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Expressional analysis of MLH1 and MSH2 in breast cancer

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ABSTRACT

Background: Mismatch repair proteins are ubiquitous keys in diverse cellular functions and protects the genome by correcting mismatch as post replication error correction machinery. Mismatch repair deficiency was associated with tumor development and progression therefore, current study was aimed to investigate MLH1 and MSH2 expression in breast cancer and correlate patients' clinicopathological factors with status of mismatch repair genes.

Material and methods: Breast cancer tissues with adjacent normal tissue along with clinical details were collected during surgery from 80 cases. Immunohistochemistry was performed with primary and secondary antibodies for expressional analysis. Results were analyzed using SPSS version 24.

Results: Immunohistochemical analysis revealed that both MLH1 and MSH2 were crucial in maintaining DNA repair system and loss of these 2 mismatch repair proteins may lead to adverse outcomes in breast cancer. Statistically significant association was found between loss of MLH1 ($P = 0.0004$; odds ratio 13.8; 95% confidence interval 4.6-41.1), MSH2 ($P = 0.0002$; odds ratio 14.0; 95% confidence interval 4.7-

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42.2) and breast cancer. Statistical analysis demonstrated that MLH1 and MSH2 deficiency may lead breast cancer progression to advanced stage, correlated with tumor focality (MLH1 $P = 0.001$; MSH2 $P = 0.002$) and chemotherapy (MLH1 $P = 0.01$; MSH2 $P = 0.04$). Presence of CK7, GATA 3, and E cadherin tends to increase in mismatch repair deficient breast cancer. Whereas, no association of mismatch repair deficiency was observed with age, tumor grade, positive lymph nodes, menopause, and ER and/or PR status.

Conclusion: Loss of mismatch repair proteins in breast cancer highlights its potential role in DNA repair mechanisms and helps tumor cells to become resistant against chemotherapeutic drugs. Therefore, mismatch repair deficiency may contribute to breast cancer progression.

Introduction

Breast cancer is the most commonly diagnosed malignancy and is responsible for huge number of cancer related deaths throughout the world. Despite immense advancement in the knowledge available regarding molecular basis of breast cancer development, no biomarker is used in disease management except BRCA1 and BRCA2.[1,2] Various genetic changes at molecular level have been considered as prognostic indicators but clinical data is limited.[3] In recent years, growing number of evidences support implications of microsatellite instability and mismatch repair deficiency in breast cancer etiology. Genetic alterations of 2 very important mismatch repair genes ie, MLH1 and MSH2 have been associated with breast cancer development.[3,4]

Functioning of mismatch repair proteins involves 2 central dimers MutLα (MLH1 and/or PMS2) and MutSα (MSH2 and/or MSH6). Intact MSH2 and MLH1 are compulsory to maintain the stability of MSH6 and PMS2 respectively. However, MLH1 and MSH2 stability can be maintained in the absence of MSH6 or PMS2 because role is compensated by other mismatch repair proteins. Therefore, expression analysis of particular proteins by immunohistochemistry can lead to precise estimation of affected mismatch repair deficiency.[5]

Estrogen (ER) and progesterone (PR) receptor status is an intrinsic component for clinical management of disease and many of post-menopausal breast cancer patients are ER or/and PR positive.[6] However, association between expression of these receptors and mismatch repair deficiency is not fully explored yet.

Breast cancer is treated with hormonal therapy, anticancer drugs, radiotherapy, and surgery. Some of the patients receive chemotherapy before surgery.[7] It was reported that loss of mismatch repair activity manifests cells resistant to various chemotherapeutic agents like 5-fluorouracil, cisplatin, topoisomerase inhibitors, and alkylating agents. Mismatch repair deficient phenotype may be developed during chemotherapeutic treatment.[3,8] Therefore, literature is lacking in the field weather mismatch repair deficiency is responsible for breast cancer development or mismatch repair deficient phenotype is a result of any chemotherapeutic agent.

To address these issues, current study was designed to evaluate the relationship between patients clinicopathological history, expressional analysis of mismatch repair proteins (MLH1 and MSH2) and breast cancer development.

