

THE STUDY OF MISMATCH REPAIR IN ENDOMETRIAL CANCER PATIENTS WITH A FAMILY HISTORY OF CANCER

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Aim: To assess the expression of mismatch repair (MMR) proteins MSH2 and MLH1 and carry out microsatellite analysis in patients with endometrial cancer (EC) with regard to the family history of cancer. **Materials and Methods:** Morphological and immunohistochemical study was performed on tumor tissue samples of 49 EC patients. Microsatellite instability was determined using PCR with primers which flank microsatellite region BAT-26. **Results:** A tendency to a decreased expression of both MSH2 and MLH1 markers in a group of EC patients with a family history of cancer as compared with a group without aggregation of cancer in family history was observed (labeling index — LI — was $36.1 \pm 8.1\%$ and $20.7 \pm 9.1\%$ versus $LI 48.0 \pm 5.8\%$ and $33.8 \pm 5.8\%$, respectively). It was determined that the number of EC patients with tumors deficient by expression of MMR markers was reliably higher in a group of patients with a family history of cancer than in a group of patients without aggregation of cancer in family history ($p < 0.05$). It was shown that in a group of EC patients with a family history of cancer, MMR-proficient tumors were detected in 38.5% of cases. Microsatellite instability was determined in 10.7% of EC patients including one patient with aggregation of Lynch-associated tumors in family history. **Conclusion:** Family history of cancer of EC patients is associated with malfunctioning of the MMR system as well as may be related to alternative molecular mechanisms.

Key Words: endometrial cancer, mismatch repair, microsatellite instability, family history of cancer, Lynch syndrome.

It is known today that approximately 5–10% of malignant neoplasms occur in the result of hereditary predisposition to cancer caused by the germinal mutations in genes which are associated with increased risk of cancer [1, 2]. Endometrial cancer (EC) may occur due to the series of hereditary cancer syndromes, among which Lynch syndrome (hereditary non-polyposis colorectal cancer) is the most prevalent. The specific feature of Lynch syndrome is a presence of germinal mutations in genes *MSH2*, *MLH1*, *MSH6*, and *PMS2*, which belong to the mismatch repair (MMR) system [3].

MMR provides improvement of non-complementary base pairs, which in high amount emerge in the process of DNA replication. Genetic or epigenetic disorders of this system cause the development of microsatellite instability (MSI) that manifests itself by microdeletions and microinsertions in the regions of location of mono-, di-, tri- and tetranucleotide repeats of DNA. At accumulation of non-repaired microdeletions and microinsertions in microsatellites, which are located in coding and regulatory regions of proto-oncogenes and suppressor genes, shift of the reading frame and change of expression of these genes occurs that can be the reason of malignant transformation [4]. According to some data, effectiveness of functioning of MMR significantly determines the aggressiveness of tumor process that can be used at the choice of EC treatment strategy [5].

In 1913, Lynch syndrome was first time described by Warthin on the example of family, in the history of which aggregation of endometrial tumors and gastrointestinal cancer was observed. Today Lynch-associated neoplasms include tumors of colon, endometrium, gastric, ovary, pancreas, bile ducts, and urogenital system as well as tumors of some other localization [3]. Lynch syndrome in patients with colorectal cancer is diagnosed basing on the Amsterdam criteria [6, 7] and Bethesda recommendations [8, 9]. The last ones, along with the assessment of family history of proband, require carrying out of molecular-genetic tests. Recently specified recommendations concerning detection of individuals with Lynch syndrome among patients with EC were published [3, 10]. It should be noted that number of researchers have evaluated the dissemination of Lynch syndrome via determination of hereditary mutations and it was detected that its frequency was within the limits of 2.0–4.6% [11–15].

Some authors assume the existence of particular hereditary syndrome of EC, at which proband has a family history of this disease and no mutations in MMR genes [16]. The last argues the necessity of carrying out of further studies concerning the detection of peculiarities of cancer pathology aggregation in family history of patients with EC and its molecular mechanisms.

