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Expression of Mismatch Repair Proteins in Invasive and *In Situ* Carcinoma of the Breast

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Abstract. *Background: Genetic instability is a characteristic feature of familial and sporadic breast carcinomas. It is not clear whether defects in the mismatch repair system accompany this instability. The purpose of this study was to explore the expression of two of the proteins encoded by the DNA mismatch repair genes, namely MLH1 and MSH2, in sporadic in situ and invasive breast carcinomas of various types and grades occurring in Greek patients. Materials and Methods: MLH1 and MSH2 expression was monitored immunohistochemically in 60 breast carcinomas (20 in situ and 40 invasive). Results-Conclusion: Although we did not detect loss of MLH1 or MSH2 expression, we do believe that our data will contribute to a better understanding of the role of the mismatch repair (MMR) system in breast cancer.*

Tumorigenesis is a multistep process, each step reflecting specific genetic alterations that drive the progressive transformation of normal to highly malignant human cells (1). The mutation rate of somatic cells is too low to account for the number of mutations required for a cell to undergo carcinogenesis. Thus, the development of genetic instability is a critical early step towards carcinogenesis. Genetic instability, which is characteristic of most solid tumors, is broadly classified into microsatellite instability (MSI) associated with mutator phenotype, and chromosome instability (CIN) recognized by gross chromosomal abnormalities (2-4). CIN is the hallmark of aneuploid tumors and is usually associated with activation of oncogenes and/or inactivation of tumor suppressor genes. MSI leads to a positive replication error (RER+) phenotype, which is characterized by the instability and consequent frameshift mutations of small repetitive DNA sequences (microsatellites) scattered throughout the human genome in both coding and noncoding sequences (3-5). Such errors occurring during DNA replication remain undetected due to silencing of tumor DNA mismatch repair genes (MMR). The identification of the human homologues of the yeast MMR genes, hMSH2, hMLH1, hMSH6, hPMS1 and hPMS2, has prompted new interest in the field (5). Germ-line mutations in the genes MLH1, MSH2 or MSH6 are implicated in the pathogenesis of the majority of hereditary nonpolyposis colorectal cancers (HNPCC) (6). Mutations of the MMR genes are associated with lack of the corresponding gene product as assessed by immuno-histochemistry in tumor specimens. Studies have shown that 12-35% of sporadic colorectal carcinomas have also acquired defects in the DNA repair system, mostly due to epigenetic silencing of MLH1 by hypermethylation of the promoter region (7-9). Similar epigenetic changes have been reported in sporadic endometrial and gastric carcinomas (10,11). Moreover the RER+ phenotype has been described in ovarian, cervical, pulmonary, prostate and skin carcinomas (12-16).

Breast cancer is the most common malignancy affecting women in Western countries today and, therefore, is a field of intense investigation for potential predictive and prognostic genetic markers. Microsatellite instability appears to be one of the various genetic alterations found in breast tumors, however the results differ considerably in various studies concerning sporadic as well as familial breast cancer (17-35). Studies in colon cancer have shown that there is a high correlation between the presence of MSI and loss of MLH1 and MSH2 expression, as determined by immuno-histochemistry (36,37). Reports on the expression of these genes in breast cancer are contradictory (17-19, 38-40).

The aim of the present study was to investigate the expression of MLH1 and MSH2 in sporadic *in situ* and invasive breast carcinomas of various types and grades occurring in Greek patients.

Materials and Methods

Samples. We studied 20 *in situ* carcinomas (15 DCIS and 5 LCIS) and 40 invasive carcinomas: 20 ductal NST (2 Grade I, 12 Grade II and 6 Grade III), 8 papillary, 2 mucinous and 10 lobular. The median age of the patients was 54 years (range 44-79).

