

Expression of Genes Responsible for the Repair of Mismatched Bases of the DNA (MLH1) in Invasive Ductal Breast Carcinoma

Rudolf Milanović¹, Sanda Stanec², Mladen Stanec³, Anđelko Korušić⁴, Ino Husedžinović⁴
and Jasminka Jakić Razumović⁵

¹ University of Zagreb, University Hospital »Dubrava«, Zagreb, Department for Plastic, Reconstructive and Aesthetic Surgery, Croatia

² »Edumed« Polyclinic, Zagreb, Croatia

³ University of Zagreb, University Clinical Hospital »Sestre milosrdnice«, Department of Surgical Oncology, Zagreb, Croatia

⁴ University of Zagreb, University Hospital »Dubrava«, Department of Anesthesiology, Reanimatology and Intensive Care Medicine, Zagreb, Croatia

⁵ University of Zagreb, University Hospital Center Zagreb, Department of Pathology, Zagreb, Croatia

ABSTRACT

Breast cancer is a heterogeneous group of diseases determined and distinguished by cellular type, gene expression and clinical signs and symptoms. Identification of histological and biological markers is of great value in predicting the progression of tumor growth and anticipating the expected response to various treatment options. Due to a high degree of cell proliferation in breast tumors and high genetic instability of these tumors, as a consequence of defective DNA repair mechanisms, chemotherapy as a treatment option often renders very successful results. During our scientific research we wanted to determine the involvement of the genetic polymorphisms of DNA mismatch repair system (MLH1 gene) and the subsequent development of breast carcinoma. This study included 108 patients who were surgically treated for invasive breast cancer at the Department of Plastic, Reconstructive and Aesthetic Surgery, University Hospital »Dubrava«. The expression of the MLH1 gene was determined by immunohistochemical methods. The results showed that 82.9% of tumor cells expressed the MLH1 gene. Analysis of survival rate for patients with invasive ductal breast cancer showed a statistically significant ($p=0.043$) correlation with the expression of MLH1 genes. The overall five year survival rate of our patients was 78.7%. These results indicate that there is a possible involvement of MLH1 gene in the progression and development of breast cancer.

Key words: breast tumor, DNA mismatch repair, genome replication, immunohistochemistry, MLH1 gene

Introduction

Breast carcinoma is the leading cause of cancer related deaths among women in the entire world. Every ninth woman can at some point in her lifetime develop this disease.

Breast carcinoma has a multifactorial etiology where exogenous and endogenous factors increase the risk of developing disease. Women in Croatia are at a high risk of developing breast carcinoma and there is a growing trend, with an increased number of young females developing aggressive subtypes when compared to other western countries. Although there have been many develop-

ments in the diagnosis and treatment of breast carcinoma, this disease still remains the leading cause of death in women aged 35 to 59¹.

Breast carcinoma is the result of abnormal genetic and epigenetic changes that occur, specifically in the BRCA and BCRA1 genes². There are more than 40 genes that have been shown to be inactive in carcinoma cells including the genes responsible for DNA repair, cycle regulation, cell adhesion and cell signalization. These specified genes serve as potential targets for molecular studies and therapy opportunities. During replication, DNA po-

lymerase is responsible for base pair integration and while this process is prone to errors, incorrectly matched base pairs are repaired via the mismatch repair system (MMR). The decreased activity of the MMR protein leads to a mutated phenotype with an increased degree of spontaneous mutations. This results in microsatellite instability (MSI) in the repeating mono and di-nucleotide regions, causing a high percentage of mutated locations, genome hypervariability, decreased apoptosis and a predisposition for developing breast carcinoma. It has been proven that in breast carcinoma the gene responsible for recognizing the mismatched base pairs, MSH2 gene, can be mutated into many polymorphic variants³.

This research was conducted with the aim to compare the profile of the MMR gene with patient clinical-pathological status, the degree of hormone receptors and survival rate. Therefore, patients with breast carcinoma from Croatia were observed to determine the involvement of the genetic polymorphisms of the MLH1 gene and the subsequent development of breast carcinoma.

