Msh2, Mlh1, Fhit, p53, Bcl-2, and Bax Expression in Invasive and in Situ Squamous Cell Carcinoma of the Uterine Cervix¹

Enrico Giarnieri, Rita Mancini, Tiziana Pisani, Mauro Alderisio, and Aldo Vecchione²

Department of Experimental Medicine and Pathology, Cytopathology, School of Medicine, University of Rome La Sapienza, 00161 Rome, Italy

ABSTRACT

To analyze relevant factors and their effects on neoplastic progression in cervical carcinoma, a panel of genetic markers was studied. Paraffin-embedded tissue sections were obtained from 37 patients with carcinoma of the uterine cervix, 14 noninvasive squamous cell carcinomas (NISCCs), and 23 invasive squamous cell carcinomas (ISCCs). Immunoreactivity of Msh2, Mlh1, Fhit, p53, Bcl-2, and Bax proteins was examined by immunohistochemical staining with appropriate antibodies. Positive staining of Msh2 was detected in 13 of 14 (92.9%) NISCCs and in 13 of 23 (56.5%) ISCCs (P < 0.02). Mlh1 immunoreactivity was observed in 10 of 14 (71.4%) NISCCs and in 8 of 23 (34.8%) ISCCs (P < 0.04). Overexpression of p53 protein was found in 4 of 14 (28.6%) NISCCs and in 16 of 23 (69.6%) ISCCs (P < 0.02). Bcl-2 overexpression was detected in 2 of 14 (14.3%) NISCCs and in 15 of 23 (65.2%) ISCCs (P < 0.003). No significant difference in the two types of lesion was found for Bax and Fhit expression. The relationship between Mlh1, Msh2, and p53 protein expression was significant (P < 0.001 and P < 0.001, respectively), as was that between Fhit and Bax immunoreactivity (P < 0.02). In conclusion, we consider that altered expression of Msh2, Mlh1, p53, and Bcl-2 may be a critical event during cervical cancer progression, whereas Fhit may be a component of a proapoptotic pathway.

INTRODUCTION

Since the introduction of cytological screening in the 1940s, the incidence of cervical carcinoma has declined in many developed countries (1). Considerable efforts have been made to classify preneoplastic and neoplastic lesions (2), and an association between HPV³ infection, especially high-risk HPV types 16 and 18, and the development of precancerous lesions has been proposed (3–6). Nevertheless, prospective studies of cervical neoplasia suggest that HPV infection alone is not responsible for tumor development (7, 8).

MMR genes Msh2 and Mlh1, located respectively on chromosomes 2p21–22 and 3p21, are involved in tumors that develop in patients with hereditary nonpolyposis colon cancer (9); germ-line mutations of MMR genes are responsible for the replication error-positive phenotype and microsatellite instability (9–11). Cells with defective MMR function show a mutant phenotype characterized by an increased mutation rate (12).

Deletions in the FHIT gene located on chromosome 3p14.2 have been observed in several types of tumor, particularly those resulting from exposure to environmental carcinogens such as lung, kidney, esophageal, head and neck, stomach, and cervical cancer. A possible role as a tumor suppressor gene has been considered (13, 14).

The p53 gene located on chromosome 17 encodes a M₅₃,000 protein that is involved in cell growth regulation and is frequently found to be mutated in human cancers. In addition, p53 can induce apoptosis in some cells and can down-regulate Bcl-2 (15). Cells expressing mutant p53 have lost the ability to arrest the cycle and show enhanced genomic instability (16).

It is commonly accepted that apoptosis is important in the death of normal and neoplastic cells (17). It has become clear that carcinogenesis can be caused by loss of growth suppression and changes in apoptotic cell death pathways (17, 18). Members of the gene family that includes BCL-2 and BAX are involved in the control of apoptosis in a range of different cell types. The BCL-2 gene was cloned initially from a t(14;18) translocation in a follicular B-cell lymphoma (19). The overexpression of Bcl-2 seems to be associated with the blocking of apoptosis (20). The ability of Bcl-2 to inhibit apoptosis is dependent on expression of Bcl-2 and on the formation of hetero- and homodimers between members of the Bcl-2 family (21). On the other hand, the BAX gene is an apoptosis-promoting member of the BCL-2 family, and apoptosis is known to be accelerated when the BAX function is predominant (22). The aim of the current study was to investigate the relationship among expressions of MMR proteins Msh2 and Mlh1, Fhit, p53, Bcl-2, and Bax by immunohistochemistry in ISCC and in situ SCC.

