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# Analysis of hMLH1 and hMSH2 expression in cisplatin-treated ovarian cancer patients

Analiza ekspresji hMLH1 i hMSH2 u pacjentek z rakiem jajnika leczonych za pomocą pochodnych platyny

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## Abstract

**Background:** Loss of DNA mismatch repair may result in resistance to platinum- based anticancer drugs. The hMLH1 and hMSH2 proteins play a critical role in the maintenance of genome integrity and are involved in resistance to platinum-based therapy in colorectal cancer, which is deficient in hMLH1 protein and endometrial cancer, as well as in hMSH2 protein. However, the predictive value of MLH1 and MSH2 expression in ovarian cancer cisplatin-resistance is still to be determined.

**Objective:** The aim of this study was to investigate the expression of hMLH1 and hMSH2 proteins in ovarian carcinoma specimens and to evaluate their prognostic significance by means of overall survival (OS) and progression-free survival rates (PSF).

**Material:** Ovarian cancer tissues were obtained from 61 patients: 45 platinum-sensitive and 16 platinum-resistant. hMLH1 and hMSH2 proteins expression was evaluated by immunohistochemistry, with the use of mouse monoclonal antibodies clone 14 for hMLH1 and clone FE11 for hMSH2. The log-rank test and Kaplan-Meier statistics were used to analyze the relationship between proteins expression and progression free survival, as well as the overall survival.

**Result:** No significant correlation was found between hMLH1 and hMSH2 expression and overall survival and progression free survival in the group of patients sensitive and resistant to cisplatin. No significant difference was found in proteins expression intensity between the two compared groups of patients.

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*Age of patients, type of cancer histology, FIGO staging, grading, clinical response and CA 125 did not reveal correlation with the expression of the analyzed proteins.*

**Conclusion:** *The immunohistochemical expression of hMLH1 and hMSH2 proteins in ovarian cancer has no predictive value in resistance to cisplatin.*

Key words: **ovarian cancer** / **drug resistance** / **cisplatin** / **DNA mismatch repair** /
/ **hMLH1** / **hMSH2** / **immunohistochemistry** /

## Streszczenie

**Wstęp:** *Białka hMLH1 i hMSH2 pełnią kluczową rolę w naprawie genomu. Utrata tej zdolności może powodować zwiększoną oporność na leki przeciwnowotworowe oparte na pochodnych platyny. Jakkolwiek wartość prognostyczna oznaczania ekspresji białek hMLH1 i hMSH2 w raku jajnika w celu przewidywania oporności na cisplatynę pozostaje wciąż nieokreślona.*

**Cel pracy:** *Celem tego opracowania było zbadanie ekspresji białek hMLH1 i hMSH2 w komórkach raka jajnika pochodzących od 61 pacjentek, w tym: 45 wrażliwych i 16 opornych na cisplatynę oraz ocena wpływu tych białek na ogólne przeżycie i czas wolny od wznowy.*

**Materiał i metody:** *Ekspresję białek hMLH1 i hMSH2 oceniono metodą immunohistochemiczną przy użyciu mysich przeciwciał monoklinalnych, klon 14 dla hMLH1 i klon FE11 dla hMSH2. Do analizy zależności pomiędzy ekspresją białek a czasem wolnym od wznowy i przeżyciem ogólnym użyto testu log rank i statystyki Kaplana-Meiera. Wartość p<0,05 uznano za istotną statystycznie.*

**Wyniki:** *Nie znaleziono istotnej statystycznie zależności pomiędzy ekspresją hMLH1 i hMSH2 a przeżyciem ogólnym i czasem wolnym od wznowy, zarówno w grupie pacjentek opornych jak i wrażliwych na leczenie cisplatyną. Nie znaleziono istotnej różnicy w ekspresji badanych białek pomiędzy dwiema porównywanymi grupami. Nie znaleziono również korelacji pomiędzy ekspresją badanych białek a cechami kliniczno-patologicznymi, takimi jak: wiek, rozpoznanie histopatologiczne, stopień zaawansowania wg FIGO, stopień zróżnicowania G, odpowiedź na leczenie cisplatyną.*

**Wnioski:** *Immunohistochemiczna ocena ekspresji białek hMLH1 i hMSH2 w raku jajnika nie jest czynnikiem prognostycznym wystąpienia oporności na cisplatynę.*