This research aims to assess the expression of MMR proteins and analyze MSI in EC patients regarding the family history of cancer.

MATERIALS AND METHODS

Morphological, immunohistochemical tests and microsatellite analysis were carried out on samples of tumor tissue of 49 patients with EC of I and II stages

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Abbreviations used: CRC – colorectal cancer; EC – endometrial cancer; GC – gastric cancer; LI – labeling index; MMR – mismatch repair; MSI – microsatellite instability.

who underwent surgery in the Research Department of Cancer Gynecology of the National Cancer Institute of Ministry of Health of Ukraine. Mean age of patients was 59.0 ± 1.7 years. All patients have given written informed consent for participation in the study.

To analyze family history of probands, special genealogical questionnaire, which contained information on diseases of relatives, life conditions of the patient and concomitant diseases, was used. As criteria for attributing of the EC patients to the group of individuals with a family history of cancer was the presence of malignant tumors of female reproductive system and/or other Lynch-associated tumors in relatives of probands of I–II degree of the relationship [3].

Morphological study was carried out on the specimens dyed with hematoxylin and eosin. Assessment of expression of key markers of the MMR-system was conducted using immunohistochemical method with monoclonal antibodies MSH2 (clone 25D12) and MLH1 (clone G168–15) (“Diagnostic BioSystems”, USA). To visualize mentioned proteins, detection PolyVue system (“Diagnostic BioSystems”, USA) was used. Results of immunohistochemical reaction were assessed by calculation of the number of stained cells, which was determined in percentage (labeling index — LI, %). Expression of markers was assessed in 800–1000 tumor cells.

Microsatellite analysis was carried out using PCR with primers that flank microsatellite region BAT-26 [17]. Genomic DNA was isolated from tumor tissues and peripheral blood lymphocytes via phenol-chloroform extraction. Nucleotide sequence of primers was the following: Forward: 5'-TGACTACTTTTGACTTCAGCC-3' and Reverse: 5'-ACCATTCAACATTTTAACCC-3'. PCR was carried out in 20 μ L of PCR buffer that contained 2.5 mM $MgCl_2$, 200 mM of each dNTP, 0.3 mM of each primer, 100 ng of DNA and 2 U Taq-polymerase. The mixture was warmed up to 95 °C for 5 min and 33 cycles of amplification were carried out with parameters: denaturation 94 °C — 30 s, annealing 58 °C — 30 s and elongation 72 °C — 30 s.

Products of PCR were separated by electrophoresis in 15% polyacrylamide gel at 120 V during 12 h and were stained with SYBR Green. MSI was confirmed at emergence of alleles, length of which differed from the normal ones, which were detected in DNA from peripheral blood lymphocytes.

Statistical processing of data was carried using program package Statistica 8.0 (StatSoft, Inc.) with the help of Mann — Whitney nonparametric criterion and Fisher criterion. Reliable were considered differences at $p < 0.05$.

RESULTS AND DISCUSSION

Verification of morphological diagnosis has determined that all studied endometrial tumors were endometrioid adenocarcinomas of various differentiation grades: 6 — well, 25 — moderately and 18 — poorly differentiated tumors. Immunohistochemical study has detected that positive expression of MSH2 protein was found in 75.5%

(37 out of 49 cases), and MLH1 protein — 57.1% (28 out of 49 cases) of endometrial tumors and mean number of cells, which express these proteins, was $44.6 \pm 4.8\%$ and $30.1 \pm 5.0\%$, respectively (Fig. 1).