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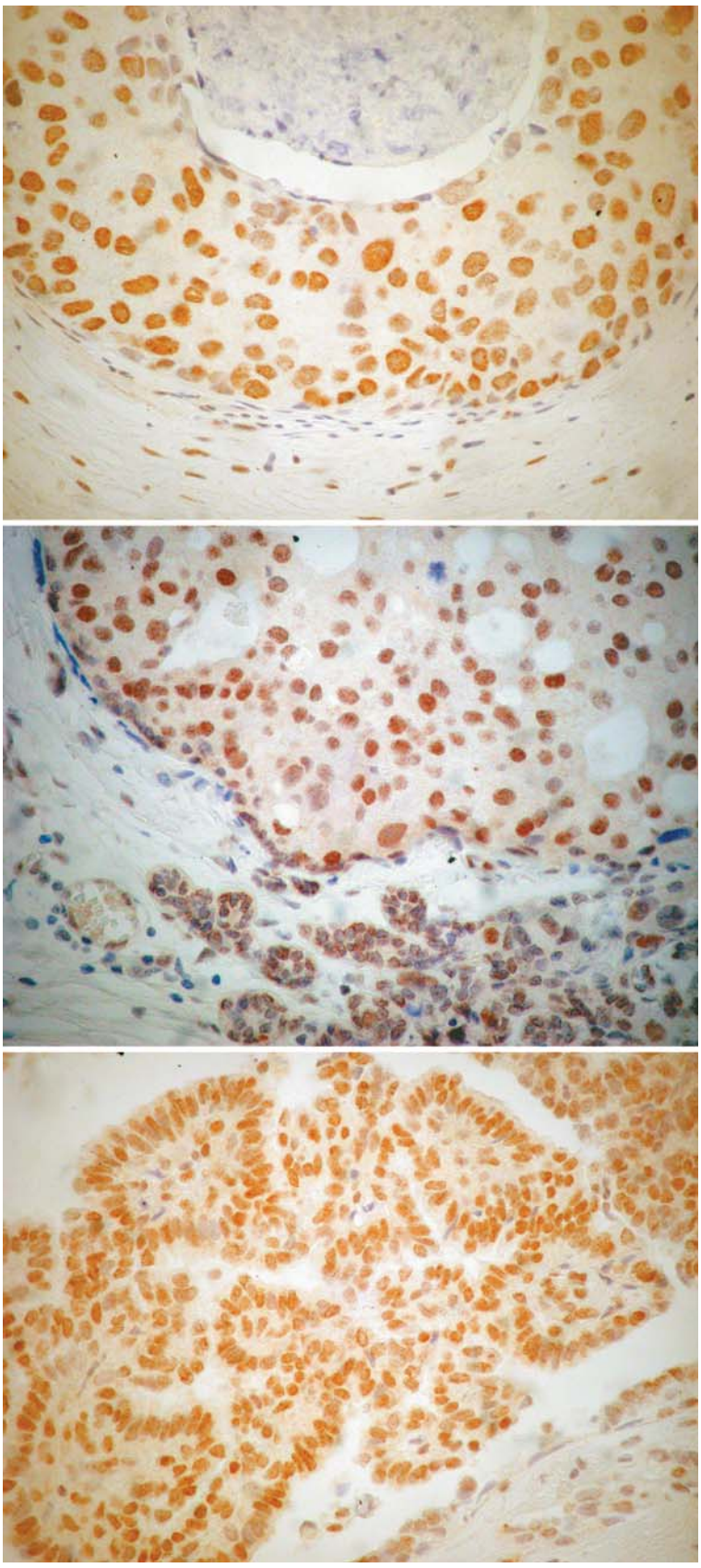

Figure 1. *A: Ductal carcinoma in situ, comedo type, showing intense nuclear immunostaining for hMLH1. (DABX400). B: Ductal carcinoma in situ, cribriform type, showing nuclear immunostaining for hMSH2, comparable to that in adjacent non neoplastic tissue. (DABX400). C: Papillary carcinoma in situ, exhibiting specific positive staining with anti-hMLH1. (DABX400).*