Słowa kluczowe: **rak jajnika** / **oporność lekowa** / **cisplatyna** / **naprawa DNA** / **hMLH1** /
/ **hMSH2** / **immunohistochemia** /

## Introduction

A primary laparotomy with maximal attempt at optimal cytoreduction is a treatment standard in ovarian cancer. Patients who have undergone surgery should receive adjuvant chemotherapy. The combination of a paclitaxel with cisplatin or carboplatin is recommended as a first choice proceeding. Ovarian cancer is a disease which responds well to chemotherapy based on platinum. Unfortunately, about 55 to 75% of patients who responded positively to the first line platinum based treatment, develop recurrence within 2 years. Owing to various tumor sensitivity to platinum containing drugs, we stratify patients into three groups: platinum-refractory (do not respond to first-line therapy), platinum-resistant (initially respond to the first-line therapy but relapse occurs within 6 months of the treatment completion), platinum-sensitive (relapse occurs 6 months or more after the completion of the first-line treatment) [2]. Current studies aim at understanding the aspects of cisplatin resistance and the indication of molecular factors responsible for these processes.

Mismatch repair (MMR) system is a correction mechanism for nucleotide mismatches or insertion/deletion of the mistakes that arise during DNA replication. It is composed of a few proteins: heterodimer hMSH2/hMSH6 recognizes small insertion/deletion loops, whereas heterodimer hMSH2/hMSH3 binds to larger insertion/deletion loops.

hMLH1/PMS2 heterodimer binds to hMSH2/hMSH6 or hMSH2/hMSH3 complex and supports initiated repair [3]. hMSH2 is required for all mismatch corrections and displays special affinity for adducts of clinically effective platinum-based drugs [4]. The deficiency in this system contributes to carcinogenesis processes by accumulation of uncorrected/unrepaired mutations in the oncogenes and tumor suppressor genes. It results in microsatellite genomic instability (MSI) and causes a loss of apoptotic mechanism. Furthermore, germ line mutations of hMLH1 and hMSH2 cause higher susceptibility to cancer. In particular, 70% of all hereditary nonpolyposis colon cancer (HNPCC) cases are associated with the loss of MMR system [5]. The inactivation of the MMR genes is connected with the loss or low immunohistochemical expression of encoded proteins [6].

The hMLH1 and hMSH2 proteins are involved in resistance to platinum based therapy. The colorectal cancer, which is deficient in hMLH1 protein, as well as endometrial cancer, deficient in hMSH2 protein, are both four times more resistant to cisplatin than cells without defects in MMR system [7]. However, the clinical significance of these proteins expression in ovarian cancer remains unknown. Our study was designed to investigate the predictive value of hMLH1 and hMSH2 expressions in platinum resistant ovarian cancer cases.