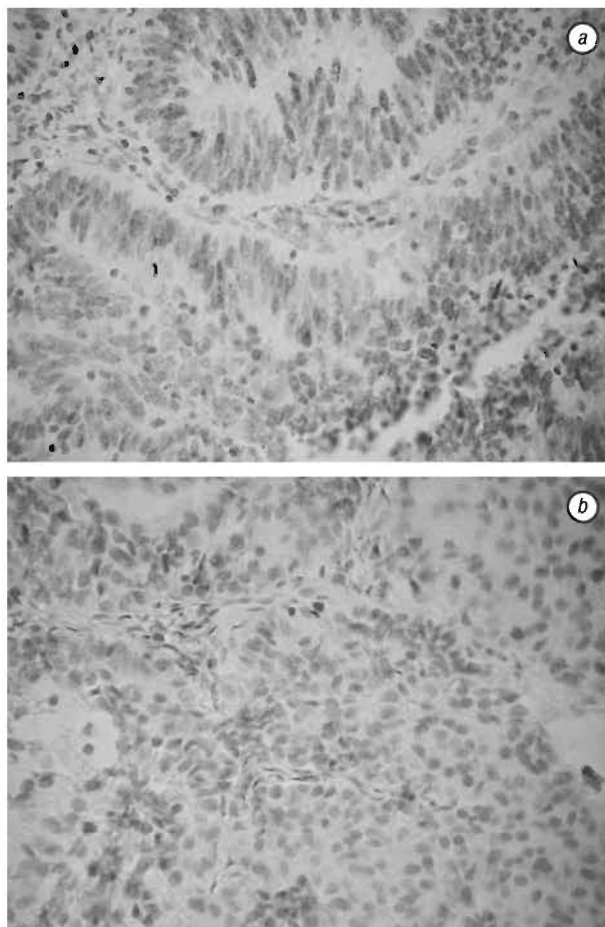


Fig. 1. Expression of MMR proteins MSH2 and MLH1 in moderately differentiated endometrial adenocarcinoma (a); poorly differentiated endometrial adenocarcinoma (b). Immunohistochemical method, stained with Mayer's hematoxylin, $\times 400$

Clinical and genealogical analysis of family histories of EC patients has showed that 13 (26.5%) out of 49 probands had a family history of cancer. It was determined that mostly tumors of female reproductive system and gastrointestinal tract were accumulated in families (Table 1, Fig. 2) that fits our results obtained in previous studies [18]. Tumors were observed in 5 (71.4%) mothers and 2 siblings (28.6%) among first degree relatives of EC patients and in 6 aunts (54.7%), 3 grandmothers (27.3%), 1 uncle (9.0%) and 1 niece (9.0%) among second degree relatives.

Table 1. Malignant tumors in relatives of EC patients

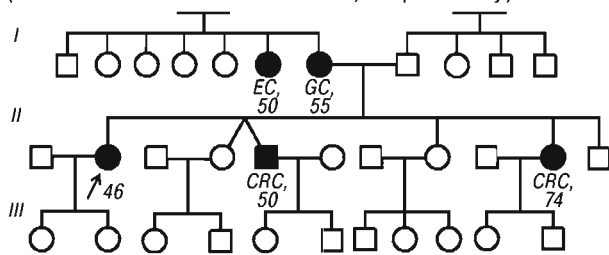
Degree of relationship	Localization of tumors and their quantity in proband families				Total
	Endometrium	Breast	Gastro-intestinal tract	Ovary	
I (mother, father, sister, brother, children)	1	2	3	1	7
II (aunt, uncle, grandmother, grandfather, nephew, niece)	5	4	2	0	11
Total	6	6	5	1	18

Comparison of expression of MSH2 marker in patients with EC from the families with a family history of cancer has not determined reliable difference in the

Table 2. Assessment of expression of MMR markers in tumors of EC patients depending on familial aggregation of cancer

