMR). Mutations in genes of the MMR are a predisposition to hereditary non-polyposis colorectal cancer (HNPCC). In women, MMR gene mutations may lead to primary endometrial cancer (EC). The important function of the MMR for the integrity of the DNA during replication makes it probable that the MMR might also be involved in the development and the course of sporadic carcinomas. Insufficient MMR activity or expression levels could be prognostic markers of the disease. Patients and Methods: Immunohistochemical analysis of MSH2 was performed in 86 tumor samples from patients with EC. Results: Compared to known tumor markers, namely estrogen and progesterone receptors, histopathological grading, TNM stage and FIGO classification, no significant correlation between MSH2 immunoreactivity and EC was found. Conclusion: MSH2 immunohistochemical analysis is not of prognostic value for endometrial carcinoma.

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Key Words: Endometrial cancer, DNA mismatch repair, MSH2, immunohistochemistry.
Here, the expression levels of MSH2 were correlated with the tumor suppressor p53, the proliferation marker Ki-67, the appearance of apoptosis and with clinical data from 86 patients with EC.

Patients and Methods

Tumor tissue. Tumor specimens from 86 carcinoma of the corpus uteri or the endometrium were collected between 1985 and 1990 from patients during inter-surgery diagnosis at the female hospital of the University of Homburg/Saar. The patients were informed about and gave their written consent to their participation in this study. The study was approved by the Ethics Committee of the University of Homburg/Saar. Residues of tumor samples were collected after histological examination by pathologists. Grading and staging of the tumors as well as the status of steroid receptors were taken from the pathologists’ reports. The tumor specimens were fixed in 4% neutral buffered formalin and embedded in paraffin for storage.

Slide preparation. Glass slides were silane-coated, pretreated with 0.5% ovarian albumin and dried at 60°C overnight. Serial sections of the tumor specimens of 6-7 μm thickness were cut on a rotation microtome (Leitz 1516, Wetzlar, Germany). After floating on 37°C distilled water the sections were mounted on the pretreated microscopic slides.

For the staining procedure the slides were deparaffinized in a xylene-bath and rehydrated in decreasing concentrations of ethanol. Finally they were washed in Tris-buffer. Endogenous peroxidase was blocked by incubation in a freshly prepared solution of 3% hydrogen peroxide in methanol for 10 min at room temperature. The sections were pretreated in a solution of 10 mM citrate-buffer, pH 6.0 by heating in a microwave-oven at 500 watts for 10 min followed by two washes in distilled water.

Immunohistochemistry. Nonspecific antibody reactions were blocked in a blocking-solution of Tris-buffer with 0.2% Triton-X-100 and 1% fish gelatine. The tumor specimens were incubated with monoclonal MSH2 antibody clone FE-11 (Calbiochem, Merck Biosciences, Schwalbach, Germany) diluted 1:50 in blocking solution. The sections were covered with a coverslip and the reaction took place at 4°C overnight in a humid chamber. Against p53, the monoclonal mouse antibody clone DO-7 at 1:150 dilution and for Ki-67 the polyclonal rabbit antibody A-047 at 1:50 dilution were used (both Dako, Hamburg, Germany).

For detection of the primary antibody, the avidin-biotin-peroxidase complex (ABC) method (Dako) was used. The secondary biotinylated canine anti-mouse antibody was applied at 1:200 dilution in fish-gelatine for 20 min at room temperature followed by incubation with the streptavidin-biotin complex for 30 min at 37°C. After each incubation, two washes were performed. The peroxidase activity was visualized with 3,3’-diaminobenzidine (DAB, Dako). The sections were counterstained in a hematoxylin solution for 10 min followed by two washes in distilled water. The slides were dehydrated in increasing ethanol concentrations and a final xylene bath before they were covered with Entellan® (Merck, Darmstadt, Germany).

Immunoreactive detection of apoptosis after TdT-mediated dUTP nick-end labelling (TUNEL) assay. For the detection of apoptotic activities in tissue sections of the tumor samples, we used the In Situ Cell Death Detection Kit, with alkaline phosphatase (AP; Roche, Mannheim, Germany). The slides were deparaffinated and rehydrated. Proteinase-K digestion was performed for 25 min at room temperature. The TUNEL reaction was undertaken at 37°C for 1 h under a coverslip. For the detection of fluorescein-dUTP-labelled cells, we used an AP-linked anti-fluorescein antibody. The immunoreaction was visualized with the New Fuchsin Substrate Kit (Dako, Hamburg, Germany).

Semi-quantitative analysis of immunoreactivity. Microscopic analysis was performed by two independent observers (R.M.D. and A.W.-H.). The immunoassayed specimens were viewed under a Leica DM4000 B microscope (Leica Microsystems, Wetzlar, Germany).