Materials and Methods

This research analyzed the prognostic parameters and used paraffin preserved biopsies of invasive ductal breast carcinoma and subsequent lymph node metastasis. 108 female patients were included who were operated between 1999 and 2002. 18 patients were excluded due to the lack of biopsy material in the Clinical Hospital Dubrava in Zagreb, Croatia archive. The majority of the sample group, 70 patients (77.8%) were from the first two stages of disease classification. In the first stage there were 34 patients (37.8%) while in the second there were 36 (40.0%), in the third stage of disease there were 16 (17.8%) while in the fourth stage only 4 (4.44%) patients. All the patients were initially surgically treated with axillary lymph node resection as well. Afterward, standard adjuvant therapy was indicated and initiated. In all of the patients, information regarding tumor size, TNM stage, age, menopausal status, type of adjuvant therapy used, lymph node status, residual disease, length of disease remission and overall survival rates were collected. Only patients that complete that statistical information were included in the study.

The main ethical committee at the Clinical Hospital Dubrava in Zagreb, Croatia approved the methods and research conducted. The conducted procedures were in accordance with the ethical standards of the institutional, regional, or national committee on human experimentation, and with the Helsinki Declaration of 1975.

The analysis of variance between quantitative variables such as the degree of differentiation with clinical and laboratory standards was compared using the ANOVA method while qualitative variables were analyzed using the χ^2 -test.

In order to determine the expression of the MLH1 gene, biopsy samples were analyzed after fixation in 10% formalin and preserved in paraffin blocks. Afterward, 4

micrometer samples were cut stained with hematoxylin and eosin and specific MLH1 antigen targeting antibody was applied. Immunohistochemical detection for estrogen and progesterone receptors was applied as well as for the hMLH receptor. Positive results were identified by light microscopy as samples which had more than 5% of the tumor cell stained. In order to determine positive results, the number of positive immunoreactive cells was calculated^{4,5}. The degree of MLH1 gene expression is shown semiquantitatively, (negative (0 cells), slightly positive (1–50 cells) and positive (>50 cells)). Relative degrees of marker expression are compared with other histological and clinical factors with relation to overall survival time and survival time without signs of disease. The second method of identifying positive samples involved recognizing the percentage of positive tumor cells in a sample of 100 cells.

Both methods of cell identification were used for statistical analysis. In all invasive ductal carcinoma samples, the degree of histological differentiation (poorly, medium and well differentiated tumors) and the degree of local tumor advancement (pathological T and N stage) was noted. The overall MLH1 gene expression in samples of invasive ductal carcinoma tissue and lymph node metastasis samples were positively identified based on a positive reaction of the monoclonal antibody defined by the manufacturer. The degree of MLH1 molecule expression in tumor and lymph node samples was compared with the overall survival time of the patient. The collected data was analyzed and results about the possible association of MLH1 gene in invasive ductal carcinoma in Croatian women were documented.

Results

Experimental samples were obtained from female patients with an average age at diagnosis of 56.1 year. In this study, a family history of breast carcinoma was positive in 14 patients (15.6%), and negative in 76 patients (84.4%). The average size of the tumor in the sample population was 22.7 mm (standard deviation 13,6). All tumors were between 5 and 70 mm. Patients were organized according to the size as governed by the TNM classification system; tumors up to 2 cm were found in 53 (58,9%) patients, tumors between 2–5 cm in 35 (38,9%) patients, and tumors larger than 5 cm in 2 patients (2,2%). The majority of patients had tumors in the left breast 59 (65,6%) while 31 (34,4%) patients had a tumor in the right. Well differentiated tumors were found in 22 patients (24,4%), the majority of the patients 34 patients (37,8%), had an medium differentiated tumor, while poorly differentiated tumors were found in 29 (32,2%) patients. 5 patients (5,6%) out of the original sample lacked sufficient data needed and were not included. Lymphovascular invasion was documented in 10 patients (11,1%), while the majority of the patients (88,9%) lacked lymphovascular invasion at the time of initial diagnosis. In 87 (96,7%) patients, the tumor did not possess an area