MATERIALS AND METHODS

Tissue Specimens. Cervical tissue from archival paraffin blocks was available from 37 patients, 14 of whom had in situ carcinoma and 23 ISCC, according to the WHO grading hist-

3 The abbreviations used are: HPV, human papillomavirus; MMR, mismatch repair; Fhit, fragile histidine triad; NISCC, noninvasive squamous cell carcinoma; ISCC, invasive SCC.
topathological classification. Eight cases were well differentiated (G1), 12 cases were moderately differentiated (G2), and three cases were poorly differentiated. The 23 cases were at stage Ib of the FIGO classification (International Federation of Gynaecology Obstetrics). The mean age of patients was 47.79 (SD, ± 11.62; range, 31–77 years). Informed consent was obtained from each subject after the purpose and nature of the study had been explained.

Immunohistochemistry. Sections 5 µm thick were cut onto silanized glass slides and air-dried overnight at room temperature. Sections were dewaxed in xylene and rehydrated through graded alcohol. Incubating the slides for 10 min in 3% hydrogen peroxide quenched endogenous peroxidase activity. Sections for microwave antigen retrieval pretreatment (Fhit, p53, Msh2, and Mlh1 antibodies) were immersed in citrate buffer (Antigen Retrieval; Biogenex, San Ramon, CA). They were irradiated twice in a domestic microwave oven (800 W) at full power for 4 min and then left to cool for 15 min in the hot buffer at room temperature. All primary antibodies used are reported in the Table 1 (Fhit antiserum was generously provided by Dr. Kay Huebner, Kimmel Cancer Center, Jefferson Medical College, Philadelphia, PA). All sections were immunostained with the universal labeled streptavidin-biotin method (Immunotech) according to the manufacturer’s instructions. Positive

<table>
<thead>
<tr>
<th>Protein</th>
<th>Antibody</th>
<th>Dilution and incubation</th>
<th>Antigen pretreatment</th>
<th>Positive control</th>
<th>Stain</th>
</tr>
</thead>
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<tr>
<td>Bax</td>
<td>Dako</td>
<td>1:1500, 1 h</td>
<td>None</td>
<td>Tonsil</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Dako</td>
<td>1:40, 1 h</td>
<td>Microwave</td>
<td>Tonsil</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>p53</td>
<td>Biogenex</td>
<td>1:135, 2 h</td>
<td>Microwave</td>
<td>Breast cancer</td>
<td>Nuclear</td>
</tr>
<tr>
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<td>Pharmingen</td>
<td>1:100, overnight</td>
<td>Microwave</td>
<td>Colon tissue</td>
<td>Nuclear</td>
</tr>
<tr>
<td>Msh2</td>
<td>Oncogene</td>
<td>1:100, overnight</td>
<td>Microwave</td>
<td>Colon tissue</td>
<td>Nuclear</td>
</tr>
<tr>
<td>Fhit</td>
<td>Polyclonal (36, 38)</td>
<td>1:5000, 30 min</td>
<td>Microwave</td>
<td>Keratocytes ME180</td>
<td>Cytoplasmic</td>
</tr>
</tbody>
</table>

*a The primary antibody was omitted for the negative control.

![Fig. 1 Lack of Mlh1 nuclear protein expression in an invasive cervical cancer case showing immunoreactivity in dysplastic tissue (×200).](clincancerres.aacrjournals.org)
staining was detected with diaminobenzidine substrate solution, and nuclei were counterstained with hematoxylin.

**Interpretation of Immunoreactivity.** The immunoreactivity was classified as follows: −, 0; +, weak with <10% positive cells; ++, >10% but <50% strongly positive cells; and ++++, >50% strongly positive cells. Negative immunoreactivity was scored as either − or +, and positive immunostaining as ++ or ++++ (23). Slides were scored independently by two pathologists; the discordant cases were reviewed and reassigned scores based on consensus of opinion.

