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CLINICAL RELEVANCE OF STUDIES ON MICROSATELLITE INSTABILITY AND DNA MISMATCH REPAIR PROTEIN EXPRESSION IN ENDOMETRIAL CARCINOMAS

Summary of Doctoral Dissertation
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ABBREVIATIONS

CI – confidence interval
DNA – deoxyribonucleic acid
FIGO – International Federation of Gynecology and Obstetrics
G1 – well-differentiated grade
G2 – moderately differentiated grade
G3 – low differentiated grade
HNPCC – Hereditary Nonpolyposis Colorectal Cancer
IHC – immunohistochemistry
MMR – mismatch repair
MSI – microsatellite instability
MSI-H – high-frequency microsatellite instability
MSI-L – low-frequency microsatellite instability
MSS – microsatellite stability
P – significance
PCR – polymerase chain reaction
SD – standard deviation

1. INTRODUCTION

Endometrial cancer is the seventh most frequently diagnosed malignancy worldwide and the most common female gynecologic cancer [Siegel R. et al., 2013]. Endometrial cancer is most prevalent in economically developed European and North American countries, and the incidence rates in these countries are 10-fold higher than those in economically developing countries [Jemal A. et al., 2011]. In Central and East Europe, the incidence and mortality rates are 15.6 and 3.4 per 100 000 women, respectively [Globocan, 2012]. According to the EUROCARE data, the 5-year survival in Europe reaches 75% [De Angelis R. et al., 2014].

In Lithuania, endometrial cancer is one of the most common malignancies of the female reproductive tract as well: the incidence and mortality rates are 18.8 per 100 000 and 3.4 per 100 000 women, respectively [Cancer in Lithuania, 2013].

Recent achievements in modern molecular biology have enabled the development of state-of-the-art genomic methods, the application of which allows to analyze mechanisms of carcinogenesis, to evaluate the diseases prognosis and response to treatment [Gilmore S. at al., 2003].

Microsatellite instability (MSI) is studied in tumors of different localization. Microsatellites are short repetitive nucleotide sequences in the human genome [Ellegren H. at al., 2004; Li Y.C. et al., 2004]. The key factor that causes microsatellite instability is defects in the DNA mismatch repair (MMR) system. The phenomenon of microsatellite instability and mutations in DNA MMR genes were initially described in 1993 in studies investigating the cases of hereditary nonpolyposis colorectal cancer (Lynch syndrome) [Lynch H.T., Lynch J.F., 1993; Peltomaki P. et al., 1993]. Microsatellite instability has also been identified in Lynch syndrome-related tumors of other localization (most commonly in endometrial cancer) [Meyer L.A., et al., 2009]. It is worth noting that microsatellite instability is seen in sporadic cancers as well (especially colorectal and endometrial).

A large body of data on microsatellite instability and DNA MMR protein expression in colorectal tumors (both Lynch syndrome-related and sporadic) is available, and its prognostic and predictive value has been evaluated. However, the data on the

frequency and status of microsatellite instability in endometrial cancer are still being accumulated, and the need for systemization of these data is recognized. The prognostic value of microsatellite instability in endometrial cancer has not been elucidated yet. Contradictory data have been reported while studying associations between microsatellite instability and clinicopathologic characteristics in this cancer [MacDonald N.D. et al., 2000; An H.J., 2007].

Various markers are used for the evaluation of microsatellite instability. The U.S. National Cancer Institute recommends to investigate 5 genomic loci and to use a panel of the following 5 markers: BAT-25, BAT-26, D2S123, D5S346 and D17S250 [Boland C.R. et al., 1998]. Other marker panels produced by various manufacturers are available on the market. Thus, more markers can be involved and used in studies on microsatellite instability, and more precise mononucleotide repeat markers are constantly being developed. For example, Promega Corporation (USA) created a panel that consists of mononucleotide BAT-25, BAT-26, MONO-27, NR-21, and NR-24 markers [Murphy K.M. et al., 2006; Wong Y.F. et al., 2006]. Moreover, new BAT-52, BAT-55, BAT-56, BAT-57 and BAT-59 markers created by Promega Corporation have recently been introduced, and it is expected that they will be more sensitive in the detection of microsatellite instability in other (extracolonic) cancers [Bacher J. et al. 2013]. Therefore, in order to validate a new panel of BAT-52, BAT-55, BAT-56, BAT-57 and BAT-59 markers for the detection of microsatellite instability in other (extracolonic) cancers, it is necessary to carry on comparative studies involving these markers.

1.1. Aim and objectives of the study

The aim of this study was evaluate the frequency and status of microsatellite instability and DNA mismatch repair protein expression in endometrial cancer and to relate the obtained results to clinicopathologic characteristics as well as patient survival.

The objectives of the study

1. To determine the frequency and status of microsatellite instability among patients with endometrial cancer by using two Promega Corporation (USA) marker panels (BAT-25, BAT-26, NR-21, NR-24, MONO-27 and newly created markers BAT-52, BAT-55, BAT-56, BAT-57, BAT-59).
2. To compare the frequency and status of microsatellite instability with clinicopathologic characteristics of endometrial cancer patients.
3. To investigate the expression of DNA mismatch repair proteins (MLH1, PMS2, MSH2, and MSH6) in endometrial carcinomas with high-frequency microsatellite instability.
4. To evaluate the impact of microsatellite instability on the survival of patients with endometrial cancer.

1.2. Statements to be defended

1. A new panel of BAT-52, BAT-55, BAT-56, BAT-57, and BAT-59 markers more precise detection of microsatellite instability in endometrial cancer as compared with already used panel of BAT-25, BAT-26, NR-21, NR-24, and MONO-27 markers.
2. The frequency and status of microsatellite instability is associated with clinicopathologic characteristics of endometrial cancer patients.
3. In order to objectively evaluate genomic instability in endometrial carcinomas, it is not sufficient to perform immunohistochemical testing alone, which is most frequently employed in numerous other cancers. The study of molecular markers such as microsatellite instability is also recommended.

1.3. Scientific novelty and practical relevance of the study

1. A new panel of BAT-52, BAT-55, BAT-56, BAT-57, and BAT-59 markers created by Promega Corporation (USA) was used for the detection of microsatellite instability in endometrial cancer, and high-frequency microsatellite instability was detected two times more frequently with this panel than using a set of BAT-25, BAT-26, NR-21, NR-24, and MONO-27 markers.
2. Immunohistochemical testing only is not sufficient for evaluation of genomic instability, therefore studies of microsatellite instability are needed.
3. Analysis of immunohistochemical/molecular genomic instability studies allows the selection of patients for further molecular genetic testing when hereditary endometrial cancer (Lynch syndrome) is suspected.

2. OBJECT AND METHODS OF THE STUDY

2.1. Study population and its characteristics

This prospective observational study enrolled 109 patients with endometrial carcinomas, who were treated at the Institute of Oncology, Vilnius University (now National Cancer Institute) from 2010 to 2011. Sample size calculations were done making an assumption that nearly 150 women with endometrial cancer will visit the Outpatient Clinic, during the study period. Estimating an event rate of microsatellite instability of approximately 40%, a total number of 107 cases were considered to be sufficient with a significance level of 0.05. Before the study, approval from Regional Biomedical Research Ethics Committee was granted (protocol No. 158200-05-180-43).

All women were questioned according to the Amsterdam II criteria. Also, anthropological measurements of height and weight were made.

The mean age of the patients enrolled to the study was 64±10 years (range, 40 to 83 years). The patients aged 60−69 years made up the largest proportion of the study population − 32.11% (n=35); 50−59-year and 70−79-year olds accounted for 27.52% (n=30), respectively; and 80−89-year-old patients made up 7.34% (n=8). The patients aged 40−49 years comprised the smallest proportion − 5.50% (n=6).

The overwhelming majority − 93.58% (n=102) of the patients were postmenopausal and only 6.4% (n=7) were premenopausal. Seven premenopausal patients were aged from 40 to 50 years old.

The mean body mass index (BMI) of the patients investigated was 31.1±5.6 kg/m^2 (range, 19.4 to 46.1 kg/m^2). Obese women with a BMI of 30.0 kg/m^2 and more comprised the largest proportion − 54.1% (n=59); overweight women with a BMI of 25.0−29.9 kg/m^2 accounted for 35.78% (n=39), and women with normal weight (BMI, 18.5−24.9 kg/m^2) made up 10.09% (n=11).

All the women enrolled to this study (n=109) had undergone surgical treatment: removal of the uterus (hysterectomy) together with the adnexa of the uterus and pelvic and para-aortic lymphadenectomy according to indications. Samples for this study were collected during the surgical treatment and later examined by an expert pathologist. The histological type was classified using World Health Organization criteria and surgical

staging were determined using the standards of the International Federation of Obstetrics and Gynecology (FIGO 2009). In our study, of the 109 cases, 100 (91.74%) were endometrioid adenocarcinomas and only 8.26% (9 of 109 cases) were of nonendometrioid histology.

Part of the patients (n=37), depending on disease stage and tumor grade, received postoperative adjuvant radiation therapy based on the treatment standards approved by the Institute of Oncology, Vilnius University: only vaginal brachytherapy (n=21) or pelvic external-beam radiation therapy along with vaginal brachytherapy (n=16); postoperative chemotherapy was given only to 4 patients who had regional spread of the disease (stage IVB according to the FIGO).

2.2. Study methods

2.2.1. DNA extraction from tumor tissue

DNA was extracted from formalin-fixed, paraffin-embedded tumor tissue specimens (sections 4−5 μm in thickness) using the MagneSil® Genomic, Fixed Tissue System kit (Promega Corporation, USA) or the organic extraction method according to the recommendations of Cancer Genetics Study Section, National Institute of Health (USA).

DNA purification from paraffin-embedded tumor tissue specimens was done using the MagneSil® Genomic, Fixed Tissue System kit according to the manufacturer's recommendations and protocols.

2.2.2. DNA extraction from blood

Patients' blood (healthy tissue) was drawn to 2−5 mL vacuum tubes with EDTA and stored at 4ºC for 2 h or was stored frozen at –20ºC for a longer time. DNA was extracted using the commercial QIAamp Blood Midi kit (Qiagen, Germany) or the organic extraction method according to the methodology approved at the Institute of Oncology, Vilnius University.

2.2.3. Microsatellite instability analysis

A multiplex microsatellite marker system (MSI Analysis System, Version 1.2, Promega Corporation, USA) was used for the detection of microsatellite instability in human cells. Two Promega Corporation marker sets were used: the already used marker panel and the newly created marker panel. The first analysis system consists of fluorescent dye-labeled primers for the analysis of 7 genetic loci, 5 of which (BAT-25, BAT-26, NR-21, NR-24, and MONO-27) are short mononucleotide repeats and 2 (Penta C and Penta D) are pentanucleotide repeats. Two pentanucleotide repeats are used in order to determine if tumor and healthy tissue (blood in our study) DNA specimens belong to the same person. The second marker panel is comprised of 5 (BAT-52, BAT-55, BAT-56, BAT-57 and BAT-59) long polyA repeats.