of necrosis. The majority of the patients (54.4%) did not have lymph node involvement at the time of diagnosis, 24 patients (26.7%) had between 1–3 positive nodes (N1 stage), 10 patients (11.1%) had between 3–10 positive nodes (N2 stage) and more than 10 positive nodes (N3 stage) were found in 7 patients (7.8%). Distant metastases were found in 4 patients (4.4%). 35 patients (38.9%) had estrogen receptor negative disease while the rest were positive with variable degrees of intensity and percentage according to their H-score. 44 (48.9%) patients were progesterone receptor negative.

Patients with breast carcinoma were treated with surgery, adjuvant cytostatic, hormonal and/or radiotherapy according to the stage of the disease and steroid receptor status. Tamoxifen as an adjuvant therapy was given to 53 (58.9%) patients while the rest, 35 (38.9%) were not treated with adjuvant hormonal therapy. 76 (84.4%) received some other form of adjuvant chemotherapy while 13 patients did not. One patient did not have the documentation of which therapy they had received. In the sample group, 57 patients (63.3%) were treated with radiotherapy while 32 (35.6%) were not. Out of the entire sample group, only 8 (8.9%) patients relapsed with disease.

The average percentage of positive immunoreactive tumor cells was found to be 82.9% (standard deviation 24.0 while results ranged between 0% (1 patient) and 100% (Figure 1))

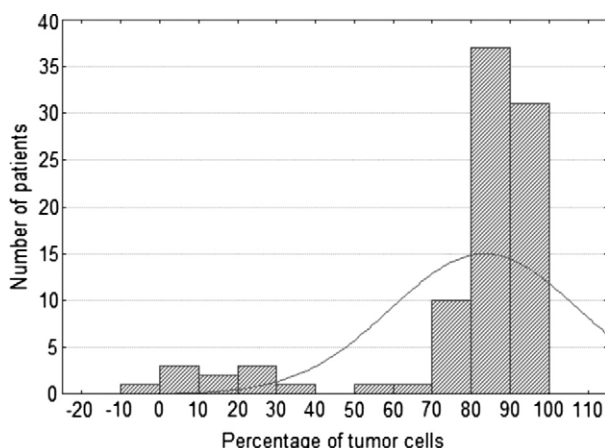


Fig. 1. Number of patients and the percentage of tumor cells with a positive expression for MLH1 gene

The majority of tumor samples were found to possess MLH1 gene with 70–100% of the cells staining positive. The degree of staining intensity was classified into 4 groups (Table 1: Figures 2, 3, 4 and 5). One sample was immunohistochemically negative, 20% weakly stained (category 1), while the rest of the samples stained between medium (category 2) and high intensity (category 3) for the MLH1 gene. Figures 2, 3, 4 and 5 display the various immunohistochemical intensities in biopsy samples when stained for the MLH1 gene.

TABLE 1
VARIOUS DEGREES OF STAINING INTENSITY IN BIOPSY SAMPLES

Staining intensity	Number of biopsies	%
0	1	1.1
1	18	20.0
2	39	43.3
3	32	35.6

When the patients were observed in regards to age and histological tumor grade, it was found the average age of patients with low grade carcinomas was 60.86 years, for medium differentiated tumors (grade 2) was 56.6 years while patients with poorly differentiated (stage 3) tumors was 54.69. In patients with medium differentiated tumors at the time of initial diagnosis, the most frequent carcinoma grade was found to be grade 1 (16 patients), while only 5 patients with poorly differentiated tumors was found to be in the first stage. Well differentiated tumors in stage 1 were observed in 9 patients. The majority of patients in the third stage of disease (n=10) were found to have poorly differentiated tumors. Estrogen receptors were not detected in poorly differentiated tumors. The highest degree of MLH1 positive tumors were seen in the medium differentiated tumor group.