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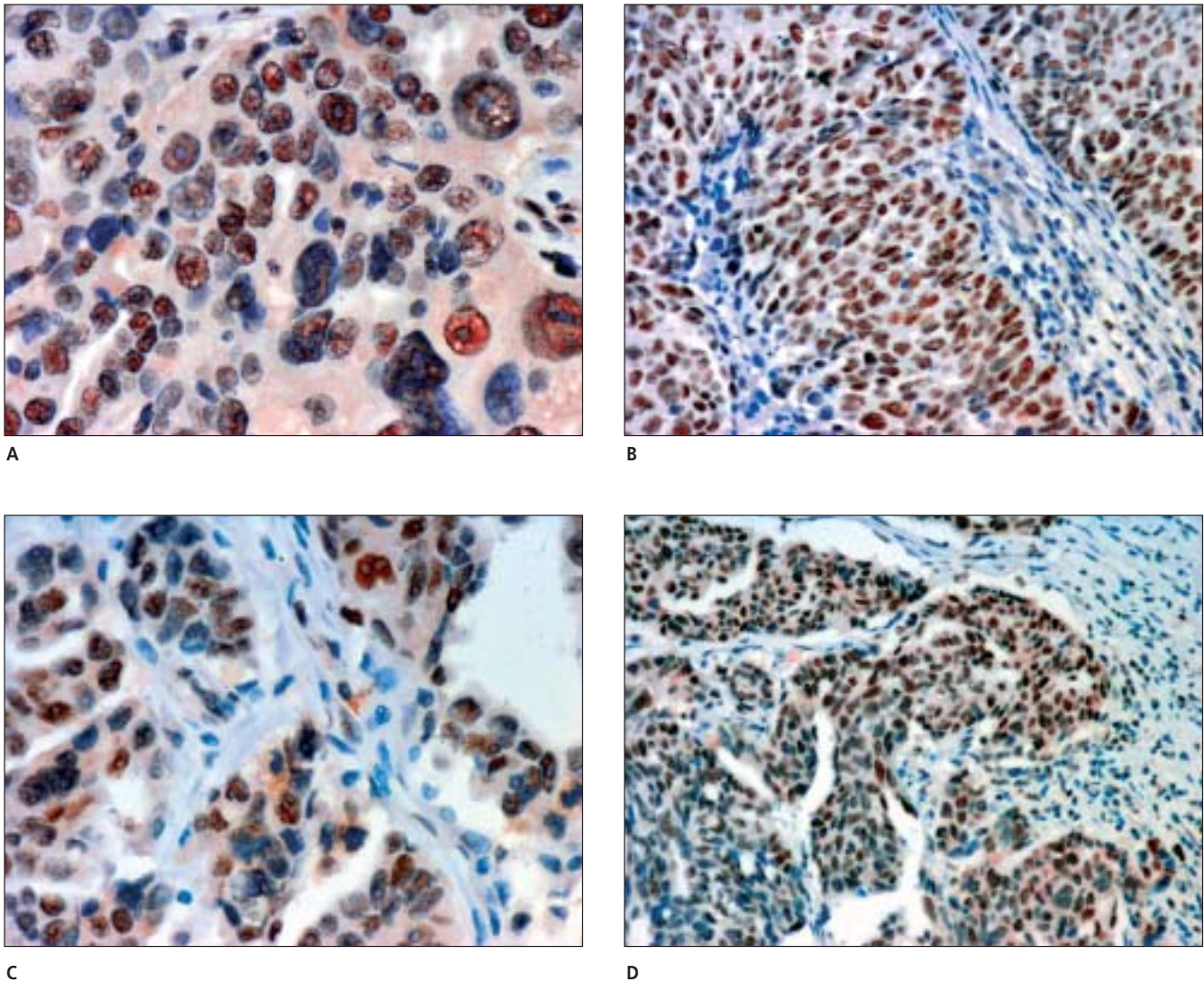


**Figure 1.** Immunohistochemical expression of analysed proteins in ovarian carcinoma specimens.
**A** – hMLH1 expression (x400), **B** – hMLH1 expression (x200),
**C** – hMSH2 expression (x400), **D** – hMSH2 expression (x200).

## Materials and methods

### Patients

Immunohistochemical examination was performed retrospectively on tissue samples taken during the first look laparotomy for routine diagnostic purposes. Sixty-one patients operated in 2000-2004 due to ovarian cancer in Department of Obstetrics and Gynecology (Poznań University of Medical Sciences, Poland) were enrolled in this study. The study was approved by the Institutional Review Board and the patients gave their informed consent before enrollment into the study. The cases were selected taking into consideration the availability of tissue and were not stratified for any known prognostic factors. Each patient underwent a primary cytoreduction surgery, but only 33 (54%) women achieved optimal cytoreduction.

In twenty-eight cases (46%) second-look operation was considered after three cycles of systemic chemotherapy. Finally, fifty (82%) patients had optimal cytoreduction and 11 (18%) suboptimal. Each patient had received cisplatin- or carboplatin-based chemotherapy as the first line treatment after surgery. The following chemotherapy regimens were administered: paclitaxel 135mg/m$^2$ over 24 hours/ cisplatin 75 mg/m$^2$ over 6 hours – in 53 cases (87%); paclitaxel 175mg/m$^2$ over 3 hours/ carboplatin AUC 6 over 1 hour – in 8 cases (13%). Patients' profiles are summarized in table I.

In order to investigate the overall survival (OS) and the progression free survival (PFS) rate, a follow-up examination was performed every six weeks for the first year and thereafter every 3 months. The mean follow-up period was 31.5 months (median 27.49 months; range 9-74).