Groups of examined patients	Number of tumors with expression of markers, %				LI, %	
	MSH2		MLH1		Mean ± SE	
	Positive expression, n (%)	Lack expression, n (%)	Positive expression, n (%)	Lack expression, n (%)	MSH2	MLH1
Patients with a family history of cancer	9 (70.2)	4 (30.8)	4 (55.5)	7 (54.5)	36.1 ± 8.1	20.7 ± 9.1
Patients without family history of cancer	29 (82.8)	6 (17.2)	29 (80.5)	7 (19.5)	48.0 ± 5.8	33.8 ± 5.8
<i>p</i>	<i>p</i> > 0.05 (F test)		<i>p</i> < 0.05 (F test)		<i>p</i> > 0.05 (Mann – Whitney test)	<i>p</i> > 0.05 (Mann – Whitney test)

number of MSH2-positive cases as compared to the group of patients without aggregation of cancer pathology in family history. However, for MLH1 protein, significant difference was determined between these indexes in groups of EC patients with aggregation and without aggregation of cancer in family ($p < 0.05$) (Table 2). Also, it was determined that in a group of EC patients with a family history of cancer, a tendency to decreased expression of MSH2 marker as compared with a group without aggregation of cancer in family was observed (LI 36.1 ± 8.1 and $48.0 \pm 5.8\%$, respectively). The expression of MLH1 changed in the same way (LI 20.7 ± 9.1 and $33.8 \pm 5.8\%$, respectively).

**Fig. 2.** Pedigree of proband with EC. In relatives of I and II degree of relationship, aggregation of Lynch-associated tumors is observed: EC, gastric cancer (GC) and colorectal cancer (CRC)

Taking into account the fact that a lack of at least one of the MMR-proteins causes the malfunctioning of the whole MMR system, we have determined two groups of tumors by expression of MSH2 and MLH1: MMR-deficient (lack of both or one of MMR-proteins) and MMR-proficient (presence of both MMR-proteins) [19, 20]. It was determined that the number of EC patients with MMR-deficient tumors was reliably higher in a group of patients with a family history of cancer (8 out of 13) while in a group of patients without family history of cancer their number was 27.8% (10 out of 36) ($p < 0.05$) (Table 3).

Table 3. Comparing number of MMR-deficient and MMR-proficient tumors in EC patients depending on familial aggregation of cancer

Groups of examined patients	Number of MMR-deficient tumors,	Number of MMR-proficient tumors,
	n (%)	n (%)
Patients with a family history of cancer	8 (61.5)	5 (38.5)
Patients with no family history of cancer	10 (27.8)	26 (72.2)
<i>p</i>	<i>p</i> < 0.05 (F test)	

Data stated above show that tumors of EC patients with a family history of cancer are characterized by the defects in expression of key proteins of the MMR system MSH2 and MLH1 to a greater extent than tumors of patients, which emerged sporadically. The further analysis of a group of patients with a family history of cancer has showed that out of 8 EC patients with MMR-deficient tumors, aggregation of cancer pathology by the type of Lynch syndrome was observed

in 5 (10.2%) of them that allows to pre-include these patients in the group of patients with suspected Lynch syndrome. According to the data of literature, for the final diagnosis of the Lynch syndrome, molecular-genetic test with detection of hereditary mutations in MMR genes in patient and her relatives has to be carried out. Such test is recommended to perform after previous immunohistochemical detection of the 4 MMR-proteins (MSH2, MLH1, MSH6 and PMS2) and assessment of promoter methylation status of *MLH1* gene in the case of lack of its expression [3].

In three examined EC patients with a family history of cancer and MMR-deficient tumors in family histories, along with tumors of gastrointestinal tract, breast tumors were observed that according to the ideas of the number of researchers indicates the possibility of referring of breast tumors to the Lynch-associated tumors [21, 22], but this issue remains disputable.

In group of 5 EC patients with MMR-proficient tumors and a family history of cancer (38.5%), 2 patients had familial aggregation of EC, 2 other patients — breast cancer, and in 1 case — aggregation of gastrointestinal cancers by the type of Lynch syndrome. Presence of EC in family histories of patients of this group fits the data of other researchers on the existence of particular hereditary syndrome of EC, which is not associated with malfunctioning of MMR-system [11, 23]. Presence of breast cancer in family histories of EC patients also may be not random and allows to assume possibility of existence of hereditary syndrome “breast cancer — endometrial cancer” that was reported by Wendt et al. [24]. At the same time, mutations at Lynch syndrome occur not only in *MSH2* and *MLH1* genes, but also in *MSH6* and *PMS2* genes. For this reason, one cannot exclude that in patients with MMR-proficient EC, hereditary aggregation is caused by germinal mutations in genes *MSH6* and *PMS2*, expression of which was not analyzed in this study.