The polymerase chain reaction (PCR) assay was employed for the detection of microsatellite instability.

2.2.4. Assessment of microsatellite instability

Tumors were characterized based on the recommendations of the U.S. National Cancer Institute: if more than 2 of the 5 markers demonstrated size alterations or shifts in tumor DNA with respect to normal tissue DNA, such tumors were defined as tumors with high-frequency microsatellite instability (MSI-H). Tumors with only one marker showing instability were classified as tumors with low-frequency microsatellite instability (MSI-L), while tumors with none of the markers showing instability were classified as microsatellite stable (MSS) tumors.

2.2.5. Determination of DNA mismatch repair protein expression

DNA mismatch repair protein expression in paraffin-embedded specimens was analyzed by employing immunohistochemical methods on a Dako Autostainer (AutostainerLink 48, DAKO, Glostrup, Denmark).

Microscope slides were digitized with an Aperio ScanScope XT slide scanner (Aperio Technologies, Vista, California, USA) at 20× magnification.

2.2.6. Statistical data analysis

Statistical data analysis was made using "SPSS 20.0" and "Stata 11.0" software. For quantitative characteristics averages, standard deviations, minimum and maximum values were calculated. For qualitative characteristics – value frequencies and relative frequencies in percent were calculated. Shapiro-Wilk test was used to evaluate quantitative data for normality. Data averages of two groups were compared using Student's t test. Fisher's exact test and χ^2 test were applied to evaluate the independence of qualitative characteristics. Confidence intervals were calculated for binomial qualitative variables. Kaplan-Meier and Cox regression methods were applied to evaluate the survival of patients. Long rank criterion was used to compare survival curves. Evaluation differences were considered statistically significant for p values lower than α (α=0,05).

3. RESULTS

3.1. Microsatellite instability in endometrial cancer

Using both Promega Corporation marker panels – already used (BAT-25, BAT-26, NR-21, NR-24, and MONO-27) and new (BAT-52, BAT-55, BAT-56, BAT-57, and BAT-59) – microsatellite instability analysis was performed in all the patients enrolled to the study (n=109).

3.1.1. Analysis of microsatellite instability using BAT-25, BAT-26, NR-21, NR-24, and MONO-27 markers

Using BAT-25, BAT-26, NR-21, NR-24, and MONO-27 markers, high-frequency microsatellite instability (≥ 2 markers of 5) was detected in 17 of the 109 cases (15.60%; 95% CI 9.36−23.79). Tumors with one or no unstable markers were categorized as being microsatellite stable – 92 of 109 cases (84.40%).

Microsatellite instability was analyzed in endometrial tumor and healthy tissue (blood) specimens of the same person. Using the abovementioned panel of 5 markers, the following alterations between the genomic loci investigated were identified:

- alterations in 2 of the 5 markers were identified in 6 of the 17 cases (35.29%);
- alterations in 3 of the 5 markers in 5 of the 17 cases (29.41%);
- alterations in 4 of the 5 markers in 5 of the 17 cases (29.41%);
- alterations in 5 of the 5 markers in 1 of the 17 cases (5.88%).

The abovementioned alterations were identified most frequently using BAT-26 and MONO-27 markers.

Table 3.1.1.1 summarizes patients' and tumor characteristics of each case with high-frequency microsatellite instability.

Table 3.1.1.1. Characteristics of each case with high-frequency microsatellite instability (n=17)

Patients' characteristics		Tumor characteristics			Number of identified markers
Age, years	**BMI, kg/m^2**	**Tumor grade**	**FIGO stage**	**Myometrial invasion**	
71	25.2	G2	IA	None	2/5
68	28.3	G2	IA	None	4/5
54	28.7	G1	IA	None	3/5
74	36.8	G3	IA	< 1/2	4/5
72	46.1	G1	IA	< 1/2	2/5
61	36.3	G2	IA	≥ 1/2	5/5
63	30.1	G2	IA	≥ 1/2	2/5
72	36.6	G3	IB	≥ 1/2	3/5
67	26.0	G3	IB	≥ 1/2	2/5
58	23.1	G2	IB	≥ 1/2	3/5
80	26.0	G2	IB	≥ 1/2	2/5
66	19.4	G2	IB	≥ 1/2	3/5
73	21.3	G3	IB	≥ 1/2	2/5
61	36.0	G3	IIIA	≥ 1/2	4/5
63	27.3	G2	IIIC	≥ 1/2	4/5
53	24.7	G3	IIIC	≥ 1/2	4/5
61	37.2	G2	IVB	≥ 1/2	3/5

BMI, body mass index; G1, well-differentiated tumor; G2, moderately differentiated tumor; G3, poorly differentiated tumor; FIGO, International Federation of Gynecology and Obstetrics; IA, tumor limited to the endometrium or invades less than half of the myometrium; IB, tumor invades half or more of the myometrium; IIIA, tumor involves the serosa of the corpus uteri and/or adnexa of the uterus; IIIC, metastasis to pelvic and/or para-aortic lymph nodes; IVB, distant metastasis; none, tumor limited to the endometrium;

< 1/2, tumor invades less than half of the myometrium; ≥ 1/2, tumor invades half or more of the myometrium.

The mean age of the women with endometrial cancer showing high-frequency microsatellite instability was 66±7 years (range, 53−80 years) and did not differ significantly from that of the patients with microsatellite-stable endometrial cancer, i.e., 64±10 years (range, 40−83 years) (P=0.534). The patients aged 60 to 70 years comprised the largest proportion (47.06%, 8 of 17). It should be noted that there were no women

aged 50 years and less in the group of patients with high-frequency microsatellite instability, and only 3 (17.6%) of the 17 patients were aged from 53 to 60 years.

The mean BMI of the patients with high-frequency microsatellite instability did not differ significantly from that of the patients with the microsatellite-stable phenotype (29.9±7.1 kg/m^2 and 31.3±5.2 kg/m^2, respectively; *P*=0.360). Obese women with a BMI of 30.0 kg/m^2 and more comprised the largest group (7 of 17, 41.18%); 6 patients (35.29%) were overweight and their BMI varied from 25.0 to 29.9 kg/m^2. Of the 17 patients, 4 (23.53%) had normal body weight; their BMI ranged from 18.5 to 24.9 kg/m^2. Among the patients enrolled to the study, there was no one underweight woman with a BMI of less than 18.5 kg/m^2.

High-frequency microsatellite instability was detected only in adenocarcinomas of endometrioid histology 17 of 100 cases (17.00%; 95% CI 10.23–25.82), and none of the 9 cases of nonendometrioid-type adenocarcinoma were found to be MSI-H. It is worth noting that the number of the latter tumor was low, and no statistical analysis could be performed. Therefore, associations between microsatellite instability and clinicopathologic tumor characteristics were analyzed only in endometrioid-type adenocarcinomas, and the results of this analysis are presented in Table 3.1.1.2.

Table 3.1.1.2. Microsatellite instability and tumor clinicopathologic characteristics

Characteristics	No. of patients (n=100)	MSI-H (n=17.17%)		MSS (n=83.83%)		*P*
Tumor grade						
G1	40	2	5.00	38	95.00	0.005*
G2	46	9	19.57	37	80.43	
G3	14	6	42.86	8	57.14	
Myometrial invasion						
< 1/2	62	5	8.06	57	91.94	0.049*
≥ 1/2	38	12	31.58	26	68.42	
FIGO stage						
I	84	13	15.48	71	84.52	0.100
II	6	–	–	6	100.0	
III-IV	10	4	40.00	6	60.00	

MSI-H, high-frequency microsatellite instability; MSS, microsatellite stable; G1, well-differentiated tumor; G2, moderately differentiated tumor; G3, poorly differentiated tumor; FIGO, International Federation of Gynecology and Obstetrics; stage I, tumor confined to the corpus uteri; stage II, tumor invades the cervical stroma but does not extend beyond the cervix; stage III, local or regional tumor spread; stage IV, tumor invades bladder mucosa and/or bowel mucosa, and/or distant metastasis; none, tumor limited to the endometrium; < 1/2, tumor limited to the endometrium or invades less than half of the myometrium; ≥ 1/2, tumor invades half or more of the myometrium; n, number of cases; *statistically significant difference.

The data presented in Table 3.1.1.2 show that there was a significant difference in tumor grade (*P*=0.005) and myometrial invasion (*P*=0.049) between the MSI-H and MSS groups.

In the group that was found to have high-frequency microsatellite instability, moderately and poorly differentiated tumors accounted for 15 of the 17 cases (88.24%; 95% CI 63.56–98.54) and in the group with the microsatellite-stable phenotype, these tumors made up 45 of the 83 cases (54.22%; 95% CI 42.92–65.21).

Tumors that invaded half or more of the myometrium was determined in 12 of the 17 cases (70.59%; 95% CI 44.04–89.69) and in 26 of the 83 cases (33.7%; 95% CI

21.59–42.44) in the groups with high-frequency microsatellite instability and with the microsatellite-stable phenotype, respectively.

There was no significant association between the disease stage according to the FIGO and microsatellite instability (*P*=0.100).

The results of our study show that using the panel of Promega Corporation markers (BAT-25, BAT-26, NR-21, NR-24, and MONO-27), 15.60% of the patients with endometrial cancer (n=109) had high-frequency microsatellite instability, and in all cases, endometrioid-type adenocarcinomas were diagnosed. Statistically significant differences in tumor grade (*P*=0.005) and myometrial invasion (*P*=0.049) between the groups (MSI-H and MSS) were found.

3.1.2. Analysis of microsatellite instability using BAT-52, BAT-55, BAT-56, BAT-57, and BAT-59 markers

Using a new set of BAT-52, BAT-55, BAT-56, BAT-57, and BAT-59 markers created by Promega Corporation, high-frequency microsatellite instability (≥ 2 markers of 5) was found in 30 of the 109 cases (27.52%; 95% CI 19.40−36.90).

Using these 5 markers, the following alterations between the genomic loci investigated were identified:

- alterations in 2 of the 5 markers were identified in 7 of the 30 cases (23.33%);
- alterations in 3 of the 5 markers in 5 of the 30 cases (16.67%);
- alterations in 4 of the 5 markers in 13 of the 30 cases (43.33%);
- alterations in 5 of the 5 markers in 5 of the 30 cases (16.67%).

It should be noted that similar alterations were found while using the following 4 of the 5 markers: BAT-52, BAT-55, BAT-56, and BAT-59.

Table 3.1.2.1 summarizes patients' and tumor characteristics of each case with high-frequency microsatellite instability.