Table 1
Summary of primary antibodies used.

Antibody	Company	Dilution/ready to use	Polyclonal or monoclonal	Origin
MLH1	Leica microsystems, UK	Ready to use	Monoclonal	Rabbit
MSH2	Leica microsystems, UK	Ready to use	Monoclonal	Rabbit
ER	Dako, USA	Ready to use	Monoclonal	Mouse
CK7	Leica microsystems, UK	Ready to use	Monoclonal	Mouse
E caderin	Leica microsystems, UK	1:25	Monoclonal	Mouse
GATA-3	Bio SB, USA	1:100	Monoclonal	Mouse

Material and methods

Cases, tissue specimens, and ethical considerations

Histopathologically confirmed 80 breast cancer tissues along with adjacent normal tissue as control were collected from Armed Forces Institute of Pathology (AFIP), Rawalpindi and Holy Family Hospital, Surgical Unit 1, Rawalpindi, Pakistan between November 2016 and August 2017 after taking approval from hospitals and institutional ethical committees (IRB, AFIP & IRF, RMC respectively). All the patients underwent surgery (mastectomy or lumpectomy) at respective hospitals and signed informed consent forms.

Morphological analysis and clinical data collection

Breast tumor staging was done by using TNM staging system whereas tumor type and grade were determined by following World Health Organization Criteria.[9] Tubular formation, nuclear pleomorphism and mitotic rate were counted to calculate a total score by following Elston–Ellis grading system. All these 3 features were assigned with a score ranging from 1 to 3 (from slower cell growth to faster). Later, these scores were added up to calculate total score ranging between 3 and 9 representing tumor grade.[10] Immunohistochemical staining of some specific biomarkers like hormone receptor studies including ER (estrogen receptor), PR (progesterone receptor), E cadherin, CK7 (Cytokeratin 7), and GATA-3 was done for particular patients.[11] Primary antibodies detail is given in Table 1. Tissues with scanty tumor cells and poor fixation were excluded from the study. Other information like patients age, tumor focality, microcalcifications, lympho-vascular invasion, number of lymph nodes involved in tumor, and response to therapy were collected through histopathology reports. All the morphological analysis was confirmed by a histopathologist.

Immunohistochemical analysis

Immunohistochemistry was performed on 80 breast cancer tissues along with adjacent controls. The procedure was followed by Masood and Kayani, (2013) with minor modifications.[12] Tissue sections were incubated with ready to use monoclonal anti-hMLH1 and anti-hMSH2 overnight at 4°C (7 mL MLH1 Bond RTU Primary and 7mL MSH2 Bond RTU Primary, Leica microsystems, UK). Staining intensity was calculated as weak, moderate and strong and given scores 1, 2, and 3 respectively.[13] Tumors represented with loss of MLH1 and MSH2 expression were classified as negative whereas, nuclear immunostaining of normal epithelial cells and lymphocytes served as internal positive controls. All cases were counter evaluated by a pathologist.

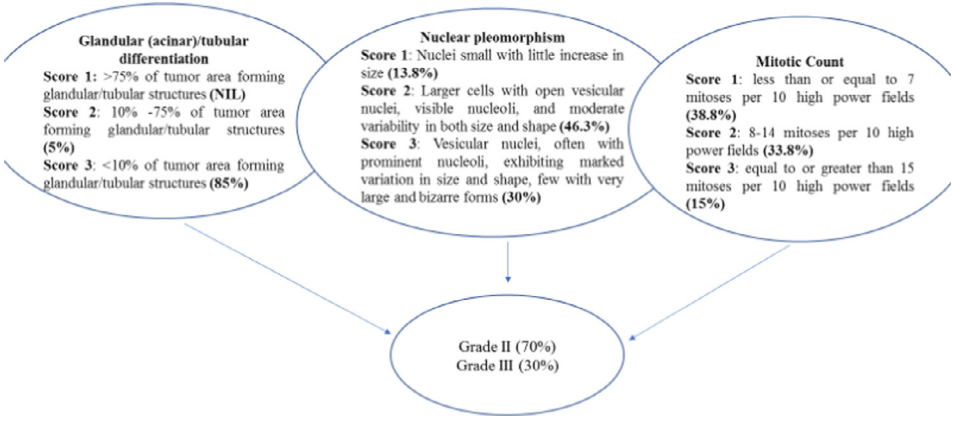

Fig. 1. Histopathological grading for selected (80) breast cancer cases.