Taking into account that genetic and epigenetic changes of MMR genes cause MSI, we have carried out microsatellite analysis by marker BAT-26 in endometrial adenocarcinomas of 28 patients. MSI was detected in 3 cases (10.7%) (Fig. 3). This rate turned out to be lower as compared with frequency of MSI, which was determined by other researchers that probably is associated with different prevalence of MSI in different ethnic groups [28]. For instance, according to the literature, MSI is detected in 25–30% of all endometrial carcinomas [25]. It should be noted that studies of Ichikawa et al. [26], and Risinger et al. [27] have showed that 75% of Lynch-associated endometrial adenocarcinomas are characterized by pre-

sence of MSI. Among EC patients, in whom MSI was determined, Lynch-associated tumors were observed in family history of one patient. Our data on frequency of MSI among EC patients with a family history of cancer may be confirmation of existence of alternative molecular mechanisms, which cause the development of hereditary forms of cancer.

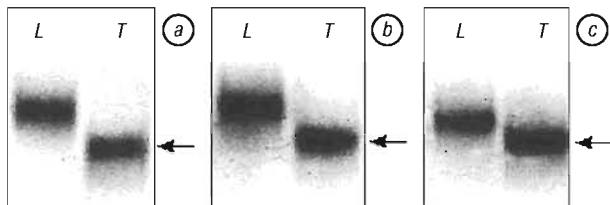


Fig. 3. Results of microsatellite analysis using BAT-26 marker. MSI is confirmed in 3 cases (a, b, c) that is evidenced by the emergence of alleles of other length in tumor DNA (marked by arrow) as compared with DNA from peripheral blood lymphocytes. L – PCR product from lymphocyte DNA, T – PCR product from tumor DNA

Thus, the results of our study show that family history of cancer in EC patients is associated with malfunction of the system of MMR. At the same time, obtained data allow to assume the existence of other molecular mechanisms, which stipulate the development of hereditary forms of cancer. It also has to be taken into account that a family history of cancer may be caused not only by genetic factors, but also by environmental exogenous factors, which influence the members of the same family. For instance, the results of studies of Seger et al. [29] show that risk of EC was higher in relatives with EC with high body mass index as compared with patients, whose indexes were low or average.

It should be noted that prognostic impact of MMR deficiency on outcome of EC remains unclear. Some studies didn't find any association between MMR deficiency and outcome in EC patients [30, 31]. By contrast, Nout et al. [32] reported association with decreased survival. In addition, de Jong et al. [33] showed that patients with the loss of MMR protein expression had a worse disease specific survival compared to patients with its expression. Further studies are needed to clarify impact of MMR status on outcome of the disease in EC patients with family history of cancer.

Understanding of mechanisms of development of familial cancers is a basis for the search of new biomarkers, by which hereditary predisposition to cancer may be determined. Such approach contributes to the effective formation of groups with high risk of cancer and primary prophylaxis of cancer diseases. Detection of individuals with suspected Lynch syndrome or other hereditary syndromes among EC patients will allow physicians to provide these patients and their relatives with required recommendations concerning the lifestyle and use adequate procedures to prevent cancer diseases.

CONCLUSIONS

Family history of cancer in EC patients is accompanied with the malfunctioning of the MMR-system that is associated with decreased expression of MMR-proteins and increase of number of MMR-deficient tumors among EC patients with family history of cancer.

MSI was determined in 10.7% of EC patients. MMR-proficient tumors were found in 38.5% of EC patients with aggregation of cancer in family history that may indicate existence of alternative molecular mechanisms, which cause the development of hereditary forms of cancer.

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