**Statistical Analysis.** The statistical correlation among Msh2, Mlh1, Fhit, p53, Bax, and Bcl-2 protein expression and histopathological features were analyzed by Fisher’s exact and Spearman’s rank (r) correlation coefficient (SPSS version 7.5.2, Chicago, IL; 1996). The statistical significance was set at \( P < 0.05 \).

**RESULTS**

**Immunohistochemical Analysis of Protein Expression.** Msh2 and Mlh1 protein expression was exclusively nuclear. Positive staining of Msh2 was detected in 13 of 14 (92.9%) NISCCs and in 13 of 23 (56.5%) ISCCs. Mlh1 immunoreactivity was observed in 10 of 14 (71.4%) NISCCs and in 8 of 23 (34.8%) ISCCs (Fig. 1).

Fhit protein expression was prevalently cytoplasmic (Fig. 2). Strong expression was detected in 2 of 23 (8.7%) ISCC cases, moderate in 6 of 23 (26%) ISCCs and 6 of 14 (42.9%) NISCCs, and low immunoreactivity in 8 of 14 (57.1%) NISCC cases. Fifteen of 23 (65.2%) ISCC cases proved negative.

Nuclear staining for p53 immunoreactivity was detected (Fig. 3). Eleven of 23 (47.8%) ISCCs and 4 of 14 (28.6%) NISCCs cases showed strong expression, 5 of 23 (21.7%) ISCC cases were found to have moderate positivity, whereas low expression was found in 4 of 23 (17.4%) ISCCs and 10 of 14 (71.4%) NISCC cases. Three of 23 (13%) ISCC cases were negative. Normal cervical epithelium adjacent to tumor did not stain overexpression of p53 protein.

Immunostaining specific for Bax was cytoplasmic. Five of 23 (21.7%) ISCC and 10 of 14 (71.4%) NISCC cases showed strong positive expression. Moderate positive staining was found in 6 of 23 (26%) ISCC cases. Low expression was found in 10 of 23 (43.4%) ISCC and 2 of 14 (14.3%) NISCC cases. Two of 23 (8.7%) ISCC and 2 of 14 (14.3%) NISCC cases were found.

Immunostaining for Bcl-2 was prevalently cytoplasmic. Eight of 23 (34.8%) ISCC cases showed strong expression, whereas 7 of 23 (30.4%) ISCC and 2 of 14 (14.3%) NISCC cases showed moderate expression. Low expression was found in 1 of 23 (4.3%) ISCC and 10 of 14 (71.4%) NISCC cases. Seven of 23 (30.4%) ISCC and 2 of 14 (14.3%) NISCC cases were negative.

**Expression of Specific Proteins and Histological Tumor Grading.** Msh2 and Mlh1 expression correlated significantly with NISCC (\( P < 0.02 \) and \( P < 0.04 \), respectively; Table 2); p53 and Bcl-2 overexpression was found to be significantly correlated with ISCC versus NISCC (\( P < 0.02; P < 0.003 \), respectively; Table 3), whereas no significant relationship was
observed between other biological markers and histopathological features. No significant correlation was observed among histopathological grading and biological markers.

Statistical analysis between biological markers showed significant correlation between Msh2 and Mlh1 protein absence and overexpression of p53 ($r = 0.481$; $r = 0.513$ (P = 0.001 and P = 0.001, respectively)). The relationship between Fhit protein expression and Bax was also statistically significant: ($r = 0.344$ (P < 0.02)). No correlation was found for any other parameters.