Table 3.1.2.1. Characteristics of each case with high-frequency microsatellite instability (n=30)

Patients' characteristics		Tumor characteristics			Number of identified markers
Age, years	**BMI, kg/m^2**	**Tumor grade**	**FIGO stage**	**Myometrial invasion**	
71	25.2	G2	IA	None	2/5
68	28.3	G2	IA	None	4/5
54	28.7	G1	IA	None	3/5
54	29.3	G1	IA	None	2/5
53	37.2	G1	IA	None	2/5
74	36.8	G3	IA	< 1/2	4/5
72	46.1	G1	IA	< 1/2	2/5
61	36.3	G3	IA	< 1/2	5/5
63	30.1	G2	IA	< 1/2	2/5
75	25.7	G2	IA	< 1/2	2/5
46	40.2	G2	IA	< 1/2	3/5
53	34.2	G1	IA	< 1/2	2/5
51	27.3	G1	IA	< 1/2	4/5
67	30.1	G2	IA	< 1/2	3/5
53	22.6	G2	IB	≥ 1/2	2/5
64	31.5	G3	IB	≥ 1/2	4/5
52	29.8	G2	IB	≥ 1/2	3/5
72	36.6	G3	IB	≥ 1/2	3/5
67	26.0	G3	IB	≥ 1/2	2/5
58	23.1	G2	IB	≥ 1/2	3/5
80	26.0	G2	IB	≥ 1/2	2/5
66	19.4	G2	IB	≥ 1/2	3/5

73	21.3	G3	IB	≥ 1/2	2/5
63	26.0	G1	IB	≥ 1/2	2/5
70	26.0	G2	IB	≥ 1/2	4/5
74	30.5	G2	IB	≥ 1/2	5/5
61	36.0	G2	IIIA	≥ 1/2	4/5
63	27.3	G2	IIIC	≥ 1/2	4/5
53	24.7	G3	IIIC	≥ 1/2	4/5
61	37.2	G2	IVB	≥ 1/2	3/5

BMI, body mass index; G1, well-differentiated tumor; G2, moderately differentiated tumor; G3, poorly differentiated tumor; FIGO, International Federation of Gynecology and Obstetrics; IA, tumor limited to the endometrium or invades less than half of the myometrium; IB, tumor invades half or more of the myometrium; IIIA, tumor involves the serosa of the corpus uteri and/or adnexa of the uterus; IIIC, metastasis to pelvic and/or para-aortic lymph nodes; IVB, distant metastasis; none, tumor limited to the endometrium; < 1/2, tumor invades less than half of the myometrium; ≥ 1/2, tumor invades half or more of the myometrium.

The mean age and BMI of the women who were found to have endometrial cancer with high-frequency microsatellite instability was 63±9 years (range, 46-80 years) and 30.0±6.1 kg/m^2 (range, 19.4–46.1) respectively. There were no significant differences in patients' age (*P*=0.413*)*, body mass index (*P*=0.204), and microsatellite instability status between the groups (MSI-H and MSS).

High-frequency microsatellite instability was detected only in adenocarcinomas of endometrioid histology – 30 of 100 cases (30.00%; 95% CI 21.24−39.98), the same as using the first marker panel, and none of the 9 cases of nonendometrioid-type adenocarcinoma were found to be MSI-H. Therefore, associations between clinicopathologic tumor characteristics and microsatellite instability were analyzed only in endometrioid-type adenocarcinomas, and the results of this analysis are displayed in Table 3.1.2.2.

Table 3.1.2.2. Microsatellite instability and clinicopathologic tumor characteristics

Characteristics	No. of patients (n=100)	MSI-H (n=30.30%)		MSS (n=70.70%)		*P*
Tumor grade, n (%)						
G1	40	7	17.50	33	82.50	0.042*
G2	46	16	34.78	30	65.22	
G3	14	7	50.00	7	50.00	
Myometrial invasion, n (%)						
< ½	62	14	22.58	48	77.42	0.046*
≥ ½	38	16	42.11	22	57.89	
FIGO stage, n (%)						
I	84	26	30.95	58	69.05	0.234
II	6	–	–	6	100.0	
III-IV	10	4	40.00	6	60.00	

MSI-H, high-frequency microsatellite instability; MSS, microsatellite stable; G1, well-differentiated tumor; G2, moderately differentiated tumor; G3, poorly differentiated tumor; FIGO, International Federation of Gynecology and Obstetrics; stage I, tumor confined to the corpus uteri; stage II, tumor invades the cervical stroma but does not extend beyond the cervix; stage III, local or regional tumor spread; stage IV, tumor invades bladder mucosa and/or bowel mucosa, and/or distant metastasis; none, tumor limited to the endometrium; < 1/2, tumor limited to the endometrium or invades less than half of the myometrium; ≥ 1/2, tumor invades half or more of the myometrium; n, number of cases; *statistically significant difference.

The data in Table 3.1.2.2 show that differences in tumor grade (*P*=0.042) and myometrial invasion (*P*=0.046) between the MSI-H and MSS groups were significant. There was no significant association between the disease stage (FIGO) and microsatellite instability (*P*=0.234).

The analysis of 109 patients with endometrial cancer revealed that high-frequency microsatellite instability was detected 2 times more frequently while using the newly created Promega Corporation markers – BAT-52, BAT-55, BAT-56, BAT-57, and BAT-59 – than already used markers – BAT-25, BAT-26, NR-21, NR-24, and MONO-27 (27.52% and 15.60% of the cases, respectively).

3. 2. Expression of DNA mismatch repair proteins (MLH1, MLH2, MSH6, and PMS2) in endometrial cancer

3. 2. 1. Immunohistochemical testing of DNA mismatch repair protein expression in tumors, in which microsatellite instability was identified by using BAT-25, BAT-26, NR-21, NR-24, and MONO-27 markers

At this study stage, immunohistochemical testing of DNA mismatch protein (MLH1, MLH2, MSH6, and PMS2) expression was carried out only in those cases when high-frequency microsatellite instability was identified. As mentioned above, microsatellite instability using early created panel of Promega Corporation markers was determined in 17 of the 109 cases. The results of immunohistochemical testing showed that even in 16 of the 17 cases (94.12%; 95% CI 71.31–99.85), there was no expression of at least one protein and only in 1 (5.9%) of the 17 cases (5.88%; 95% CI 0.15–28.69), the expression of all the proteins investigated was positive. The data of protein expression and its distribution is depicted in Fig. 3.2.1.1.

Protein	Patients																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
MLH1																	
PMS2																	
MSH2																	
MSH6																	
Age, years	71	72	72	63	61	58	80	66	68	61	74	63	73	54	53	67	61
Grade	G2	G2	G1	G2	G2	G2	G2	G2	G2	G2	G2	G2	G3	G1	G3	G2	G2
FIGO stage	IA	IB	IA	IA	IIIA	IB	IB	IB	IA	IA	IA	IIIC	IB	IA	IIIC	IB	IVB

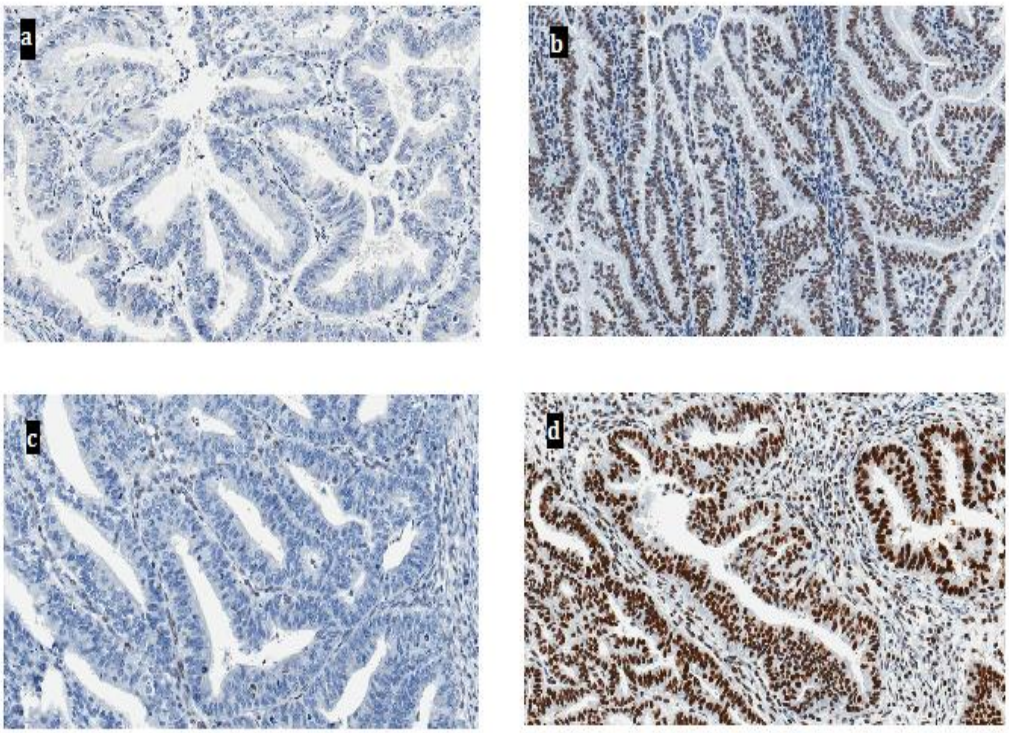
G, tumor grade; red color, absence of protein expression; green color, presence of protein expression.

Fig. 3.2.1.1. Distribution of DNA mismatch repair protein expression in endometrial cancer

The data presented in Fig. 3.2.1.1 show that of the 17 cases, 12 (70.59%; 95% CI 44.04–89.69) lacked MLH1 protein expression; 14 (82.35; 95% CI 56.57–96.20), PMS2 protein expression; 3 (17.65%; 95% CI 3.80–43.43), MSH6 protein expression, and 1 (5.88%; 95% CI 0.15–28.69), MSH2 protein expression.

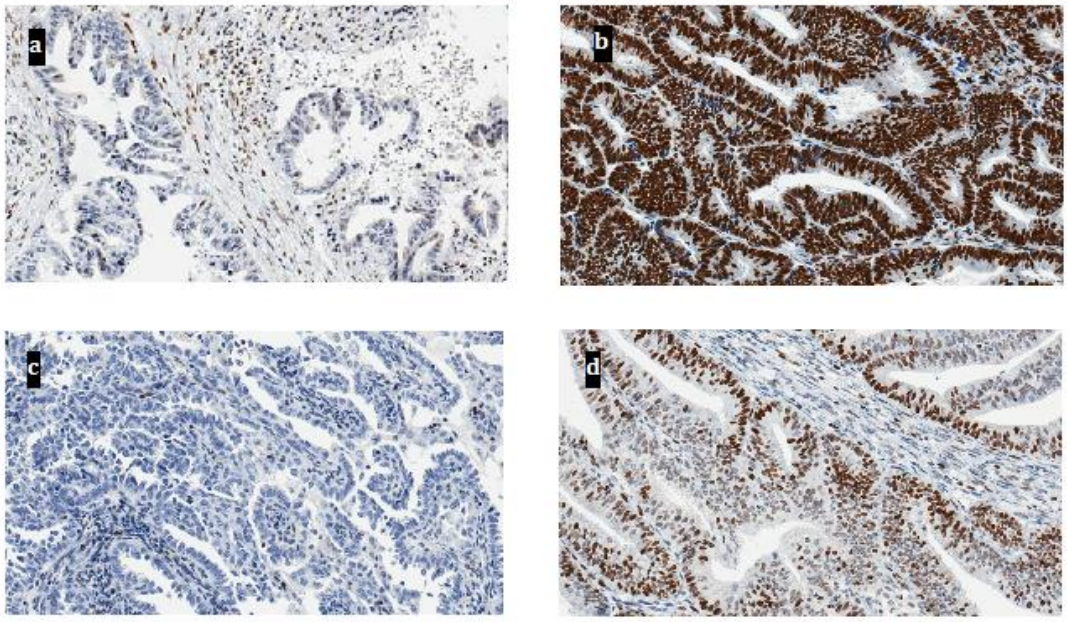
Both pairs of MLH1/PMS2 and MSH2/MSH6 proteins are needed for the DNA MMR system to function properly. In our study, concurrent loss of MLH1/PMS2 protein expression was determined most frequently 12 of the 16 cases (75.00%; 95% CI 47.62–92.73), and concurrent loss of another pair of necessary proteins (MSH2/MSH6) was documented only in 1 of the 16 cases (6.25%; 95% CI 0.16–30.23). It should be noted that in this case, there was a lack of expression of all the proteins investigated. Between the abovementioned pairs of MLH1/PMS2 and MSH2/MSH6 proteins, there were 2 of the 16 cases (12.50%; 95% CI 1.56−38.35) with a lack of expression of either PMS2 or MSH6 protein.