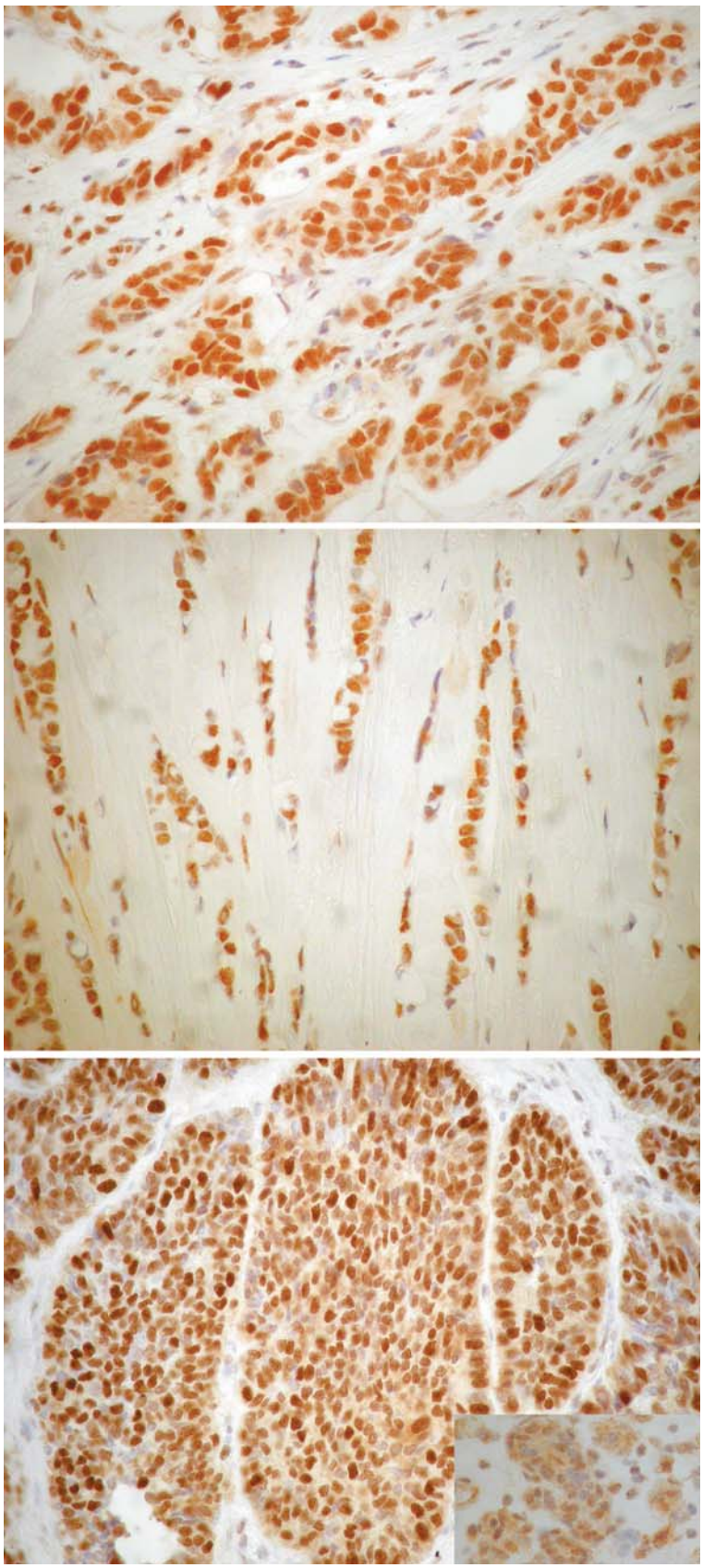

Figure 2. *A: Infiltrating ductal carcinoma, NST, showing nuclear immunostaining for hMLH1. (DABX400). B: Infiltrating lobular carcinoma showing nuclear immunostaining for hMSH2. (DABX400). C: Infiltrating papillary carcinoma exhibiting strong nuclear immunostaining for hMSH2. In insert (lower right corner) the non-neoplastic breast tissue in the same section displays only moderate staining intensity. (DABX400).*

Samples were fixed in 10% (v/v) buffered formalin and embedded in paraffin. Serial 5-μm sections were obtained for staining with haematoxylin and eosin and immunohistochemistry.

Immunohistochemistry. MLH1 and MSH2 expression was detected by immunohistochemistry using the EnVision System (DAKO, Carpinteria, CA, USA), and the monoclonal antibodies MLH1 (G168-15, BIOCARE MED, Walnut Creek, CA, USA) and MSH2 (FE-11, BIOCARE MED). Briefly 5-μm-thick, histological sections were dewaxed in xylene, rehydrated through graded alcohols, immersed in 0.01 M citric buffer (pH 6.0), and microwaved twice for 5 minutes each. Subsequently, the sections were incubated with 0.3% H_2O_2 for 30 min to block endogenous peroxidase activity. The sections were then incubated for 1 h at RT with the primary antibodies (dilution 1:20 for MLH1 and 1:100 for MSH2)). Non specific binding was blocked by incubating the sections for 30 min with Blocking Solution (DAKO). Negative controls were processed by omitting the primary antibody and substituting it with non-immune serum.

Detection was carried out using the EnVision System kit (DAKO) with diaminobenzidine as chromogen. Counterstaining was performed with hematoxylin Harris.

The evaluation of immunohistochemistry was done independently by two pathologists. Both the percentage of stained neoplastic cells as well as the intensity of staining were recorded and compared with that seen in normal breast tissue in the same section.

Results

MLH1 and MSH2 expression. The MLH1 and MSH2 staining patterns in normal cells are nuclear. Expression was noted in benign epithelium, stromal cells and lymphocytes. In cases of *in situ* carcinoma, the retained myoepithelial cells also served as internal positive controls.

Nuclear staining was observed with MLH1 and MSH2 antibodies in more than 95% of neoplastic cells in all cases of *in situ* carcinoma examined. The presence and the intensity of the staining with both antibodies was comparable to that of non neoplastic epithelial cells in 19 cases (Figure 1A, B). In only one case of DCIS, papillary type, the staining for MLH1 was stronger than that of the adjacent normal breast tissue while the staining for MSH2 was similar (Figure 1C).

The intensity of nuclear staining for MLH1 and MSH2 within invasive carcinomas was more variable (Figure 2A, B). Regarding MLH1, only one papillary carcinoma exhibited decrease in percentage and intensity of staining (70% of tumor cells stained weakly). All others exhibited staining comparable with the non neoplastic breast tissue (>95% of epithelial cells stained, moderate to strong intensity).