Results indicate that there is no significant statistical relevance between the expressions of MLH1 gene with prognostic factors such as: size of tumor, lymph node involvement, or positive steroid receptors, nor with any clinical and pathohistological signs such as age, size of the tumor, distant metastasis, stage of disease, lymphovascular invasion or with tumor necrosis. There was a relationship between the expressions of the MLH1 gene with the histological grade of tumors (Figure 6).

When the percentage immunoreactive cells were compared with the tumor differentiation, there was no statistical significance. (Kruskal-Wallis test, $H=4.96$; $p=0.084$).

When the relationship between prognostic factors and the relapse of disease were compared, there was no correlation between the relapse of disease and age, lymph node involvement, stage of differentiation, presence of estrogen or progesterone receptors, adjuvant chemotherapy and adjuvant hormonal therapy. There was a positive correlation between disease relapse and the size of the original tumor.

In the sample group, the overall 5 year survival rate was 78.7%, with 75% of these patients without any relapse of disease. The disease free survival period was calculated as the time from initial diagnosis until the relapse of disease in months. It has been found that the overall survival rate is related to the size of the tumor ($p=0.004$), involvement of axillary lymph nodes ($p=0.018$), the presence of distant metastasis ($p=0.036$) and the stage of disease ($p=0.001$). All other factors observed were not statistically significant when compared with the overall survival rate. Analysis of the overall survival

Figs. 2, 3, 4 and 5 display the various immunohistochemical intensities in biopsy samples when stained for the MLH1 gene

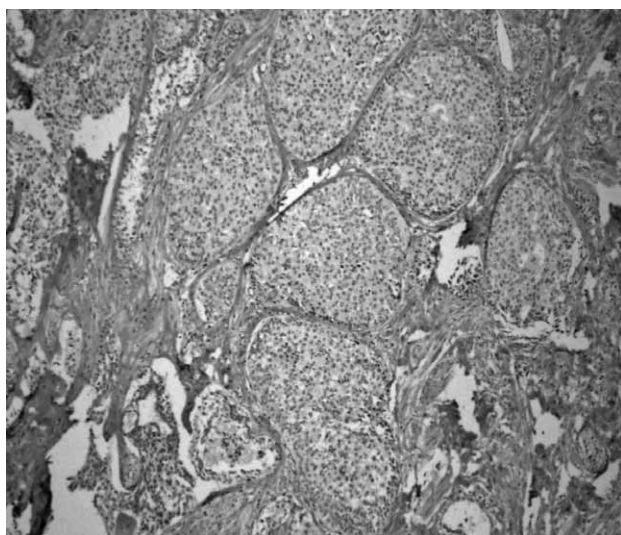


Fig. 2. Negative reaction for MLH1 (MLH1 x 100)

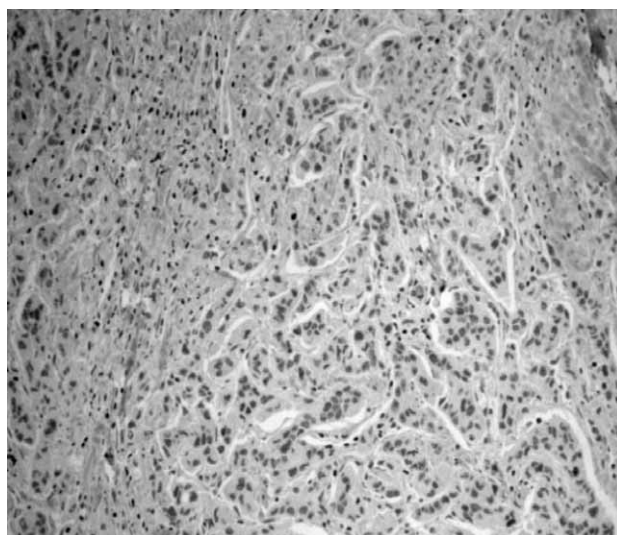


Fig. 3. Weak reaction for MLH1 (MLH1 x 200)