The examples of DNA MMR system protein expression are shown in Figures 3.2.1.2. and 3.2.1.3.



MLH1 protein expression is absent (a); MLH1 protein expression is present (b);
PMS2 protein expression is absent (c); PMS2 protein expression is present (d)

Fig. 3.2.1.2. MLH1 and PMS2 protein expression in endometrioid adenocarcinoma



MSH2 protein expression is absent (a); MSH2 protein expression is present (b);
MSH6 protein expression is absent (c); MSH6 protein expression is present (d)

Fig. 3.2.1.3. MSH2 and MSH6 protein expression in endometrioid adenocarcinoma

Due to a small sample size, there was no possibility to statistically compare altered expression of the proteins investigated considering patients' and tumor characteristics. Therefore, by presenting these characteristics in Table 3.2.1.1, we just attempted to show how the investigated cases with a defective DNA mismatch repair system caused by lack of one or two proteins distributed depending on patients' age, body mass index, tumor grade, and disease stage.

Table 3.2.1.1. Distribution of patients by loss of DNA mismatch repair protein expression (n=16)

Characteristics	Loss of two protein expression		Loss of one protein expression	
	MLH1– PSM2– (n=12, 75.00%)	**MSH2– MSH6–** (n=1*, 6.25%)	**PMS2– MLH1+** (n=2, 12.50%)	**MSH6– MSH2+** (n=2, 12.50%)
Age, mean ± SD, years	67±7	63	60±10	64±13
BMI, mean ± SD, kg/m^2	30,9±7,5	27,0	25,3±0,9	25,0±5,2
Tumor histologic type, n (%) Endometrioid adenocarcinoma	12 (100)	1(100)	2(100)	2(100)
Tumor grade, n (%) G1	1 (8.33)	0	0	1 (50.00)
G2	8 (66.67)	1 (100)	0	0
G3	3 (25.00)	0	2 (100)	1 (50.00)
FIGO stage, n (%) I	10 (83.33)	0	1 (50.00)	2 (100)
II	0	0	0	0
III-IV	2 (16.67)	1 (100)	1 (50.00)	0

n, number of cases; SD, standard deviation; *, absence of all proteins expression; –, absence of protein expression; +, presence of protein expression; BMI, body mass index.

The results summarized in Table 3.2.1.1 show that the patients with concurrent loss of MLH1/PSM2 protein expression were older (mean age, 67±7 years), and their body mass index (mean BMI, 30.9±7.5 kg/m^2) was marginally greater as compared to that of the patients in other groups. The aforementioned group was mainly comprised of patients with stage I (FIGO) (83.33%) and moderately differentiated (G2; 66.67%) endometrial cancer.

Thus, the results of our study show that on immunohistochemistry, 94.12% of the cases with high-frequency microsatellite instability lacked expression of at least one

DNA mismatch repair protein, and concurrent loss of MLH1/PMS2 protein expression was most common (75.00% of cases).

3. 2. 2. Immunohistochemical testing of DNA mismatch repair protein expression in tumors, in which microsatellite instability was identified by using BAT-55, BAT-59, BAT-52, BAT-56, and BAT-57 markers

Using a new panel of Promega Corporation markers, high-frequency microsatellite instability was detected in 30 of the 109 cases. Immunohistochemical testing was performed in all the cases (n=30), and its results showed that of the 30 cases, 22 (73.33%; 95% CI 54.11–87.72) lacked the expression of at least one DNA MMR protein (MLH1, PMS2, MSH2, or MSH6), and the remaining 8 of the 30 cases (26.67%; 95% CI 12.28–45.89) had positive expression of all the proteins being investigated.

Fig. 3.2.2.1 displays the expression of the above proteins and its distribution. The data presented in this figure show that of the 30 cases, 17 (56.67%; 95% CI 37.43–74.54) lacked MLH1 protein expression; 19 (63.33%; 95% CI 43.86–80.07), PMS2 protein expression; 4 (13.33%; 95% CI 3.76–30.72), MSH6 protein expression; and 2 cases (6.67%; 95% CI 0.82–22.07), MSH2 protein expression. Concurrent loss of MLH1/PMS2 protein expression, as in the first case, was most common – 17 of the 22 cases (77.27%; 95% CI 54.63–92.18), and the lack of expression of another protein pair (MSH2/MSH6) was documented in 2 of the 22 cases (9.09%; 95% CI 1.12–29.16). Of the 22 cases, 2 (9.09%; 95% CI 1.12–29.16) did not show expression of either MSH6 or PMS2 protein.

Protein	Patients																													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
MLH1																														
PMS2																														
MSH2																														
MSH6																														
Age, years	71	72	72	63	61	58	80	66	68	61	74	53	51	70	74	67	63	52	73	54	53	67	61	75	46	63	54	53	64	53
G	2	2	1	2	2	2	2	2	2	2	2	1	1	2	2	2	2	2	3	1	3	2	2	2	2	1	1	2	3	1
FIGO stage	IA	IB	IA	IA	IIIA	IB	IB	IB	IA	IA	IA	IA	IA	IB	IB	IA	IIIC	IB	IB	IA	IIIC	IB	IVB	IA	IA	IB	IA	IB	IB	IA

G, tumor grade; red color, absence of protein expression; green color, presence of protein expression.

Figure 3.2.2.1. Distribution of DNA mismatch repair protein expression in endometrial cancer

Table 3.2.2.1 shows the distribution of patients by loss of DNA MMR protein expression as well as patients' and tumor characteristics.

Table 3.2.2.1. Distribution of patients by loss of DNA mismatch repair protein expression (n=22)

Characteristics	Loss of two protein expression		Loss of one protein expression	
	MLH1–PSM2– (n=17, 77.27%)	**MSH2–MSH6–** (n=2*, 9.09%)	**PMS2–MLH1+** (n=2, 9.09%)	**MSH6–MSH2+** (n=2, 9.09%)
Age, mean±SD, years	66±8	58±8	60±10	64±13
BMI, mean±SD, kg/m^2	30,5±6,5	28,6±1,8	25,3±0,9	25,0±5,2
Tumor histologic type, n (%) Endometrioid adenocarcinoma	17 (100)	2 (100)	2 (100)	2 (100)
Tumor grade, n (%) G1	3 (17.65)	0	0	1 (50,00)
G2	11 (64.71)	2 (100)	0	0
G3	3 (17.65)	0	2 (100)	1 (50.00)
FIGO stage I	15 (88.24)	1 (50.00)	1 (50.00)	2 (100)
II	0	0	0	0
III-IV	2 (11.76)	1 (50.00)	1 (50.00)	0

n, number of cases; SD, standard deviation; *, absence of all proteins expression in one case; –, absence of protein expression; +, presence of protein expression; BMI, body mass index.

The results summarized in Table 3.2.2.1 show that the same as using the first panel of markers, the patients with concurrent loss of MLH1/PSM2 protein expression were older (mean age, 66±8 years) as compared with other groups. The aforementioned group was mainly comprised of patients with stage I (FIGO) (88.24%) and moderately differentiated (G2; 64.71) endometrial cancer.

3.2.3. Selection of patients for genetic counseling and molecular genetic testing

Screening of patients for suspected Lynch syndrome was performed based on the algorithms published in the literature (Fig. 3.2.3.1), where the main attention was paid to the patient's age at diagnosis, family history (Amsterdam Criteria II), and expression of DNA mismatch repair proteins.

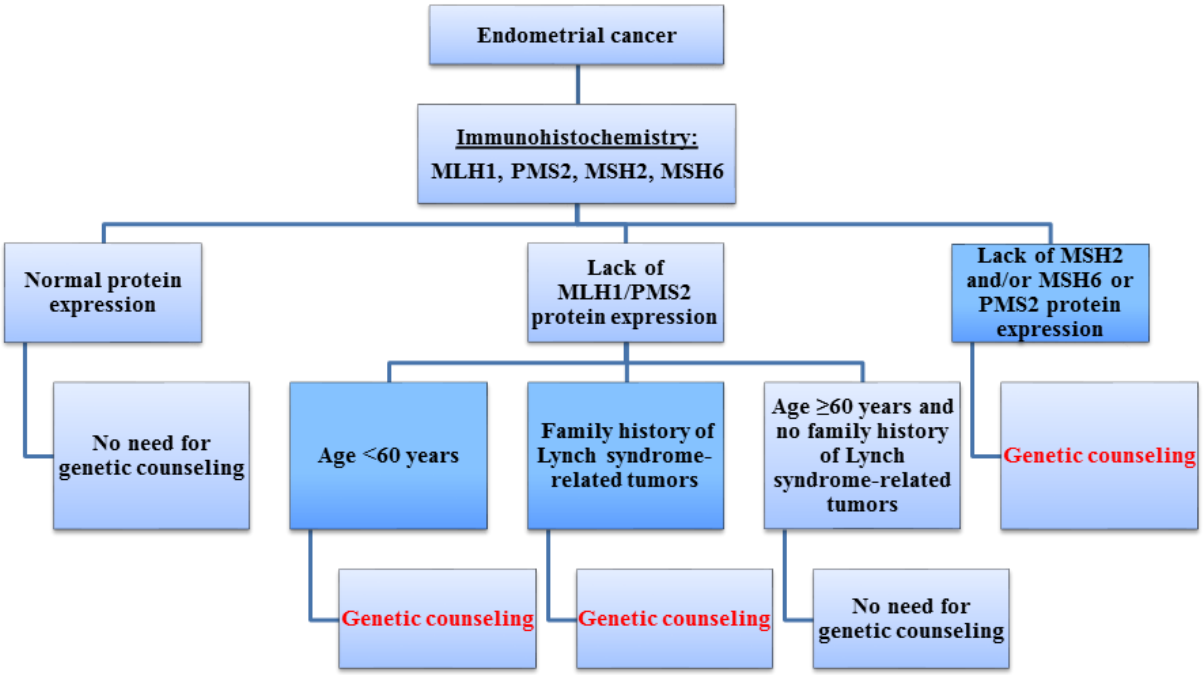


Fig. 3.2.3.1. The recommended algorithm for genetic counseling for patients with endometrial cancer

Considering the results obtained, of the 30 patients investigated, 9 (30.00%; 95% CI 14.73–49.40) were recommended a consultation with a geneticist. Their characteristics are shown in Figure 3.2.3.2. It should be noted that in the group with a loss of MLH1/PMS2 protein expression (n=17), 14 patients were older than 60 years and had no family history of Lynch syndrome-related cancers. Apparently, these cases of endometrioid adenocarcinoma were sporadic, and the patients were not advised to

consult a geneticist. Three patients younger than 60 years from the abovementioned group were recommended a geneticist's consultation.