Statistical analysis

All the available information was arranged numerically in SPSS version 24.0. *T* test, ANOVA, chi square, univariate analysis, and Fishers exact test were employed to find the association between mismatch repair proteins and available details of breast cancer cases. All the results were represented by *P* value, where less than 0.05 was considered statistically significant.

Results

General features of breast cancer cases

There were 80 breast cancer cases with mean age 46.7 ± 11.3 years (average age in years ± SD). All cases examined were females and their minimum and maximum age was 26 and 66 years respectively. Approximately 51.2% and 42.5% cases had tumor in right and left breast respectively whereas, remaining 6.3% had tumor in both. A total of 73.8% cases had invasive ductal carcinoma and 26.3% were with invasive mammary carcinoma. Most (70%) of the patients had grade II tumor evaluated with the help of detailed examination of glandular (acinar) and/or tubular differentiation, nuclear pleomorphism, and mitotic count as represented in Fig. 1. Positive lymph nodes were observed in 66% breast cancer cases. No association ($P = 0.21$) was found between age and histological tissue type. Even no association was found between tissue type and tumor grade reported individually by *t* test and Levene's test ($P = 0.35$ and $P = 0.11$). Among 66.3% cases examined for focality 61.3% were unifocal. Positive association ($P = 0.004$) was observed between tumor focality and grading. Whereas, no association ($P = 0.89$) was found among tumor focality and histological tissue typing. Neoadjuvant chemotherapy regimens included either doxorubicin, cyclophosphamide, and 5-fluorouracil, or epirubicin, cyclophosphamide, and 5-fluorouracil (CEF), 21 days apart. Number of chemotherapy cycles varied from 2 to 8 depending on tumor size. Different pathological responses were observed from 13% breast cancer cases undergoing neoadjuvant therapy. Most of the chemotherapeutic effects were hyalinization, fibrosis, calcification, hemosiderophages, loss of tumor cell organization, and necrosis.

Immunohistochemistry scoring

Table 2 showed distribution of immunohistochemical scores for MLH1 and MSH2 proteins. Out of 80 breast carcinomas examined, 46 (57.5%) showed normal or positive nuclear expression

Table 2
Immunohistochemical expression analysis for MLH1 and MSH2 proteins.

Immunohistochemical expression	MLH1 n (%)	MSH2 n (%)
Positive	46 (57.5)	43 (53.8)
Weak	15 (32.6)	14 (32.6)
Moderate	26 (56.5)	20 (46.5)
Intense	5 (10.9)	9 (20.9)
Negative	34 (42.5)	37 (46.3)

Table 3
Association of MLH1 and MSH2 with breast cancer.

Proteins expression	*P* value	Odds ratio	95% confidence interval
MLH1	0.0004*	13.8	4.6-41.1
MSH2	0.0002*	14.0	4.7-42.2

*Statistically significant values were highlighted.

for MLH1. Among which 15, 26, and 5 cases showed week, moderate and intense MLH1 staining whereas 34 (42.5%) cases had showed complete loss of MLH1 protein. Statistical analysis showed significant association between loss of MLH1 protein ($P = 0.0004$; odds ratio 13.8; 95% confidence interval 4.6-41.1) and breast cancer risk. For MSH2 expression analysis, 43 (53.8%) breast carcinomas showed positive staining comprised of 14, 20, and 9 as week, moderate and intense. Negative MSH2 staining (complete loss) was observed in 37 (46.3%) breast cancer tissues. Significant association was observed between loss of MSH2 protein ($P = 0.0002$; odds ratio 14.0; 95% confidence interval 4.7-42.2) and risk of breast cancer as illustrated in Table 3. Strong nuclear staining was observed in all noncancerous cells. Both MLH1 and MSH2 proteins showed normal nuclear expression in (53.8%) cases. Whereas, 34 (42.5%) breast carcinomas had demonstrated complete loss of both MLH1 and MSH2 proteins. Representative immunohistochemistry breast cancer cases and normal breast parenchyma for MLH1 and MSH2 are illustrated in Fig. 2.