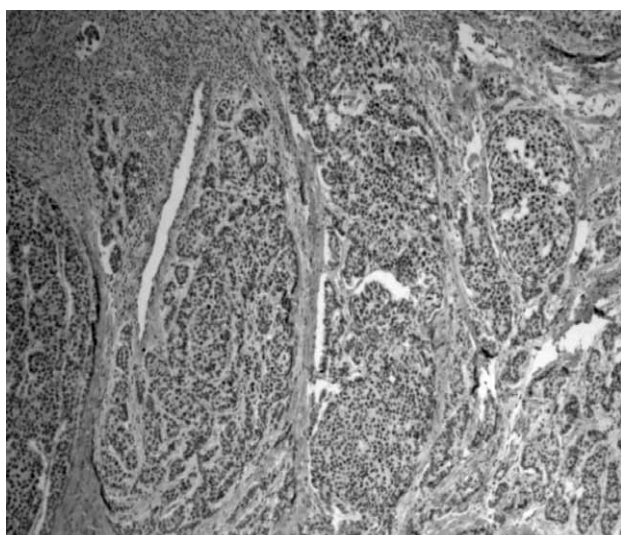


Fig. 4. Average intensity for MLH1 (MLH1x200)

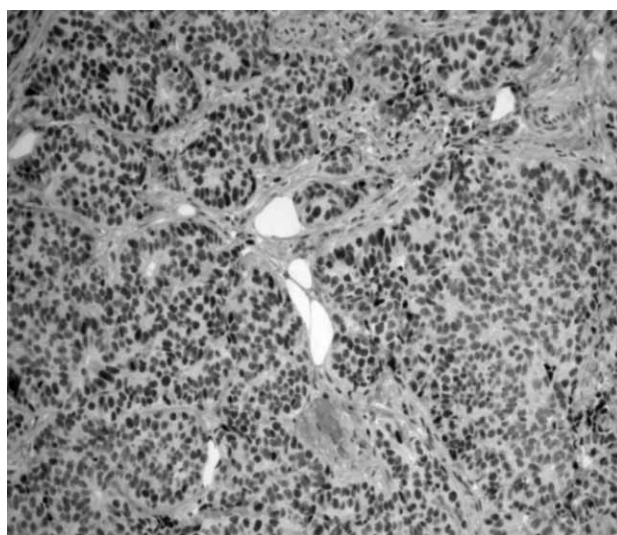


Fig. 5. Strong reaction for MLH1 (MLH1 x 200)

rate in patients with invasive ductal carcinoma of the breast was statistically related to the expression of the MLH1 gene in tumor cells ($p=0.043$) (Figure 7). There is a statistically significant relationship in survival rates when depending on the pT stage ($p=0.003$).

There was a statistically significant relationship between the overall survival rate and the presence of distant metastasis ($p=0.006$). When comparing patients with and without metastasis, it was found that patients who did not have distant metastasis statistically had a longer survival period. There was a significant difference in the overall survival rate between the various stages of disease ($p=0.001$). The 5 year survival rate in the first stage of disease was 90% while in the fourth stage was

only 25%. It is important to note that the majority of stage 4 patients die within 20 months of diagnosis.