Protein	Patients								
	1	2	3	4	5	6	7	8	9
MLH1									
PMS2									
MSH2									
MSH6									
Age, years	58	53	51	53	67	63	52	54	73
Grade	G2	G1	G1	G3	G2	G2	G2	G1	G3
FIGO stage	IB	IA	IA	IIIC	IB	IIIC	IA	IA	IB
Amsterdam II Criteria	no	yes	yes	no	no	NA	no	no	no

G, tumor grade; Amsterdam II Criteria: at least one relative had Lynch syndrome-related cancer such as colorectal, endometrial, small bowel, ureter, or kidney cancer; no, did not meet the Amsterdam criteria; yes, met the Amsterdam criteria; NA, not available; red color, protein expression is absent; green color, protein expression is present.

Fig. 3.2.3.2. Characteristics of the patients who consulted with a geneticist

The data in Figure 3.2.3.2 show that of the 9 patients who were consulted by a genetic counselor, 6 did not meet the Amsterdam II criteria, i.e., there was no relative in their families, who would had a Lynch syndrome-related cancer. Two patients met the Amsterdam II criteria as they had relatives in their families, who had a Lynch syndrome-related cancer (colorectal cancer in both cases). Therefore, they were suggested molecular genetic testing for gene mutations that cause Lynch syndrome. Among the patients selected for genetic counseling, one could not provide any information on her family history of Lynch syndrome-related cancers. However, considering the fact that this 63-year-old patient with stage IIIC (FIGO) endometrioid adenocarcinoma had no

expression of all the proteins investigated, she was also suggested to undergo molecular genetic testing.

Therefore, the results of our study show that of the 109 patients enrolled in the study, 3 (2.75%; 95% CI 0.57–7.83) were recommended to undergo molecular genetic testing for gene mutations that cause Lynch syndrome.

3.3. Impact of microsatellite instability on patient survival

In this study, the patients were followed up for 40.4 months on average (range, 5.2 to 47.9 months); therefore, the 3-year survival was analyzed. Figs. 3.3.1–3.3.5 display the dependence of survival of patients with endometrial cancer on clinicopathologic tumor characteristics, and Table 3.3.1 shows the results of 3-year survival analysis.

Table 3.3.1. Survival of patients with endometrial cancer

Characteristics	3-year survival,%	95% CI
Age, years		
< 64	92.45	81.13–97.10
≥ 64	90.96	79.63–96.14
FIGO stage		
IA	96.83	89.90–99.20
IB	92.00	71.64–97.94
II-IV	75.89	51.39–89.20
Tumor grade		
G1	100.00	–
G2	95.87	84.46–98.95
G3	64.29	34.33–83.31
Gx	50.00	5.78–84.49
Histologic tumor type		
Endometrioid adenocarcinoma	95.93	89.52–98.46
Other types*	44.44	13.59–71.93

Characteristics	3-year survival,%	95% CI
Treatment Surgical	97.06	88.75–99.26
surgical and radiotherapy	82.25	64.68–91.61
surgical and chemotherapy	85.71	33.41–97.86
Microsatellite instability MSS	90.15	81.92–94.75
MSI-H	100.0	–

*Serous adenocarcinoma, clear cell carcinoma, adenosarcoma; CI, confidence interval; MSS, microsatellite stable; MSI-H, high-frequency microsatellite instability; Gx, tumor grade cannot be determined.

Patient survival depended on clinicopathologic tumor characteristics. Most (n=100) of the patients had histologically diagnosed endometrioid adenocarcinoma, and their survival was significantly better than that of the patients with tumors of other histologic types (Fig. 3.3.1). The 3-year survival was 95.93% and 44.44%, respectively (Table 3.3.1).

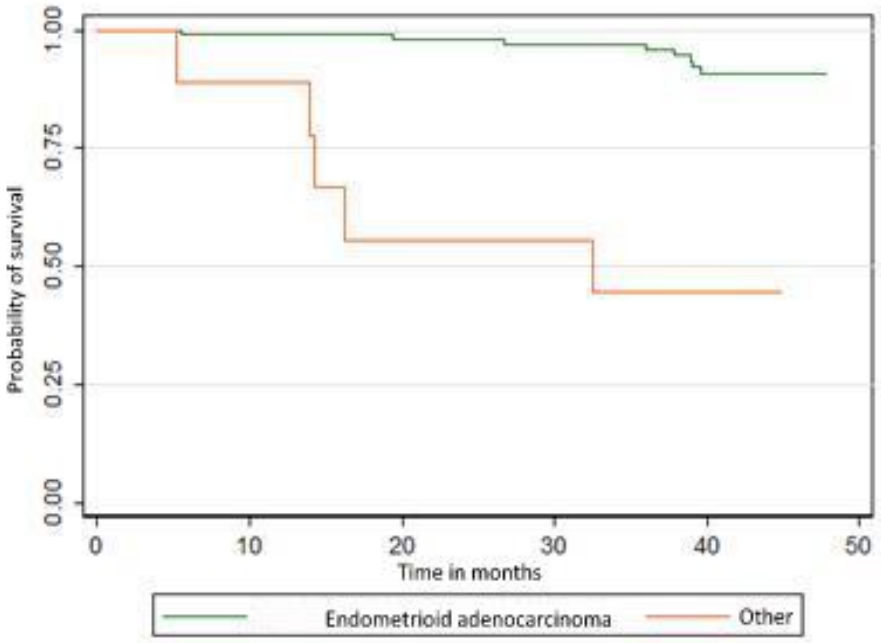


Fig. 3.3.1. Survival of patients with endometrial cancer regarding tumor histologic type (*P*<0.0001)

Patient survival significantly depended on tumor grade as well: the patients with well and moderately differentiated tumors had better survival than their counterparts with a poorly differentiated tumor (Fig. 3.3.2). In the presence of well and moderately differentiated tumors, the 3-year survival was greater than 95% (Table 3.3.1).

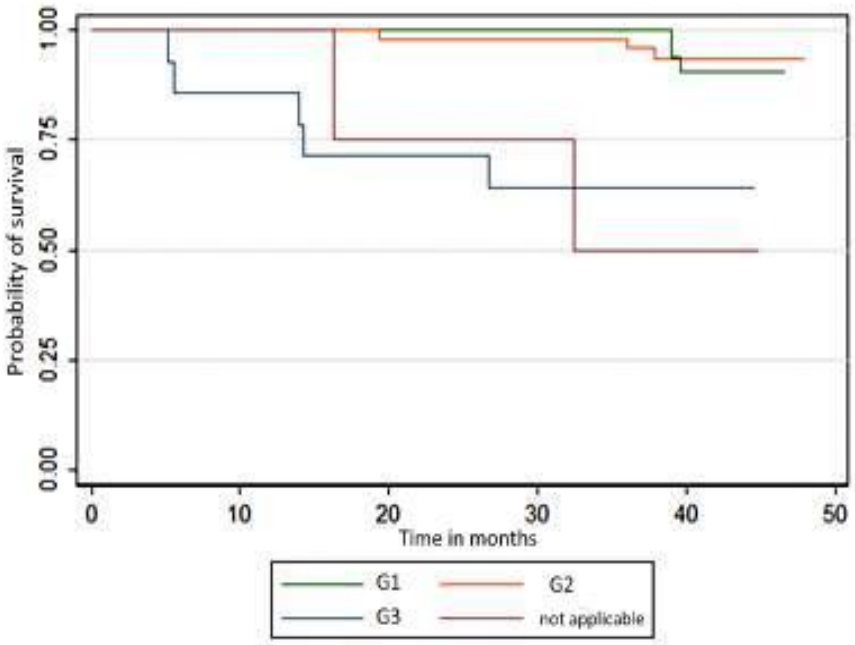


Fig. 3.3.2. Survival of patients with endometrial cancer regarding tumor grade (*P*=0.0001)

Disease stage was a significant factor having an impact on patient survival: the survival of the patients who had tumors confined to the corpus uteri (stage IA-IB according to the FIGO) was better than that of the patients diagnosed with local regional tumor or spread (stage II-IV according to the FIGO) (Fig. 3.3.3). The 3-year survival of the patients with stage IA and IB endometrial cancer exceeded 90% (Table 3.3.1).

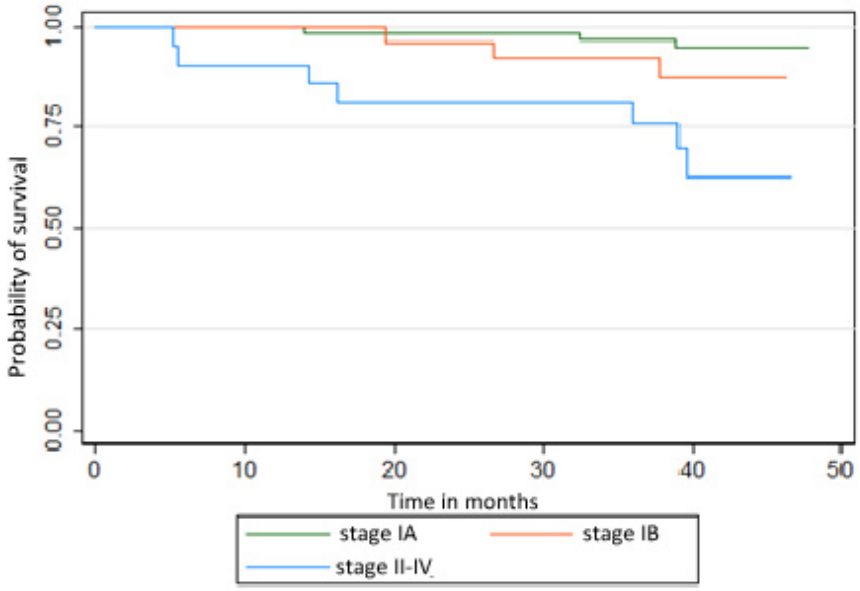


Fig. 3.3.3. Survival of patients with endometrial cancer regarding disease stage (*P*=0.001)

As previously mentioned, all the patients (n=109) enrolled in the study were subjected to surgical treatment, and part (n=37) of them, depending on disease stage and tumor grade, underwent postoperative radiotherapy based on the treatment standards approved by the Institute of Oncology, Vilnius University, and only four patients underwent postoperative chemotherapy (n = 4). The patients who underwent only surgical treatment had significantly better survival than those who had postoperative radiotherapy or chemotherapy (Fig. 3.3.4). It worth noting that the majority (65 of 68) of the patients who underwent only surgical treatment were diagnosed with an early stage (IA-IB) disease, and their 3-year survival was 97.06% (Table 3.3.1).