Estrogen and progesterone receptor was evaluated in 27 cases. Among which 19 (70.3%) cases had negative ER and PR status. Frequency distribution showed that 13 (68.4%) ER negative patients were MLH1 positive whereas 6 (31.6%) patients showed loss of MLH1 protein. Among 8 ER positive cases 4 (50%) were MLH1 negative. Meanwhile, 11 ER negative patients were MSH2 positive and 8 showed loss of MSH2 protein. ER was positive in 3/8 (37.5%) breast cancer cases with loss of MSH2 protein. Similar trend was followed by PR as well. A total of 14 (73.6%) PR negative patients were MLH1 positive whereas 5 (26.3%) had loss of MLH1 protein. Among 8 PR positive cases 3 (37.5%) were MLH1 negative. Meanwhile, 11 PR negative patients were MSH2 positive and 8 showed loss of MSH2 protein. PR was positive in 2/8 (25%) breast cancer cases with loss of MSH2 protein. These results showed that estrogen and progesterone receptors tends to be present at a lower rate in mismatch repair deficient breast cancer.

Cytokeratin 7 (CK7) was positive in 37/39 cases. Among them 27/37 (72.9%) had showed loss of MLH1 protein whereas, 21/37 (56.8%) CK7 positive breast cancer cases were MSH2 negative. These results indicated that presence of CK7 tends to increase in mismatch repair deficient breast cancer.

GATA-3 was positive in 32/35 breast cancer cases. Among them 18/32 (56.2%) and 16/32 (50%) GATA-3 positive breast cancer cases showed loss of MLH1 and MSH2 protein respectively. Positive relationship was found between presence of GATA-3 and mismatch repair deficiency in breast cancer.

E cadherin was evaluated in 27 cases and all were positive. It was found that 24/27 (88.8%) and 19/27 (70%) breast cancer cases showed loss of MLH1 and MSH2 protein respectively. E cadherin positivity was also associated with mismatch repair deficiency.