Discussion

The majority of authors agree that the successful treatment of breast carcinoma is dependent on early diagnosis and that a tumor smaller than 1 cm in the majority of cases is curable. There are many authors who state that breast carcinoma from the start is a systemic disease^{6,7}. Support of this theory can be seen with the variable biological behavior of breast cancer, especially the invasive ductal carcinoma which is the most frequently seen. Due to this variability, the difficulty in treating this

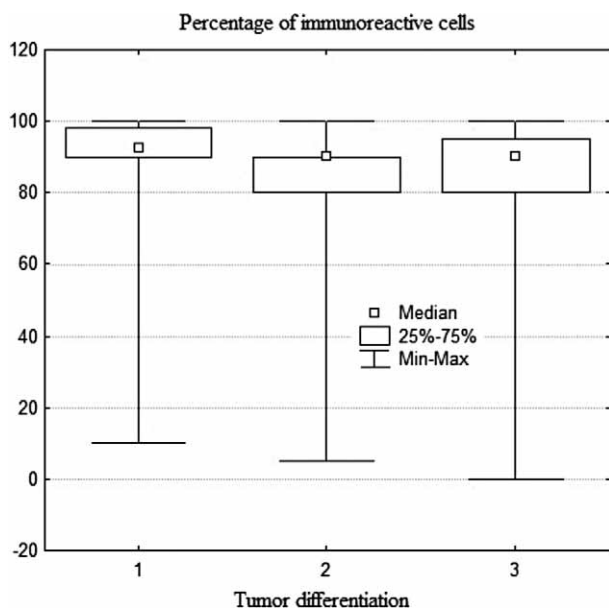


Fig. 6. The percentage of MLH1 gene expression in tumors of various differentiations

disease can be understood because of the unfamiliarity with this type of tumor. Invasive ductal carcinoma is therefore the focus of a variety of different studies^{7,8}.

With the development of new medications such as cytostatic, the treatment of this disease is becoming more successful; although there are still numerous associated side effects. It is therefore extremely important to recognize which patients will have the greatest benefit of medication therapy and to minimize the side effects as much as possible^{9,10}. The progression of disease can be foreseen using the many known signs and symptoms at the time of diagnosis. These include the size of the tumor, the involvement of axillary lymph nodes and the hormonal receptor status¹¹. These factors directly influence the choice of therapy and it is necessary to identify which patients are at a high risk of developing secondary disease¹².

Research results show that patients with better differentiated tumors most often had smaller tumors ($p < 0.019$), lower number of lymph nodes with metastasis ($p < 0.027$), and more often had estrogen receptor positive tumors ($p = 0.008$). These results coincide with other literature findings¹³.

Our research failed to show a statistically significant correlation between survival rate and tumor grade which may possibly be due to the analysis of patients with different tumor sizes and at various stages of lymph node involvement.

Our research show the relationship between the size of the tumor and the survival rate ($p = 0.004$) as well as lymph node involvement and survival rate ($p = 0.018$). These results are similar to other studies which show that tumor size is an important prognostic factor¹³. Carter et al. analyzed 24740 patients with a tumor size

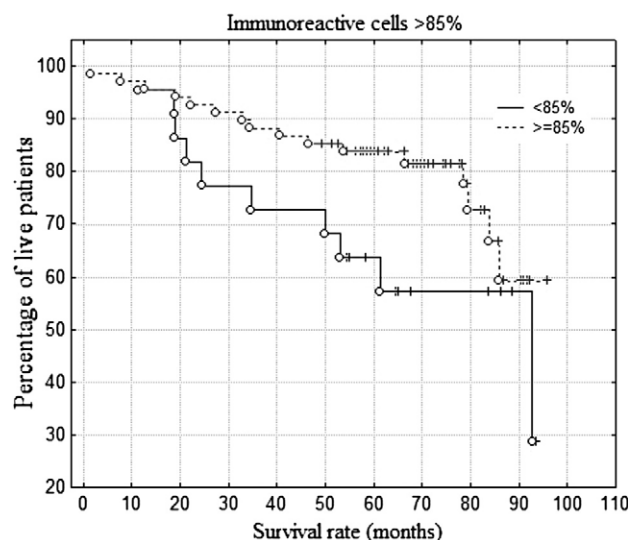


Fig. 7. Survival rates and the expression of MLH1 gene in breast cancer patients

smaller than 1 cm and found that 99% had a 5 year survival period while patients with a tumor between 1 and 3 cm, only 89% survived 5 years¹⁴. Lymph node involvement has also been found to be an important prognostic factor for survival as patients with lymph node metastasis had a lower survival rate which concurs with multiple foreign studies.