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The progression free survival time was defined as the time between the end of first line chemotherapy and the diagnosis of the recurrence. Sixteen patients were considered resistant to cisplatin as they relapsed within 6 months, forty-five women were sensitive to administered chemotherapy (recurrence after 6 months). Because the group of refractory patients amounted only 4 women, we have joined the resistant one. The recurrence was diagnosed by means of manual examination, and/or CA-125 serum level increase and/or characteristic changes in transvaginal ultrasound.

### Tissue

Tissue samples were fixed in 10% buffered formalin and embedded in paraffin. In each case, hematoxylin and eosin stained preparations were subjected to histopathological evaluation by two pathologists. The stage of the tumors was assessed according to the FIGO criteria [8]. Tumors were graded according to the Silverberg grading system [9].

### Immunohistochemistry

Formalin-fixed, paraffin embedded tissue was freshly cut (4um). The sections were mounted on Superfrost slides (Menzel Gläser, Germany), de-waxed with xylene, and gradually hydrated. Activity of endogenous peroxidase was blocked by 5min. exposure to 3% $H_2O_2$. All the studied sections were boiled for 15min. at 250W in the Antigen Retrieval Solution (DakoCytomation, Denmark). Then, immunohistochemical reactions were performed with the use of the following anti-bodies: monoclonal anti-hMSH2 mAb (mouse), clone FE11 (Calbiochem, La Jolla, CA), dilution 1:250 and monoclonal anti-hMLH1 mAB (mouse), clone 14 (Calbiochem, LA Jolla, CA), dilution 1:100 in Antibody Diluent, Background Reducing (DakoCytomation, Denmark). Tested sections were incubated with antibodies for 1h at room temperature. Subsequent incubations involved biotinylated antibodies (15min., room temperature) and streptavidin-biotinylated peroxidase complex (15min., room temperature) (LSAB+, HRP, DakoCytomation, Denmark). DAB (DakoCytomation, Denmark) was used as a chromogen (7min., at room temperature). All the sections were counterstained with Meyer's hematoxylin (DakoCytomation, Denmark). In every case, control reactions, in which specific antibody was substituted by the Primary Mouse Negative Control (DakoCytomation, Denmark), were included.

Control reactions were also performed on paraffin sections from six healthy human appendices (from the archive of the Department of Histology and Embryology, University School of Medicine, Wrocław, Poland).

### Evaluation of reaction intensity.

Intensity of immunohistochemical reactions was estimated independently by two pathologists. When in doubt, a re-evaluation was performed using a double-headed microscope and staining was discussed until a consensus was achieved. In order to evaluate the hMLH1 and hMSH2 expression, a semi-quantitative scale of ImmunoReactive Score (IRS), which took into account intensity of the colour reaction as well as proportion of positive cells (Table II), was applied. The final score represented the product of points given for individual characters and ranged between 0 and 12 [10].

### Statistical analysis

For statistical analyses, GraphPad Prism 5 (v 5.01; 2007) for Windows from GraphPad Software and Microsoft Excel were used. To estimate the significance of differences in survival time and progression-free survival time, Kaplan-Meier statistics and log-rank tests were used. The Mann-Whitney test was used for comparing the intensity of immunoreaction between two studied groups: sensitive and resistant to cisplatin. The correlation between the age of patients and the expression of hMLH1 and hMSH2 proteins was assessed by means of Spearman rang correlation. ANOVA rang Kruskall-Wallis test was used to analyze the relationship between the expression of mentioned proteins and clinicopathological factors. The outcome was recognized as statistically significant when $p < 0.05$. All p-values are given for two-sided tests.

## Results

The expression of mismatch repair proteins was evaluated by an immunoreactive score (Table II). Patients were divided into two groups, with no/low reaction intensity (IRS 0-4) and with high reaction intensity (IRS 6-12). Of the 61 ovarian carcinomas, 24 (39%) showed high immunostaining for hMLH1 protein (Fig. 1A,B) and low in 37 (61%) cases. For hMSH2 protein, 15 (25%) cases of ovarian cancer tissue showed high expression (FIG.1C,D) and 46 (75%) ones were of low expression. However, 13% of hMLH1 and 20% of hMSH2 showed complete absence of staining.