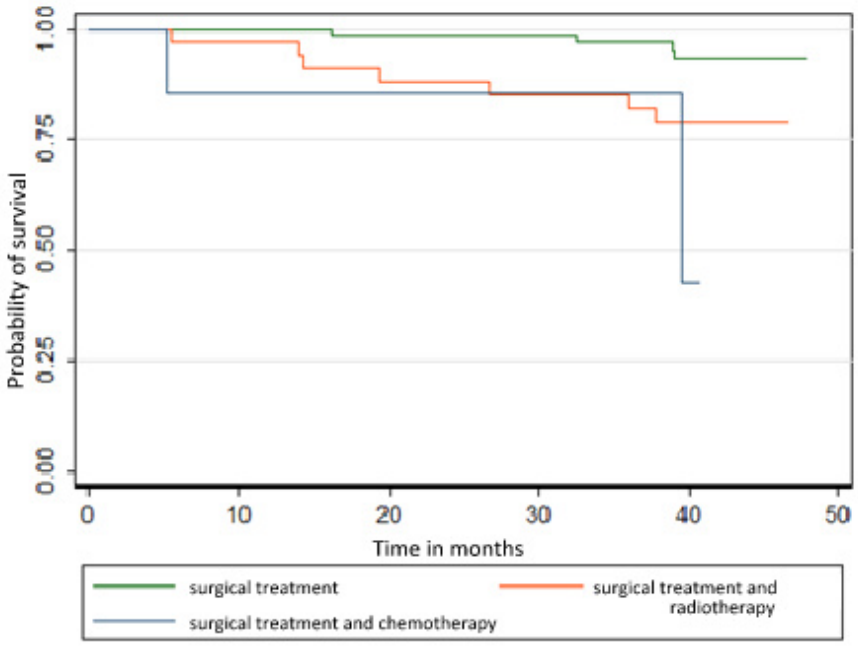
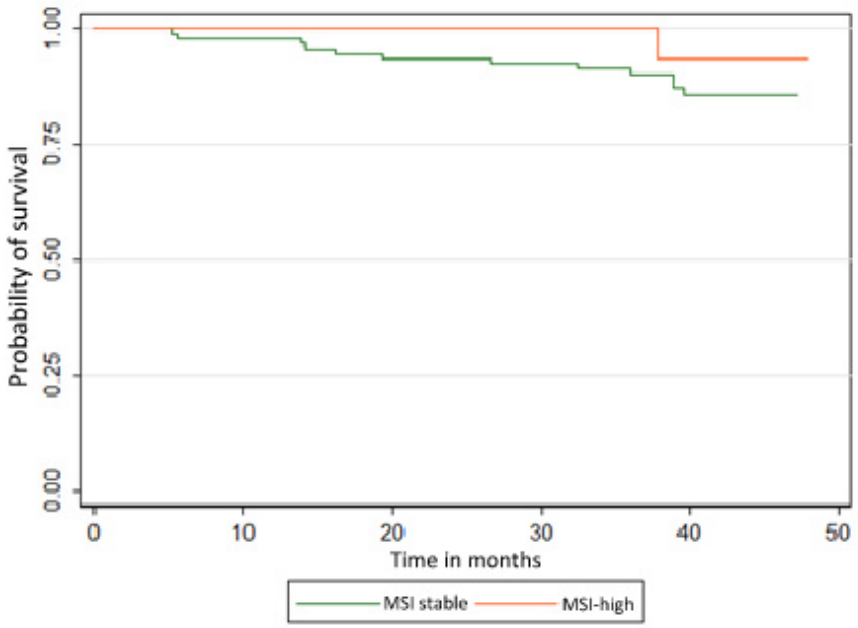


Fig. 3.3.4. Survival of patients with endometrial cancer regarding applied treatment (*P*=0.031)

The results of study showed no statistically significant association between patient survival and microsatellite stability status, but the survival of the patients with the microsatellite-stable phenotype was poorer than that of the patients with tumors showing high-frequency microsatellite instability (Fig. 3.3.5).



MSI-H, high-frequency microsatellite instability; MSS, microsatellite stable.

Fig. 3.3.5. Survival of patients with endometrial cancer regarding microsatellite stability status (*P*=0.431)

Multivariate analysis was done with the aim to determine the relevance of clinicopathologic characteristics and their interaction to patient survival (Table 3.3.2). Only factors that were significant in the univariate analysis as well as microsatellite instability were entered into the multivariate analysis model.

Table 3.3.2. Results of multivariate analysis regarding microsatellite instability status in patients with endometrial cancer

Characteristics	Hazard ratio	95% CI	*P*
FIGO stage IA	1.00	Ref.	–
IB	2.25	0.24–21.03	0.483
II-IV	4.94	0.60–40.60	0.140
Tumor grade G1	1.00	Ref.	–
G2	0.85	0.14–5.14	0.864
G3	3.88	0.73–20.51	0.112
Gx	1.46	0.17–12.56	0.354
Histological tumor type Endometrioid adenocarcinoma	1.00	Ref.	–
Other types*	4.13	0.60–40.60	0.111
Treatment Surgical	1.00	Ref.	–
Surgical and radiotherapy	0.88	0.12–6.40	0.884
Surgical and chemotherapy	1.26	0.10–16.65	0.100
Microsatellite instability MSS	1.00	Ref.	–
MSI-H	0.41	0.05–3.69	0.433

*Serous adenocarcinoma, clear cell carcinoma, adenosarcoma; CI, confidence interval; MSS, microsatellite stable; MSI-H, high-frequency microsatellite instability; Gx, tumor grade cannot be determined.

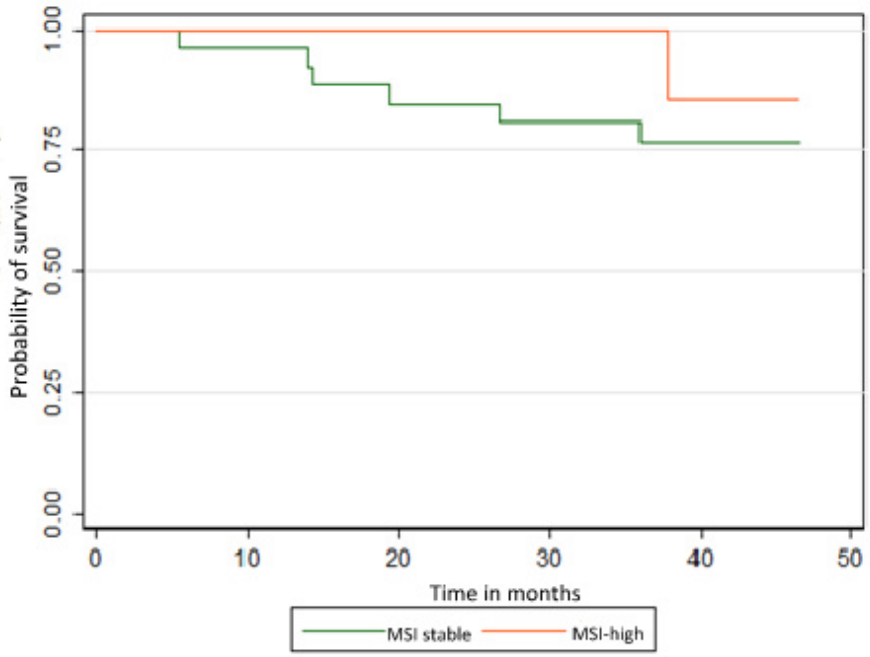
Multivariate analysis revealed that high-frequency microsatellite instability was not significantly associated with the survival of patients with endometrial cancer ($P = 0.433$), but disease stage and tumor grade had a significant impact on patient survival (Table 3.3.3).

Table 3.3.3. Factors having a significant impact on the survival of patients with endometrial cancer

Characteristics	Hazard ratio	95% CI	*P*
FIGO stage IA	1.00	Ref.	–
IB	2.50	0.50–12.43	0.264
II-IV	5.19	1.24–20.74	0.022**
Histological tumor type Endometrioid adenocarcinoma	1.00	Ref.	–
Other types*	6.18	1.84–20.74	0.003**

*Serous adenocarcinoma, clear cell carcinoma, adenosarcoma; CI, confidence interval; **statistically significant difference.

As the data on the impact of microsatellite instability on the survival of patients who underwent postoperative radiotherapy in the literature are scarce, we decided to present the survival analysis of this group (Fig. 3.3.6). Although there was no significant difference in survival between patients with high-frequency microsatellite instability and those with the microsatellite-stable phenotype who underwent postoperative radiotherapy, the survival of patients having cancers with high-frequency microsatellite instability was better than that of patients with microsatellite stable cancers. All the patients in the MSI-H group survived for 3 years, and in the MSS group, the survival was 76.73% (95% CI 55.35–88.82).



MSI-H, high-frequency microsatellite instability; MSS, microsatellite stable.

Fig. 3.3.6. Survival of patients who underwent radiation therapy regarding microsatellite stability status (*P*=0.492)

Thus, the results of our study showed no statistically significant association between the survival of patients with endometrial cancer and high-frequency microsatellite instability. It is worth noting that only 3-year survival was analyzed; therefore, patient follow-up should be continued with particular attention paid to the possible predictive relevance of microsatellite instability in terms of radiotherapy.

4. DISCUSSION

Data on the clinical relevance of microsatellite instability in endometrial cancer are scarce, and the prognostic value of this marker has not been elucidated yet. Little is known about a predictive role of microsatellite instability in endometrial cancer as well. The preliminary results of the study by C. Bilbao et al. [2010] have suggested a possible predictive value of microsatellite instability in radiotherapy response in early stage endometrioid endometrial carcinoma. In our study, most probably due to a small sample size, microsatellite instability had no significant impact on treatment response.

Contradictory data have been reported while studying associations between microsatellite instability and clinicopathologic characteristics such as patients' age, body mass index, histologic tumor type, tumor grade, disease stage, etc. In our study, the association between clinicopathologic characteristics and microsatellite instability was analyzed only in cases of endometrioid-type adenocarcinomas (n=100). The analysis revealed that a higher degree of myometrial invasion and worse tumor grade were significantly associated with high-frequency microsatellite instability. This association was confirmed by other scientists as well.