In 12 patients with invasive ductal carcinoma of the breast, 83% were found to have microsatellite instability regions in the associated gene which indicates the strong possibility of MMR in the development of breast carcinoma¹⁵. Results show that the MLH1 gene may possibly be used for the prognosis of disease and the possible opportunity for use in clinical practice. A lower degree of gene expression of the MLH1 gene was more common in carcinomas with distant metastasis as well as in larger tumors (larger than 3cm.). There was a correlation with the expression of MLH1 gene with the grade of the tumor, an important prognostic factor and therefore the MLH1 gene could also be used as a prognostic factor.

The study conducted by Sondes Karray-Chouayekh showed that tumors which were estrogen receptor negative were more malignant with less MLH1 methylations¹⁶. Our results also correlated with Layfield, who showed a relationship with positive hormone receptors and a lower tumor grade with a longer survival rate¹⁷.

Using immunohistochemical methods with MLH1 antibody, we intended to show the relationship between mismatched base pairs with other clinical-pathological factors in breast carcinoma. Quantitatively, gene involvement and the presence of protein products in the repair of mismatched bases were observed^{18,19}. Our goal was to determine the various MLH1 expressions and compare them with the other parameters associated with breast carcinoma.

It is well known that mutations within our genome are a constant occurrence and are most easily seen in base pairs which repeat (microsatellite regions). This occurrence of micro-satellite instability has been found in breast tumors by Chintamani, and Murata^{20,21}.

In order to determine the lack of MMR gene activity we tested the expression of the MMR gene protein product histochemically. The role of the MMR gene in breast carcinoma has been the focus of study for the last 20 years. It was found that the presence of MSI varies between 5–30% in certain studies and that it can be used as a determining factor for breast carcinoma. Murata and Mackay, observed 7 microsatellite regions within 20 breast carcinoma types and found variations in about 20% while Patel et al. found variations in microsatellite regions in about 85%^{21,22,24}. It is therefore necessary to determine the correlation between tumor markers and repair methods in invasive ductal carcinomas. It can be concluded that the immunohistochemically observed MMR protein is a good test for genetic MMR dysfunction in breast carcinomas^{21,22}. In the future surgery option this can also lead to the right choice of breast reconstruction method²³.

Our research agrees with the research conducted by Chintamania where no significant correlation was found between the expression of MLH1 gene and the age of the patient when diagnosed. Likewise we have observed similarities as with some authors about the correlation between the MLH1 gene expression and the histologic grade of the tumor²⁵. We observed that the gene expression and the tumor grade are inversely proportional, without statistical significance.

In one study by Ahn Byugg, 71 histological slides of stage II and stage III tumors was observed and a positive expression of MLH1 was found in 57.7% of cases²⁶. Those patients with advanced disease, axillary lymph node involvement and with little expression of MLH1 had a weaker response to chemotherapy than those patients with a high degree of expression²⁷. In breast carcinoma it was found that there is a loss of heterozygosity of the MLH1 gene in 46% of sporadic tumors. Khilko et al. (since 2007) have followed 121 patients with breast carcinoma and failed to show a loss of MLH1 expression in ever the entire patient group regardless of the various pathological and clinical signs. They have therefore concluded that this gene does not play a role in the development of breast carcinoma.

Lugman et al. have found MSI in up to 64% out of 108 patients located on the A25 microsatellite region²⁸. Orlandi et al. and Molist have not observed the presence of MSI in a series of 84, 267, 40 and 29 breast carcinomas respectively^{29,30}. They have all concluded that MSI is not common in breast tumors. Batistaton et al. has also failed to show the loss of MLH1 expression in 40 breast carcinoma samples³¹.