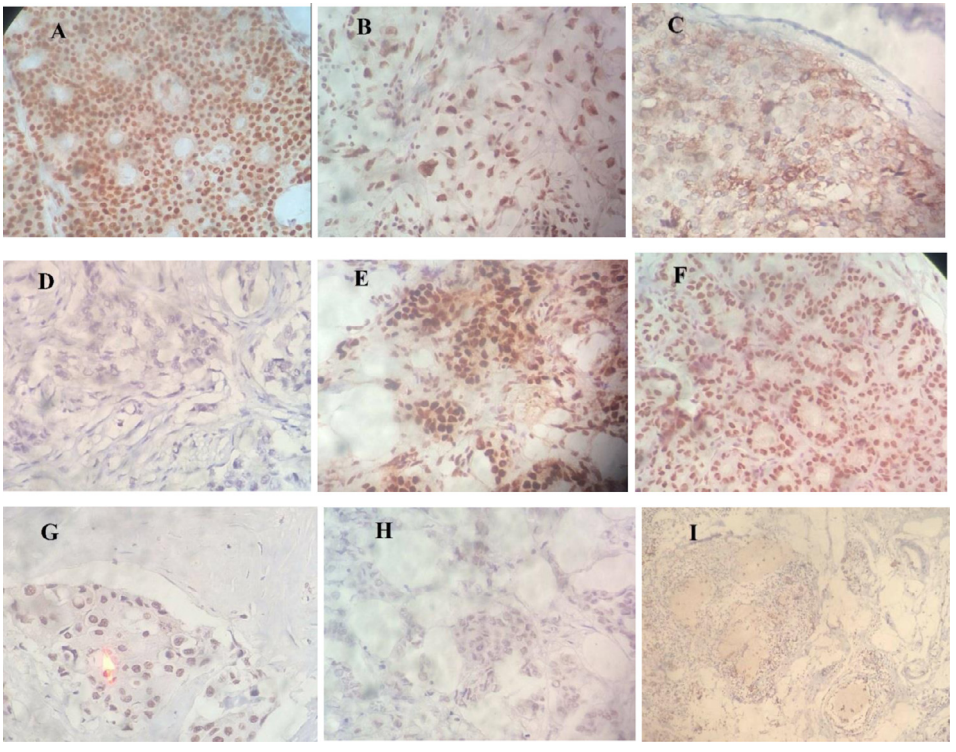

Fig. 2. Representative immunohistochemistry for MLH1 and MSH2. (A) Strong positive MLH1 staining in breast cancer (B) Moderate positive MLH1 staining in breast cancer (C) Weak positive MLH1 staining in breast cancer (D) Negative MLH1 staining in breast cancer (E) Strong positive MSH2 staining in breast cancer (F) Moderate positive MSH2 staining in breast cancer (G) Weak positive MSH2 staining in breast cancer (H) Negative MSH2 staining in breast cancer (I) Normal breast parenchyma.

Table 4
Association between mismatch repair protein expression and clinicopathological details.

Characteristics	MLH1 (P value)	MSH2 (*P* value)
Age	0.54	0.81
Histological tissue type	0.09	0.01*
Tumour Grade II/III	0.15	0.13
Lymph nodes	0.28	0.22
Tumour focality	0.001*	0.002*
Menopause	0.58	0.47
Chemotherapy	0.01*	0.04*

*Statistically significant values were highlighted.

Association between mismatch repair protein expression and clinicopathological details

Table 4 showed association between expression of 2 mismatch repair proteins MLH1 and MSH2 and breast cancer patients clinicopathological history. Characteristics were not assessed in all cases due to nonavailability of data. No difference was observed in the mean ages of patients with normal (46.1 ± 11.6) and negative (47.7 ± 11.1) MLH1 breast cancer cases. Similar pattern was observed with MSH2 normal (46.4 ± 11.5) and negative (47.0 + 11.3) cases. There was no statistical association between age and loss of 2 mismatch repair proteins (MLH1 and MSH2).

Histological tissue type was associated with the loss of MSH2 expression but no association was analyzed with MLH1 protein expression. Invasive ductal carcinoma was 32/59 found more prone to loss of MSH2 protein. Tumor grade was also not associated with loss of mismatch repair proteins. Loss of MLH1 and MSH2 was observed in 27/56 and 29/56 grade II patients. Whereas 7/24 and 8/24 grade III patients showed loss of mismatch repair proteins ie, MLH1 and MSH2 respectively. Positive lymph nodes also found not to be associated with the loss of 2 mismatch repair proteins in the selected patients as illustrated in Table 4.

Most of the breast cancer cases 49/53 were unifocal as already mentioned that every single piece of information is not available for each patient. Statistically significant association was observed with both the loss of MLH1 and MSH2 proteins with unifocal tumors. Even though sample size (3/4) is very small but still a positive relationship was obvious for multifocal tumors and loss of MLH1 expression. No such relationship was observed in case of MSH2 protein (1/4).

At the time of sampling, 32 breast cancer cases were postmenopausal while 48 had not reached menopause. Loss of MLH1 protein was observed in 14/32 (43.7%) postmenopausal cases whereas 20/48 (41.6%) premenopausal cases showed loss of MLH1 expression. Loss of MSH2 protein was reported in 14/32 (43.7%) and 23/48 (47.9%) postmenopausal and premenopausal breast cancer cases respectively. No statistical difference was evaluated between menopause and loss of mismatch repair proteins (Table 4).

Limited data is available regarding chemotherapeutic treatments. Total 11(13%) patients received chemotherapy and among them 6 and 5 cases showed loss of MLH1 and MSH2 proteins respectively. These observations suggest that chemotherapy may contribute to defective MLH1 and MSH2 expression in breast cancer cases. Significant correlation was observed between chemotherapeutic treatments and loss of both mismatch repair proteins.