Immunostaining for MSH2 revealed staining of more than 95% of neoplastic and normal epithelial cells, in all cases examined. Interestingly, 3 cases of invasive ductal carcinoma (2 Grade II and 1 Grade III) and 6 out of 8 papillary carcinomas displayed increase in staining intensity compared to normal tissue (Figure 1C). The papillary carcinoma that exhibited decreased staining with MLH1, showed specific nuclear staining for MSH2 comparable to adjacent non neoplastic breast tissue.

Discussion

The positive replication error (RER+) phenotype is associated with defects of three intracellular mechanisms involved in DNA damage repair, namely the nucleotide excision repair (NER), base excision repair (BER) and mismatch repair (MMR) (2-4). Defects in the DNA MMR are responsible for a variety of hereditary cancer predisposition syndromes including hereditary non-polyposis colorectal carcinoma (HNPCC), Bloom syndrome, ataxia-telangiectasia and Fanconi anemia (2). While MSI is classically attributed to genetic defects, there are also epigenetic factors that have emerged as being equally damaging to cell cycle control. For example, it has been shown that hypermethylation of DNA MMR gene promoter regions is an epigenetic mechanism of gene silencing that contributes to tumorigenesis (7-9).

MSI has been shown in 0-30% of sporadic breast tumors and in 0-83% of familial breast carcinomas (17-35). In breast tumors, MSI appears to be different from that of HNPCC because it occurs in fewer markers, the microsatellite foci are mainly trinucleotides and tetranucleotides and the instability leads to the generation of new alleles that have very different size from the normal ones (21,33,41). Although not all reports agree, it seems that in breast the RER+ phenotype is associated with poorer prognosis compared with its RER- counterpart, in contrast to what happens in colon carcinoma. There also appears to be no association between the RER+ phenotype and age, tumor size, lymph node status or ER status (17). In medullary carcinomas, a low frequency of MSI has been reported, while in lobular carcinomas the frequency of MSI is higher (26,40). Furthermore, Stone *et al.* (42) have suggested that mutations in the MLH1 may underlie a subset of LCIS (lobular carcinoma *in situ*) cases.

Studies in colon cancer have shown that there is a high correlation between the presence of MSI and loss of hMLH1 and hMSH2 expression, as determined by immuno-histochemistry. Conserved expression of hMLH1 and hMSH2 implies that the tumor is microsatellite stable or that the loci showing instability are less than 30% (9). Regarding breast cancer, the immunohistochemical results are few and conflicting. Walsh *et al.* (38) have reported that MLH1, MSH2 and PMS2 are expressed in all 23 cases of ductal carcinoma *in situ* of the breast, even those with detected MSI, proposing that MSI in the breast is different than that in colon cancer. Adem *et al.* (43) investigated 30 familial and 40 sporadic breast cancers and reported that none had loss of hMSH2 expression. In addition, only one DCIS from a patient with BRCA2 deleterious mutation had loss of hMLH1. It is worth

noting that, in this study, none of the explored lesions exhibited MSI. The authors conclude that DNA MMR defects involving underexpression of hMLH1 and hMSH2 are extremely rare events in sporadic and familial breast cancer. Murata *et al.* (19) investigated 32 sporadic breast tumors for MSI, possible genetic and epigenetic modifications, as well protein expression of hMSH2 and hMLH1 genes. MSI was detected in 15 cases. In the tumors examined both genetic and epigenetic modifications were identified in these genes, which were not reflected in the expression of the protein products. So, they suggested that, in breast carcinomas, the MSI status might be underestimated by immunohistochemistry due to the expression of nonfunctional proteins.

On the other hand, Yang *et al.* (39) reported loss of MSH2 expression in 47% of the 53 examined breast carcinomas. Along these lines, Bock *et al.* (18) examined 119 cases of sporadic breast cancer and have also reported a decrease in MSH2 expression during tumor progression from *in situ* to invasive cancer. It is surprising that, in the same study, increased expression of the same protein is associated with grade, higher proliferative activity and lymph node involvement.

We did not detect loss of MLH1 or MSH2 expression in our material, which included sporadic ductal as well as lobular carcinomas. It is possible that the proteins detected by immunohistochemistry are not functional due to possible epigenetic modifications. It is also conceivable that other, still undiscovered, genes may repair DNA through different pathways than the MMR system and may be involved in MSI observed in breast carcinomas. An intriguing finding is the increase in staining intensity for MSH2 in the majority of invasive papillary carcinomas examined. Taking in account that papillary lesions exhibit distinct morphological features, it is also possible that this finding is another clue to unraveling their unique differentation profile. Molecular studies with more cases are necessary for definitive conclusions. Although we found no loss of MLH1 or MSH2 expression, we do believe that our data will contribute to a better understanding of the role of the MMR system in breast cancer.

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