The key factor that causes microsatellite instability is defects in the DNA mismatch repair system. Four main proteins possessing distinct expression – MLH1, MSH2, MSH6, and PMS2 – are involved in this system. As mentioned earlier, in our study, the analysis of DNA MMR protein expression was done only for those tumors that were found to have high-frequency microsatellite instability. The results of immunohistochemical testing showed that using the first marker panel (BAT-25, BAT-26, NR-21, NR-24, and MONO-27) to detect microsatellite instability, defective expression of at least one DNA MMR protein was found to be in 94.12% of cases (16 of the 17 cases). In our study, concurrent loss of MLH1/PMS2 protein expression was most common (75.00%, 12 of the 16 cases), and concurrent loss of the expression of other pair of necessary proteins − MSH2/MSH6 – was documented only in 1 (6.25%) of the 16 cases. The loss of PMS2 or MSH6 protein expression alone occurred in 2 (12.50%) of the 16 cases. Similar results have been reported by other investigators as well. For example, according to the data of the study by L.M. Peterson et al. [2012], no protein

expression was documented in 94% of the cases with high-frequency microsatellite instability; I. Modica et al. [2007] reported 69% of tumors with high-frequency microsatellite instability when only expression of MLH1 and MSH2 proteins were analyzed, and the sensitivity of testing increased to 91% when PMS2 and MSH6 were added to the antibody panel.

The analysis of our results revealed that in the majority (94.12%) of cases, high-frequency microsatellite instability was associated with defects in the DNA MMR system, i.e., the system does not function properly if there is a lack of at least one protein expression. Among 16 cases with loss of at least one protein expression, one case lacked the expression of all the proteins investigated (MLH1, MSH2, MSH6, and PMS2). This shows that a defective DNA MMR system is not activated, and errors that occur during the replication of DNA are not repaired and their accumulation leads to greater risk of developing a malignancy. Another case when the expression of all the proteins investigated was present in the tumor with high-frequency instability is worth noting as well. Such findings could be caused by the fact that the expression of only 4 main DNA MMR proteins (MLH1, MSH2, MSH6, and PMS2) was analyzed, and the expression of proteins such as PMS1 and MSH3 as well as other proteins involved in this system was not evaluated. Most probably, analysis of DNA MMR protein expression alone is not sufficient in order to elucidate predisposition to cancer occurrence and development. According to B. Halvarsson et al. [2004], in the evaluation of genomic instability, it is not sufficient to rely only on the results of immunohistochemical testing. In such cases, other approaches to evaluate genomic instability should be employed as well.

Diverse methods for the detection of microsatellite instability are available, and these methods aim at identifying genomic alterations in the chosen genomic loci by comparison of different tissues (tumor and healthy tissue) obtained from the patient being investigated. In 1998, the U.S. National Cancer Institute recommended to investigate 5 genomic loci and to use a panel of 5 markers, which consists of 3 dinucleotide markers (D2S123, D5S346, and D17S250) and 2 mononucleotide markers (BAT25 and BAT26) in case of colorectal cancer. There are panels of other markers introduced by various manufacturers on the market. Moreover, more reliable and sensitive markers for the detection of microsatellite instability are constantly being

developed. In an attempt to increase the sensitivity and specificity of markers recommended by the U.S. National Cancer Institute for the detection of microsatellite instability, Promega Corporation (Madison, USA) suggested to use a panel of 5 mononucleotide markers (BAT-25, BAT-26, NR-21, NR-24, and MONO-27) for microsatellite instability determination and 2 pentanucleotide markers (Penta C and Penta D) for sample identification. According the data of the study by K.M. Murphy et al. [2006], mononucleotide markers are more sensitive and specific than dinucleotide markers for the detection of high-frequency microsatellite instability cancers. The abovementioned panel of 5 Promega Corporation markers (BAT-25, BAT-26, NR-21, NR-24, and MONO-27) was used in our study as well.

Considering the fact that mononucleotide repeats and especially long polyA sequences are unstable genomic units characterized by more frequent mutations in cells with a defective DNA MMR system, the use of these mononucleotide repeats for the detection of microsatellite instability in cancers of different localization (extracolonic) with endometrial cancer being among them is superior to the application of already used markers. Thus, based on this hypothesis, Promega Corporation has created a new panel of BAT-52, BAT-55, BAT-56, BAT-57, and BAT-59 markers, which was used in our study as well [Bacher J. et al., 2012].

Using a new Promega Corporation panel of markers, high-frequency microsatellite instability was detected almost 2-fold more frequently as compared to that detected with the already used panel of BAT-25, BAT-26, NR-21, NR-24, and MONO-27 markers. Analysis of the data on 109 patients with endometrial cancer revealed that using the first marker panel, high-frequency microsatellite instability was identified in 15.60% of the cases (17 of 109), while the use of the second (new) marker panel led to the detection of high-frequency microsatellite instability in 27.52% of the cases (30 of 109).

As previously noted, immunohistochemical analysis of DNA MMR protein expression (MLH1, MSH2, MSH6, and PMS2) was done only in those tumors that were found to show high-frequency microsatellite instability. When the newly created Promega Corporation marker panel was used for the detection of microsatellite instability, the loss of at least one aforementioned protein was documented in 77.33% of cases (22 of 30), and even 8 (26.67%) of the 30 cases showed the expression of all

proteins. Our results suggest that new markers not only allow more precise detection of microsatellite instability in endometrial cancer, but also more precise identification of a defective DNA mismatch repair system; therefore, the use of both methods (molecular and immunohistochemical) is advocated for the evaluation of genomic instability.

In future, studies on microsatellite instability using the newly created marker panel should be continued in order to apply these markers in diagnostics of not only endometrial cancer but also cancers of other localization (extracolonic) as well as early premalignant lesions (for example, colon polyps, endometrial hyperplasia, etc.).

5. CONCLUSIONS

1. The analysis of the data on 109 patients with endometrial cancer revealed that high-frequency microsatellite instability was detected in 15.6% of the patients while using a Promega Corporation (USA) panel of BAT-25, BAT-26, NR-21, NR-24, and MONO-27 markers and in 27.5% of the patients while using a new panel of BAT-52, BAT-55, BAT-56, BAT-57, and BAT-59 markers.
2. High-frequency microsatellite instability was determined only in cases of endometrioid-type adenocarcinomas; a statistically significant difference regarding tumor grade and myometrial invasion was found between the groups with high-frequency microsatellite instability and microsatellite-stable phenotype. High-frequency microsatellite instability was detected more frequently in higher tumor grade and deeper myometrium invasion.
3. The loss of expression of at least one DNA mismatch repair protein was detected in 94.1% of the cases while using the first panel of markers and in 73.3% of the cases while using the newly created panel of markers. The loss of MLH1/PMS2 protein expression was most common.
4. There was no significant association between the survival of patients with endometrial cancer and microsatellite instability.

6. PRACTICAL RECOMMENDATIONS

In Lithuania, based on the experience of other countries, recommendations for the detection of microsatellite instability in endometrial cancer should be prepared, the same as in cases of hereditary nonpolyposis colorectal cancer (Lynch syndrome).

7. LIST OF DISSERTATION-RELATED SCIENTIFIC PUBLICATIONS AND PRESENTATIONS

Articles:

1. Didžiapetrienė J., **Kanopienė D**., Gudlevičienė Ž., Sužiedėlis K., Uleckienė S., Vidugirienė J. Mikrosatelitinis nestabilumas sergant onkologinėmis ligomis. Lietuvos akušerija ir ginekologija. 2010; 13(2): 170-7.
2. Didžiapetrienė J., **Kanopienė D**., Valuckas K. P., Uleckienė S., Atkočius V., Mickė I., Sužiedėlis K. Mikrosatelitų nestabilumas ir heterozigotiškumo praradimas sergant vėžiu. Medicina (Kaunas). 2011; 47(2): 125-35.
3. **Kanopiene D**., Vidugiriene J., Valuckas K. P., Smailyte G., Uleckiene S., Bacher J. Endometrial cancer and microsatellite instability status. Centr. Eur. J. Med. 2014 (in press).
4. **Kanopienė D**., Meškauskas R., Laurinavičienė A., Schveigert D., Chvatovič G., Sužiedėlis K. DNR klaidingai suporuotų nukleotidų taisymo sistemos baltymų (MLH1, MSH2, MSH6 IR PMS2) raiška endometriumo navikuose. Lietuvos akušerija ir ginekologija. 2014; 7(2): 113-21.
5. **Kanopiene D**., Smailyte G., Vidugiriene J., Bacher J. Impact of microsatellite instability on survival of endometrial cancer patients. Medicina (Kaunas). 2014; DOI: 10.1016/j.medici.2014.09.002 (in press).

Abstracts:

1. Bacher J., Vidugiriene J., **Kanopiene D**. New markers for improved detection of MSI in endometrial cancers. J. Mol. Diagn. 2012; 14(6): 708.
2. Bacher J., Grimes I., Albrecht D., Weiss J., Sievers C., Richie K., Agni R., Reichelderfer M., Vidugiriene J., **Kanopiene D**. New markers for improved detection of MSI in early and extra-colonic tumors. J. Mol. Diagn. 2013; 15(6): 916.
3. **Kanopiene D.**, Laurinaviciene A., Meskauskas R., Uleckiene S., Valuckas K. P. Evaluation of DNA mismatch repair system protein expression in endometrial

carcinomas. Book of Abstracts of 6th Baltic Congress of Oncology "Multidisciplinary approach in cancer care". Vilnius (Lithuania), Oct. 3-4, 2014, 68.

Presentations:

1. **Kanopiene D.** Microsatellite instability in cervical and corporis uteri cancer. International conference “Modern radiation oncology: economical aspects and innovations in the treatment”. Palanga (Lithuania), Sept. 14-15, 2012.
2. Bacher J., Vidugiriene J., **Kanopiene D.** New markers for improved detection of MSI in endometrial cancers. Annual Meeting of the Association-for-Molecular-Pathology. Long Beach, CA, Oct. 25-27, 2012.
3. Bacher J., Grimes I., Albrecht D., Weiss J., Sievers C., Richie K., Agni R., Reichelderfer M., Vidugiriene J., **Kanopiene D**. New markers for improved detection of MSI in early and extra-colonic tumors. Annual Meeting of the Association-for-Molecular-Pathology. Phoenix, AZ, Nov. 14-16, 2013.

8. SUMMARY IN LITHUANIAN

Darbo aktualumas

Pasaulyje gimdos kūno vėžys užima septintąją vietą tarp visų piktybinių navikų ir yra labiausiai paplitęs moterų lyties organų navikas. Gimdos kūno vėžys ypač dažnas Europos ir Šiaurės Amerikos šalyse: jose sergamumas apie 10 kartų didesnis, palyginti su sergamumu ekonomiškai silpnose šalyse. Centrinėje ir Rytų Europoje sergamumo ir mirtingumo rodikliai yra atitinkamai 15,6/100 000 ir 3,4/100 000 moterų. Remiantis *EUROCARE* (angl. *European Cancer Registry based study on survival and care of cancer patients*) duomenimis, penkerių metų išgyvenamumas Europoje siekia 75 proc.

Gimdos kūno vėžys yra vienas dažniausių tarp moterų lyties organų piktybinių navikų ir Lietuvoje: sergamumo ir mirtingumo rodikliai atitinkamai − 18,8/100 000 ir 3,4/100 000 moterų.

Šiuolaikinės molekulinės biologijos pasiekimai įgalino sukurti modernius genomo tyrimo metodus, kuriuos panaudojus galima skirtingais aspektais aiškintis kancerogenezės mechanizmus, prognozuoti ligos eigą bei parinkti individualų gydymą.