The interpretation of immunoreactivity in the tumor sections was performed using the immunoreactive score (IRS) ranging from 0 to 12 (negative=0; weak=1–3; moderate=4–8; strong=9–12) described by Remmele and Stegner (27). The IRS results from the multiplication of a staining intensity (SI) score (negative=0; weak=1; moderate=2; strong=3) and the values of percentage of positive tumor cells (PP-score: 0-10% = 1; 11-50% = 2; 51-80% = 3; 81-100% = 4). The immunoreactivity for Ki-67 was evaluated and compared with other immunoreactivities with a scoring after Friedrich et al. (24) that creates a ranking of the percentages of immunopositive cells (PP) (rank 0=0%; 1=1–10%; 2=11–25%; 3=26–25%).

Statistical analysis of correlation. The statistical correlations between MSH2, p53 and apoptosis immunoreactivity and with patient data were analyzed by the Spearman’s rank correlation coefficient in SPSS version 10.0 (SPSS GmbH Software, München, Germany).

Results

Expression of MSH2, p53 and apoptosis. The antibody against MSH2 reacted positively in 72.1% of the tumor specimens: 21% with strong IRS, 48.8% with moderate, 23.3% with weak and 27.9% were negative. The average IRS score was 4.78±3.48. Among the 86 EC specimens, 47.7% did not react with the p53 antibody, 23.3% were strongly positive, 25.5% were moderately and 3.5% were weakly stained. The average IRS score was 3.59±3.83. The immunoreaction after the TUNEL assay was positive in 80.2% of the specimens, with a mean IRS of 3.57±2.25. Strong reactivity was observed in 35.9% of the tumor samples, moderate in 62.6% and weak in 13.9%; 19.8% of the specimens did not show staining for apoptosis.

Correlation of MSH2 expression with p53, Ki-67 and apoptosis. No correlation of MSH2 expression was observed with p53, apoptosis or the proliferation marker Ki-67 (Table I). Furthermore, no correlations were found of p53 expression with apoptosis, p53 expression with Ki-67 or between apoptosis and Ki-67.

Correlation of MSH2 immunoreactivity with clinical parameters. Regarding the histopathological differentiation grade of the tumor cells, the highest expression of MSH2
was found in the group of tumors with grading G3 and an IRS score of 5.48 (Table II). Nevertheless, Spearman’s correlation rank test over all the samples did not show a statistically significant correlation between the grade of differentiation and the expression of MSH2.

The MSH2 immunoreactivities were tested for correlation with further clinical parameters (Table III). The IRS of the immunostainings for estrogen and progesterone receptors (ER and PR) did not correlate with the IRS for MSH2. With regard to the TNM classification neither the tumor size (T stage), nor lymph node infiltration (N stage), nor the appearance of metastases (M stage) correlated with the immunoreactivity for MSH2. Furthermore, the FIGO (Fédération Internationale de Gynécologie et d’Obstétrique) stages of the tumors did not correlate with the MSH2 immunostaining.

Table I. Correlation between MSH2 p53 and Ki-67 expression and apoptosis following TUNEL assay.

<table>
<thead>
<tr>
<th></th>
<th>IRS [r_s]</th>
<th>PP-score [r_s]</th>
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<tbody>
<tr>
<td>MSH2 – p53</td>
<td>0.091 (p=0.497)</td>
<td>0.085 (p=0.435)</td>
</tr>
<tr>
<td>MSH2 – apoptosis</td>
<td>-0.025 (p=0.817)</td>
<td>0.064 (p=0.559)</td>
</tr>
<tr>
<td>MSH2 – Ki-67</td>
<td>0.036 (p=0.739)</td>
<td></td>
</tr>
<tr>
<td>p53 – apoptosis</td>
<td>0.133 (p=0.224)</td>
<td>0.142 (p=0.194)</td>
</tr>
<tr>
<td>Apoptosis – Ki-67</td>
<td>0.027 (p=0.807)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.119 (p=0.277)</td>
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IRS, Immunoreactive score; PP, percentage immunopositivity; r_s, Spearman’s rank correlation coefficient; statistical significance: p<0.05.

Table II. MSH2 immunoreactivities correlated with the grade of histopathological differentiation.

<table>
<thead>
<tr>
<th>Grading</th>
<th>N</th>
<th>MSH2 IRS±SEM</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>r_s: 0.108; p=0.322</td>
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<tr>
<td>G1</td>
<td>30 (37.5%)</td>
<td>4.72±3.43</td>
</tr>
<tr>
<td>G2</td>
<td>29 (33.7%)</td>
<td>4.28±3.30</td>
</tr>
<tr>
<td>G3</td>
<td>27 (31.4%)</td>
<td>5.48±3.46</td>
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r_s, Spearman’s rank correlation coefficient; IRS, immunoreactive score; SEM, standard error of the means.