A large percentage of authors state that these MMR genes are not important factors in the development of

breast tumors, although they all agree the need for further research. Soudes, Karray et al. (since 2009.) have analyzed 74 patients with breast tumors and have shown the expression of MLH1 in 24.3% of samples¹⁶. They found a connection between MLH1 and the size of the tumor and have concluded that this gene facilitates tumor cell invasion. They also state that MLH1 gene is found in advanced metastatic disease. They found that 14.3% of their patients were in the T3 or T4 stage as compared to 4.8% in T1 or T2 stages, although this difference was not statistically significant. ($p=0.27$). There is a positive relationship between the efficacy of therapy and MLH1 expression ($p=0.004$). When the survival rates were observed in 34 patients in the period of 3–119 months, it was found that the expression of MLH1 can be a significant prognostic factor for breast carcinoma.

In their study, Kostera F. et al. found that out of 200 patients with breast carcinoma, MLH1 expression was positive in 86.5%, which is concurrent with our results of over 80% positive results¹⁹. There is a sign that the variability in microsatellite regions in breast cancer is different from that in colorectal carcinoma³².

Conclusion

The primary conclusion of this research is that the lowered expression of the MLH1 gene is rare in sporadic breast carcinomas, while the second conclusion is that the mutation of the gene responsible for base pair repair can be a secondary occurrence in the progression of breast carcinoma. The results in this study are similar to the results obtained by other authors who used similar methodology. Therefore, in this study, MLH1 was positive in 73 (80.22%) and negative in 17 (19.78%) patients. This discussion signifies the need for further research in order to determine the involvement of the genetic polymorphisms of DNA mismatch repair system (MLH1 gene) and the subsequent development of breast carcinoma, which is the most common malignant tumor in woman and whose incidence and mortality rate, is constantly increasing.

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Conflicts of Interest

All of the above mentioned authors certify that there is no conflict of interest with any financial organization, governing body or individual regarding the material discussed in this manuscript.

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R. Milanović

Department of Plastic, Reconstructive and Aesthetic Surgery, University Hospital »Dubrava«, Av. Gojka Šuška 6, 10000 Zagreb, Croatia
e-mail: smilanovic@kdb.hr

EKSPRESIJA GENA ODGOVORNIH ZA POPRAVAK KRIVO SPARENIH BAZA U MOLEKULI DNA (MLH1) KOD INVAZIVNOG DUKTALNOG KARCINOMA DOJKE

SAŽETAK

Rak dojke je heterogena skupina bolesti koju definiramo tipom zahvaćenih stanica i gena, te različitim kliničkom slikom. Identifikacija histoloških i bioloških markera od velike je važnosti u predviđanju progresije rasta tumora i odgovora na različite modalitete liječenja. Zbog visokog stupnja proliferacije stanica kod karcinoma dojke i visoke genetske nestabilnosti tih tumora, kao posljedica neispravnog mehanizma popravka DNA molekule, kemoterapijom se postižu uspješni rezultati liječenja. Tijekom našeg znanstvenog istraživanja željeli smo utvrditi stupanj genetskih polimorfizama kod gena koji su odgovorni za mehanizam ispravljanja krivo sparenih baza u molekulama DNA (MLH1 gena) i naknadnog razvoja raka dojke. Ovo istraživanje uključuje 108 pacijentica koje su kirurški liječene zbog invazivnog raka dojke na Klinici za plastičnu, rekonstrukcijsku i estetsku kirurgiju, KB »Dubrava«. Ekspresija MLH1 gena je određena imunohistokemijskim metodama. Rezultati su pokazali da je 82,9% tumorskih stanica imalo ekspresiju MLH1 gen. Analiza preživljenja bolesnica s duktalnim invazivnim karcinomom dojke je pokazala statistički značajnu ($p=0,043$) korelaciju s ekspresijom gena MLH1. Ukupna petogodišnja stopa preživljenja naših pacijenata bila je 78,7%. Rezultati potvrđuju prisutnost MLH1 gena te njihovu uključenost u progresiju i razvoj raka dojke.