Plačiai tyrinėjamas mikrosatelitų nestabilumas sergant įvairių lokalizacijų navikais. Mikrosatelitai − tai trumpos iki 150 nukleotidų porų ilgio kartotinės DNR sekos, kurių atskirą pasikartojantį elementą (t. y. paeiliui pasikartojančią seką) dažniausiai sudaro 1−5 nukleotidai.

Svarbiausias veiksnys, kuris lemia mikrosatelitų nestabilumą, yra klaidingai suporuotų nukleotidų DNR pažaidų taisymo (MMR; angl. *Mismatch Repair*) sistemos sutrikimas. Veikli tokia sistema pakeičia klaidingai į grandinę įjungtus nukleotidus, užtikrina tik identiškų grandinių rekombinaciją ir yra ląstelės DNR pažaidų taisymo sistemos dalis. Mikrosatelitų nestabilumo fenomenas ir klaidingai suporuotų nukleotidų DNR pažaidų taisymo sistemos genų mutacijos pirmą kartą charakterizuoti 1993 metais tiriant paveldimo nepolipozinio storosios žarnos vėžio (Lynčo sindromo) atvejus. Mikrosatelitų nestabilumas taip pat nustatomas ir kitų lokalizacijų navikuose, susijusiuose su Lynčo sindromu. Reikia pažymėti, kad mikrosatelitų nestabilumas stebimas ir sporadiniuose navikuose, ypač storosios žarnos ir gimdos kūno.

Jei duomenų apie mikrosatelitų nestabilumą ir klaidingai suporuotų nukleotidų DNR pažaidų taisymo sistemos baltymų raišką, esant storosios žarnos navikams (tiek susijusiems su Lynčo sindromu, tiek sporadiniams), sukaupta nemažai ir įvertinta jų prognozinė bei predikcinė reikšmė, tai tokie duomenys sergant gimdos kūno vėžiu dar tik kaupiami ir nėra susisteminti. Reikia pažymėti, kad nevienareikšmiai rezultatai gauti tyrinėjant sąsają tarp mikrosatelitų nestabilumo ir naviko bei organizmo klinikinių-patologinių charakteristikų.

Mikrosatelitų nestabilumui vertinti naudojami įvairūs žymenys. JAV nacionalinis vėžio institutas rekomenduoja tirti penkias genomo sritis ir naudoti penkių žymenų rinkinį: BAT-25, BAT-26, D2S123, D5S346 ir D17S250. Įvairūs gamintojai pateikia rinkai ir kitokių žymenų rinkinius. Todėl, tiriant mikrosatelitų nestabilumą, gali būti naudojama ir daugiau žymenų, be to, nuolat kuriami patikimesni dėl jų jautrumo ir specifiškumo mononukleotidiniai žymenys. Pavyzdžiui, *Promega Corporation* (JAV) sukūrė rinkinį, kurį sudaro mononukleotidiniai BAT-25, BAT-26, NR-21, NR-24 ir MONO-27 žymenys. Taip pat pradėti tirti naujai sukurti *Promega Corporation* žymenys – BAT-52, BAT-55, BAT-56, BAT-57 ir BAT-59, kurie, kaip tikimasi, bus jautresni mikrosatelitų nestabilumui nustatyti kitų, nei storosios žarnos (ang. *extracolonic*) navikuose. Todėl, siekiant pagrįsti naujų žymenų – BAT-52, BAT-55, BAT-56, BAT-57 ir BAT-59 taikymą, būtina atlikti tų žymenų tyrimus palyginamuoju aspektu.

Darbo tikslas

Ištirti mikrosatelitų nestabilumo dažnį, pobūdį ir klaidingai suporuotų nukleotidų DNR pažaidų taisymo sistemos baltymų raišką sergant gimdos kūno vėžiu ir gautus rezultatus susieti su pacienčių klinikinėmis-patologinėmis charakteristikomis bei jų išgyvenamumu.

Darbo uždaviniai

1. Nustatyti mikrosatelitų nestabilumo dažnį ir pobūdį tarp sergančiųjų gimdos kūno vėžiu, naudojant *Promega Corporation* (JAV) sukurtus du žymenų rinkinius (anksčiau sukurtą BAT-25, BAT-26, NR-21, NR-24, MONO-27 ir naująjį BAT-52, BAT-55, BAT-56, BAT-57, BAT-59).
2. Palyginti pacienčių, sergančių gimdos kūno vėžiu, mikrosatelitų nestabilumo dažnį ir pobūdį priklausomia nuo klinikinių-patologinių charakteristikų.
3. Ištirti klaidingai suporuotų nukleotidų DNR pažaidų taisymo sistemos baltymų (MLH1, PMS2, MSH2, MSH6) raišką gimdos kūno navikuose, kuriuose nustatytas mikrosatelitų didelio dažnio nestabilumas.
4. Įvertinti mikrosatelitų nestabilumo įtaką pacienčių, sergančių gimdos kūno vėžiu, išgyvenamumui.

Ginamieji teiginiai

1. Naujasis žymenų – BAT-52, BAT-55, BAT-56, BAT-57 ir BAT-59 rinkinys tiksliau parodo mikrosatelitų nestabilumą gimdos kūno vėžio atveju, palyginti su jau naudojamu žymenų – BAT-25, BAT-26, NR-21, NR-24 ir MONO-27 rinkiniu.
2. Mikrosatelitų nestabilumo dažnis ir pobūdis yra susijęs su pacienčių, sergančių gimdos kūno vėžiu, klinikinėmis-patologinėmis charakteristikomis.
3. Siekiant objektyviau įvertinti genomo nestabilumą gimdos kūno navikuose, nepakanka atlikti vien imunohistocheminį tyrimą, kuris dažniausiai yra taikomas daugelio kitų lokalizacijų vėžio atvejais. Kartu tikslinga tirti tokius molekulinius žymenis kaip mikrosatelitų nestabilumas.

Darbo mokslinis naujumas ir praktinė reikšmė

1. Mikrosatelitų nestabilumui tirti buvo panaudoti nauji *Promega Corporation* (JAV) sukurti žymenys − BAT-52, BAT-55, BAT-56, BAT-57 ir BAT-59, kuriuos taikant dvigubai dažniau nustatytas mikrosatelitų didelio dažnio nestabilumas gimdos kūno vėžio atveju, palyginti su nustatymo dažniu naudojant BAT-25, BAT-26, NR-21, NR-24 ir MONO-27 žymenų rinkinį.

2. Genomo nestabilumui įvertinti nepakanka vien tik imunohistocheminio tyrimo, tikslinga atlikti ir mikrosatelitų nestabilumo molekulinį tyrimą.
3. Imunohistocheminių ir molekulinių genomo nestabilumo tyrimų rezultatai padeda atrinkti pacientes tolesniems molekuliniams genetiniams tyrimams įtariant paveldimą gimdos kūno vėžį (Lynčo sindromą).

Tiriamieji ir tyrimo metodai

Į stebėsenos perspektyvinį tyrimą įtrauktos 109 pacientės, kurioms Vilniaus universiteto Onkologijos institute (dabar Nacionalinis vėžio institutas) 2010−2011 metų laikotarpiu nustatytas gimdos kūno vėžys. Naudoti tyrimo metodai:

- anketinė apklausa (pagal Amsterdamo II kriterijų klausimyną);
- antropometriniai matavimai (ūgis ir svoris);
- DNR gryninimas iš navikinio audinio ir kraujo (sveikas audinys);
- mikrosatelitų nestabilumo nustatymas PGR metodu (naudojant *Promega Corporation* du žymenų rinkinius);
- klaidingai suporuotų nukleotidų DNR pažaidų taisymo sistemos baltymų (MLH1, MSH2, MSH6, PMS2) raiškos nustatymas imunohistocheminiu metodu;
- pacienčių išgyvenamumas vertintas naudojant Kaplano-Mejerio bei Kokso regresijos metodus, išgyvenamumo kreivėms palyginti – *log rank* kriterijus;
- statistinė duomenų analizė atlikta naudojant „SPSS 20.0“ ir „Stata 11.0“ programinės įrangos paketus.

Išvados

1. Ištyrus 109 sergančiąsias gimdos kūno vėžiu, mikrosatelitų didelio dažnio nestabilumas nustatytas 15,6 proc. pacienčių, naudojant *Promega Corporation* (JAV) žymenų rinkinį − BAT-25, BAT-26, NR-21, NR-24, MONO-27 ir 27,5 proc. − naudojant naująjį BAT-52, BAT-55, BAT-56, BAT-57, BAT-59 žymenų rinkinį.
2. Mikrosatelitų didelio dažnio nestabilumas nustatytas tik endometrioidinės adenokarcinomos atvejais; statistiškai reikšmingi skirtumai konstatuoti tarp mikrosatelitų didelio dažnio nestabilumo ir stabilaus fenotipo priklausomai nuo

naviko diferenciacijos laipsnio bei invazijos į miometriumą gylio. Mikrosatelitų didelio dažnio nestabilumas dažniau nustatytas blogesnės diferenciacijos navikuose ir esant gilesnei naviko invazijai į miometriumą.

3. Klaidingai suporuotų nukleotidų DNR pažaidų taisymo sistemos bent vieno baltymo raiškos sutrikimas nustatytas 94,1 proc. atvejų, kai mikrosatelitų nestabilumo analizei naudotas pirmasis žymenų rinkinys, ir 73,3 proc. atvejų − kai naudotas antrasis (naujas) žymenų rinkinys. Dažniausiai konstatuotas MLH1/PMS2 baltymų raiškos sutrikimas.
4. Sergančiųjų gimdos kūno vėžiu išgyvenamumas nebuvo statistiškai reikšmingai susijęs su mikrosatelitų nestabilumu.

PADĖKA

Nuoširdžiai dėkoju Nacionalinio vėžio instituto (anksčiau Vilniaus universiteto Onkologijos institutas) administracijai ir direktoriui prof. dr. Narimantui Evaldui Samalavičiui už suteiktą galimybę ir paramą vykdant šį mokslinį darbą.

Esu ypač dėkinga mokslinio darbo konsultantams − prof. habil. dr. Konstantinui Povilui Valuckui ir dr. Jolantai Vidugirienei. Prof. K. P. Valuckui dėkoju už diskusijas bei konsultacijas klinikiniais darbo aspektais, nuostatas siekti kuo geresnių darbo rezultatų ir nuolatinį skatinimą tobulėti. Dr. J. Vidugirienei ir kolegai iš JAV dr. Jeff Bacher labai dėkinga už galimybę realizuoti mokslinę idėją bei pagalbą organizuojant tyrimus *Promega* korporacijoje.

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Labai dėkinga Valstybinio patologijos centro darbuotojams − prof. dr. Arvydui Laurinavičiui, doc. dr. Aidai Laurinavičienei ir gydytojui patologui konsultantui Raimundui Meškauskui už histologinių ir imunohistocheminių tyrimų vertinimą bei konsultacijas.

Taip pat esu dėkinga savo šeimai už visapusišką palaikymą ir supratimą.

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