**DISCUSSION**

Inactivation of DNA repair genes results in an increased rate of mutation in gatekeeper genes that control cell proliferation and death (24), increase in genomic instability (25), and acceleration of tumorigenesis (26). Defects in the MMR genes Msh2 and Mlh1 were detected in hereditary nonpolyposis colon cancer patients and in some sporadic colorectal cancer, and no expression of either Msh2 and Mlh1 protein has been reported in tumors with microsatellite instability (27, 28). In cervical cancer, the literature reported different results with regard to microsatellite instability and mutator phenotype (29–33). Larson et al. (31) reported an attenuated mutator phenotype in cervical carcinoma, which suggests in cervical carcinogenesis an improbable MMR deficiency. By immunohistochemistry, in this preliminary study, we detected the absence of Msh2 and Mlh1 immunoreactivity in 1 of 14 (7.1%) and 4 of 14 (28.6%) cases, respectively, of NISCC. Conversely, in ISCC, it was found in 10 of 23 (43.5%) and 15 of 23 (65.2%) cases, respectively (Table 2). The lack of protein expression observed by immunohisto-

<table>
<thead>
<tr>
<th>Table 2</th>
<th>p53, Msh2, and Mlh1 expression in cervical carcinoma</th>
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<tbody>
<tr>
<td></td>
<td>p53 Positive (++) Negative (-) Msh2 Positive (+) Negative (-) Mlh1 Positive (+) Negative (-)</td>
</tr>
<tr>
<td>NISCC (n = 14) (%)</td>
<td>4* (28.6) 10 (71.4)</td>
</tr>
<tr>
<td>G1* (n = 8) (%)</td>
<td>4 (50.0) 4 (50.0)</td>
</tr>
<tr>
<td>G2 (n = 12) (%)</td>
<td>9 (75.0) 3 (25.0)</td>
</tr>
<tr>
<td>G3 (n = 3) (%)</td>
<td>3 (100) 0 (0)</td>
</tr>
<tr>
<td>ISCC (n = 23) (%)</td>
<td>16* (69.6) 7 (30.4)</td>
</tr>
</tbody>
</table>

*–c Fisher’s exact test: * P < 0.02; * P < 0.02; * P < 0.04.

G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated.

Fig. 3 p53 overexpression in invasive cervical cancer (×400).
chemistry in these cases could be speculative for somatic alterations in both Msh2 and Mlh1.

Alteration of the FHIT gene was observed in several types of carcinoma (34–38). Aneuploid FHIT transcripts were detected in cervical carcinoma cell lines and primary carcinomas (39) and correlated with a significant loss of Fhit protein by immunohistochemistry (37). In previous studies, the loss of Fhit expression was detected in 33–76% of cervical carcinomas, whereas high levels of Fhit expression were detected in normal cervical epithelium but less frequently in squamous intraepithelial lesions (37, 38, 40–42). Nakagawa et al. (43) suggest that the alteration of the FHIT gene may be an early genetic event during the generation of cervical carcinoma but not the consequence of cancer progression. On the other hand, Yoshino et al. (42) suggest that alterations of the Fhit gene can occur as later events in tumorigenesis. Hendricks et al. (44) reported abnormal Fhit expression in 63% of cervical cancer cell lines. Later, Birrer et al. (45) detected an abnormal staining in 61% of primary cervical carcinoma, confirming that FHIT gene dysfunction is a frequent event in cervical carcinoma. In agreement with Birrer et al. (45) in our study, we actually observed an absence of Fhit protein expression in 15 of 23 (65.2%) ISCCs and 8 of 14 (57.1%) NISCCs (Table 2). Moreover, we found that the Fhit protein was strongly expressed in dysplastic koilocytosis tissue but was reduced or actually disappeared in areas of invasive carcinoma (Fig. 2).

Recently, Sard et al. (46) found that the FHIT gene is apparently associated with a proapoptotic function. We observed a significant correlation between proapoptotic Bax and Fhit protein expression that was independent of tumor grading (P < 0.02).

In cervical cancer, the frequency of p53 mutation is rather low compared with mutations in other tumors, such as lung or colon carcinomas (47). Different results concerning p53 protein expression percentage are reported in the literature. Rajkumar et al. (48) reported only 4 of 40 (10%) cases of SCC positive for p53 protein expression; Crawford et al. (49) observed 16 and 34% of cases p53 positive in cervical carcinoma using semi-quantitative (cutoff at 5%) and quantitative methods of scoring, respectively.