Table III. MSH2 immunoreactivities correlated with clinical data.

<table>
<thead>
<tr>
<th>ER</th>
<th>PR</th>
<th>T stage</th>
<th>N stage</th>
<th>M stage</th>
<th>Histopath. grading</th>
<th>FIGO stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSH2 IRS [r_s]</td>
<td>0.074</td>
<td>0.117</td>
<td>0.048</td>
<td>-0.330</td>
<td>0.030</td>
<td>0.108</td>
</tr>
<tr>
<td>p-Value</td>
<td>0.497</td>
<td>0.284</td>
<td>0.668</td>
<td>0.767</td>
<td>0.790</td>
<td>0.322</td>
</tr>
</tbody>
</table>

ER, Estrogen receptor; PR, progesterone receptor; r_s, Spearman’s rank correlation coefficient; statistical significance: p<0.05; FIGO, Fédération Internationale de Gynécologie et d’Obstétrique.

Discussion

As expected, a high percentage of the tumor samples were found to express MSH2 protein and most of them exhibited moderate to strong immunoreactivities. The mean IRS of 4.78 was relatively lower than that analyzed in previous studies in EC (mean IRS of about 9.00) (24, 25). These immunohistochemical studies used frozen tissue instead of paraffinized tumor material. The finding of 27.9% of MSH2 negative probes was in a sharp contrast to other studies e.g. by Peiro et al. who found only one case in 89 patients with the total loss of MSH2 expression in paraffinized material of EC (28). By contrast, Cohn et al. reported 29% of endometrial cancer patients with MMR deficiency, where either MSH2 or MLH1 expression was lacking (26). A total loss of MSH2 immunoreactivity could be caused by a mutation in one of the genes of the Mut-S heterodimer, namely MSH2 or MSH6. When one of the binding partners for the MMR heterodimers is missing, the other will be undetectable as well. For the interpretation of our results, we used the IRS values for correlation statistics instead of differentiating strictly between MSH2 expressing and non-expressing patients.

No statistically relevant correlation was found between the MSH2 IRS and that for tumor suppressor p53, the proliferation marker Ki-67 and the TUNEL assays. In previous studies using samples from breast cancer or cervical cancer, we have observed correlations between MSH2 immuno-reactivity with that scored for p53 and apoptosis (12, 13). However, Giarnieri et al. working with samples from the uterine cervix found a correlation of high p53 immunoreactivity and the loss of MSH2 reactivity (29). The interpretation of p53 immunoreactivity is difficult because of the fast degradation of the protein. P53 mutations frequently occur in tumors and are associated with unfavorable prognosis (30, 31). Of note, a marked p53 expression and activity may be associated with MSH2 expression since a binding site for p53 has been detected in the promoter region of the MSH2 gene (32, 33). The interpretation of apoptosis after the TUNEL assay in paraffinized material can be associated with unspecific labeling reactions. In accordance with the results from Labat-Moleur et al., we used formalin.
fixation and a proteinase-K pretreatment because this methodical combination achieves the highest specificity (34).

No correlation was found in this study between the immunoreactivity of MSH2 and the proliferation marker Ki-67. Because the MMR is highly active in the posttranscriptional phase of proliferating cells, one could speculate that a strong expression of MSH2 is exclusively associated with the proliferative activity of these tumor cells. Due to the non-existence of correlation between MSH2 and Ki-67 an unknown mechanism in the neoplastic tissue must be suggested for the regulation of MSH2 expression.

The correlations of MSH2 immunoreactivities with the clinical data of the patients did not show any significant result. The highest IRS value of MSH2 was found in the patients with a histopathological grading of G3 according to the worst prognosis, but the correlation in patients overall did not reach statistical significance. This result was in agreement with the study of Peiro et al. who were also unable to find a correlation between MSH2 immunohistochemical analysis and histopathological grading (28).

With regard to other classical prognostic and predictive tumor markers, namely ER and PR, TNM stage and FIGO classification, no significant correlations were detected with the immunohistochemical analysis of MSH2 expression. Cohn et al. analyzed the MSH2 and MLH1 expression in 336 patients with EC on a tissue microarray. They found a correlation between the lack of MSH2 or MLH1 and negative prognostic factors, as well as a worse progression-free survival (26). Garg et al., searching for HNPCC-screening criteria for patients with EC, found that the age of patients and a family or personal history of HNPCC-associated carcinomas will underestimate the risk for HNPCC. Therefore, they suggest that tumor morphology and the analysis of MMR protein defects by immunohistochemistry for should be included (7).

These results indicate that the immunoreactive score of MSH2 is not a useful prognostic test for endometrial carcinoma.

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