Discussion

Breast cancer is one of the most common malignancy among women throughout the world. Mismatch repair pathway is an important part of DNA replication machinery and corrects base substitution and insertion-deletion mismatches. MLH1 and MSH2 are most amply expressed mismatch repair proteins and their activation have complex pathological outcomes on DNA, particularly in case of microsatellite instability. Therefore, they should be considered as well-known biomarkers for defective mismatch repair activities in cancerous cells.[12] Individual characteristics, behavior, biology, and outcomes of defective mismatch repair proteins in breast cancer are poorly understood. In the current study, loss of MLH1 and MSH2 protein expression found to be associated with breast cancer. Large number of cases showed defected expression of mismatch repair proteins suggesting that loss of mismatch repair proteins is pivotal for progression of sporadic breast cancer. Similar findings have been reported in different cancers.[3,12,14-16] Results showed that 42.5% same breast cancer cases showed loss of expression in both MLH1 and MSH2 proteins. However, underlying mechanism is not explored yet.

Current study explored association of unifocal and multifocal breast tumors with loss of MLH1 and MSH2. Like others, it was observed that tumor focality might play an important role in mismatch repair deficient breast cancer.[17]

It was observed that 41.6% and 47.9% premenopausal breast cancer cases had loss of mismatch repair proteins (MLH1 and MSH2) respectively. Although results were not statistically significant, but it can be easily seen that hormones play an important role in regulating expression of mismatch repair proteins. Similar findings have been reported that hormones together with deficiency of DNA damage repair proteins provides an insight into breast cancer development.[18-20]

ER and PR are most common immunohistochemical prognostic and therapeutic markers for breast cancer. Aberrant methylation of ER gene particularly at 1 site within CpG island has been observed in more than 25% ER negative breast cancer cell lines.[21] Therefore, we investigated the possibility that ER negative breast cancer cases might also exhibit negative mismatch repair

expression following the same epigenetic mechanism. However, no association was observed between ER and PR negative breast cancer and mismatch repair deficiency.

CK 7 is a well characterized marker for differential diagnosis and expression analysis of breast and colon cancer. It is the only specific markers which identifies breast Paget's disease and provides meaningful information about metastasis.[22,23] Therefore, we explored the possibility of relationship between CK7 positivity and mismatch repair deficiency and a positive association was observed suggesting role of mismatch repair deficiency and adverse disease outcomes.

GATA 3 is expressed in the luminal epithelia of breast and commonly expressed in breast cancer. It is a potential biomarker for metastatic cancer of mammary origin.[24] In current study (91.4%) cases were GATA 3 positive and showed potential for future research in mismatch repair deficient breast cancer.

E cadherin is used to differentiate between lobular and ductal carcinoma.[25] Most of the cases in current study were ductal carcinoma therefore, positive association was observed between E cadherin and mismatch repair deficiency.

Literature have illustrated that mismatch repair protein deficiency make tumor cells resistant against chemotherapy, as normal cells (control group) promote DNA damage induced apoptosis.[26,27] It is already reported that mismatch repair lacking phenotype is drug inducible.[3,28] Therefore, a debate is still going on whether mismatch repair deficiency is the result of chemotherapeutic drugs or cause of tumorigenesis. Current study reported positive association between mismatch repair deficiency and chemotherapy. It might be possible that chemotherapeutic drugs could selectively change gene expression patterns through various complex mechanisms like promoter hypermethylation. Whereas, loss of mismatch repair functioning represses DNA damage induced apoptosis, it is anticipated that mismatch repair deficiency will lead to breast cancer progression.

In conclusion, mismatch repair deficiency may contribute to breast cancer progression into advanced stages and aids cancerous cells to survive against chemotherapeutic drugs. MSH2 seems to be more involved in breast tumorigenesis as compared to MLH1. But the difference is not much pronounced.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.currproblcancer.2018.08.001.

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