Raju et al. (50) detected protein expression in 11 of 19 (58%) cases of SCC; Skomedal et al. (51) found elevated p53 protein in 41 of 74 cases (55%) of cervical cancer. Our study using a semiquantitative method (cutoff at 10%) indicated p53 protein overexpression in 16 of 23 (69.6%) cases of ISCCs and 4 of 14 (28.6%) cases of NISCC with a statistically significant correlation (P < 0.02; Table 3). The different percentage of p53 expression observed in literature could be explained by methodological differences, the type of antibody that recognizes wild-type and mutant p53, and different scoring and cutoff of the protein expression. Normal cervical epithelium adjacent to tumor did not stain p53 protein, which confirms reports by Cattoretti et al. (52) and Caamano et al. (53) that the steady-state level of normal p53 protein is too low to be detected immunohistochemically. A monoclonal antibody that recognized wild-type and mutant p53 (DO7) was used. The wild form of p53 protein has a very short half-life, and it cannot be detected by immunohistochemistry (52–54). However, overexpressed or mutated p53 proteins have a longer half-life and can be recognized by immunohistochemistry (55). Mutated p53 proteins complexed with other proteins, such as heat shock protein, may not be completely degraded by the protease system involving the gene E6 products of HPV and can be detected by immunohistochemistry (56).

Msh2 and Mlh1 absence protein expression correlated with p53 overexpression as P < 0.001 and P < 0.001, respectively. No studies are reported in literature on the immunohistochemistry of MMR in cervical cancer; for this reason, verification of these preliminary findings in a much larger number of cases is necessary.

It is generally accepted that the potential of tumor progression is associated with the balance between cell death and cell proliferation and that the apoptotic process plays an important role in that balance. Members of the gene family that includes BCL-2 and BAX are involved in the control of apoptosis in a range of different cell types. Because BAX induces apoptosis and BCL-2 protects cells, it has been accepted that Bcl-2 and other members of the family compete to inactivate it through binding Bax (57). It is commonly accepted that prolonged cell survival with inhibition of apoptosis is associated with carcinogenesis. Kokawa et al. (58) reported a large fraction of ISCCs immunonegative for Bcl-2, and Bax was detected weakly in a small fraction of ISCCs. Saegusa et al. (59) found that Bcl-2 immunoreactivity was significantly statistically correlated with CIN 3 in ISCC but not for Bax. We detected Bcl-2 expression in 15 of 23 (65.2%) of ISCCs and 2 of 14 (14.3%) NISCCs with a significant difference (P < 0.003). No correlation was found between Bax and NISCC and ISCC. No correlation was found between Bcl-2 and Bax expression.

Mutated p53 expression was found to be significantly

### Table 3  Fhit, Bax, and Bcl-2 expression in cervical carcinoma

<table>
<thead>
<tr>
<th></th>
<th>Fhit</th>
<th>Bax</th>
<th>Bcl-2</th>
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<tbody>
<tr>
<td></td>
<td>Positive (+/++)</td>
<td>Negative (−/+)</td>
<td>Positive (+/++)</td>
</tr>
<tr>
<td>NSCC (n = 14) (%)</td>
<td>6 (42.9)</td>
<td>8 (57.1)</td>
<td>10 (71.4)</td>
</tr>
<tr>
<td>G1 (n = 8) (%)</td>
<td>3 (37.5)</td>
<td>5 (63.5)</td>
<td>6 (75.0)</td>
</tr>
<tr>
<td>G2 (n = 12) (%)</td>
<td>3 (25.0)</td>
<td>9 (75.0)</td>
<td>3 (25.0)</td>
</tr>
<tr>
<td>G3 (n = 3) (%)</td>
<td>2 (66.7)</td>
<td>1 (33.3)</td>
<td>2 (66.7)</td>
</tr>
<tr>
<td>ISCC (n = 23) (%)</td>
<td>8* (34.8)</td>
<td>15 (65.2)</td>
<td>11* (47.8)</td>
</tr>
</tbody>
</table>

G1: well differentiated, G2: moderately differentiated, G3: poorly differentiated.

*− Fisher’s exact test: * not significant, b P < 0.003.
associated with the loss of Msh2 and Mlh1 protein expression. In conclusion, these preliminary data indicate that the aberrant expression of Msh2, Mlh1, p53, and Bcl-2 could be involved in cervical cancer through NISCC to ISCC progression. FHIT gene alteration may play a role as a component of a proapoptotic pathway.

REFERENCES


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