**Table I**. Patients' profiles.

| Characteristics | No | % |
|---|---|---|
| **All patients** | 61 | 100 |
| **Age (mean 50,7), range 31-73 ≤50** | 35 | 57 |
| **>50** | 26 | 43 |
| **Grade** | | |
| 1 | 11 | 18 |
| 2 | 22 | 36 |
| 3 | 21 | 34 |
| x | 7 | 12 |
| **FIGO** | | |
| I | 9 | 15 |
| II | 4 | 6 |
| III | 48 | 79 |
| **Histology** | | |
| Serous | 31 | 51 |
| Solidum | 16 | 26 |
| Other | 14 | 23 |
| **Response to cisplatin** | | |
| Sensitive | 45 | 74 |
| Resistant* | 16 | 26 |

*recurrence < 6 months

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**Table II**. Evaluation criteria of expression using the immunoreactive score.

| Percentage of positive cells | Points | Intensity of reaction | Points |
|---|---|---|---|
| No positive cells | 0 | No reaction | 0 |
| <10% of positive cells | 1 | Weak reaction | 1 |
| 10-50% of positive cells | 2 | Moderate reaction | 2 |
| 51-80% of positive cells | 3 | Intense reaction | 3 |
| >80% of positive cells | 4 | | |

**Table III**. The distribution of hMLH1 and hMSH2 high and low expression in the group of patients resistant and sensitive to cisplatin.

| | hMLH1 high expression 24 (39%) | hMLH1 low expression 37 (61%) |
|---|---|---|
| Sensitive 45 (74%) | 18 (30%) | 27 (44%) |
| Resistant 16 (26%) | 6 (10%) | 10 (16%) |

| | hMSH2 high expression 15 (25%) | hMSH2 low expression 46 (75%) |
|---|---|---|
| Sensitive 45 (74%) | 11 (18%) | 34 (56%) |
| Resistant 16 (26%) | 4 (7%) | 12 (20%) |

The distribution of hMLH1 and hMSH2 high and low expression in cisplatin resistant and sensitive subgroups is presented in table III.

Comparing the reaction intensity between these two groups, we noted no significant difference for hMLH1 (p=0.3882) and for hMSH2 (p=0.7031; Mann-Whitney test). Examining the hMLH1 expression in the group of resistant patients and the sensitive one, no significant differences in overall survival (Fig. 2A,B) and in progression-free survival (Fig. 2C,D) between patients with hMLH1 high and low expression were found.

Regarding the hMSH2 expression in the group of resistant patients and the sensitive one, there were also no significant differences in overall survival (Fig. 3A,B) and in progression-free survival (Fig. 3C,D) between patients with hMSH2 high and low expression. There was also no difference in the hMLH1 expression between patients who were alive or deaseased (Fisher's exact test, p=0.1723). The same situation was noted in case of hMSH2 expression (p=0.4103).

Comparing survival curves in the whole studied group, we found no significant differences between high and low hMLH1 expression (Fig 4A,C). Regarding hMSH2 expression, we also did not find any significance in overall survival and progression-free survival (Fig 4B,D).

The expression of hMLH1 and hMSH2 had no correspondence with age, p=0.3903 for hMLH1 and p=0.5903 for hMSH2 (Spearman rang correlation).

For clinicopathological factors such as: histology, FIGO staging, grading, clinical response and cytoreduction, no correlation with expression of analysed proteins was found (Table IV).

The relationship between the level of CA125 and overall immunoreactive score presented no statistical significance, p=0.9095 for hMLH1 and p=0.4302 for hMSH2 (Spearman rang correlation).

## Discussion

Data regarding the role of the mismatch repair gene changes in ovarian cancer remains unclear. Some authors suggest a relationship between the expression of hMLH1 and hMSH2 proteins and anticancer drugs resistance [11, 12].

The studies on the human ovarian cancer cell line A2780 showed that *hMLH1* gene promoter methylation may be a common mechanism for cisplatin resistance. This cancer cell line, resistant to cisplatin, revealed loss of hMLH1 expression in 90% of cases. These cells also demonstrated an increase of *hMLH1* promoter methylation.

Moreover, the treatment of the resistant cell line A2780/MCP3 with 5-azacytydine, inhibitor of methyltransferase, results in hMLH1 re-expression, as well as corresponding increase in cisplatin sensitivity [11].

This *in vitro* study raises the hope that MMR-related drug resistance could be overcome *in vivo*.
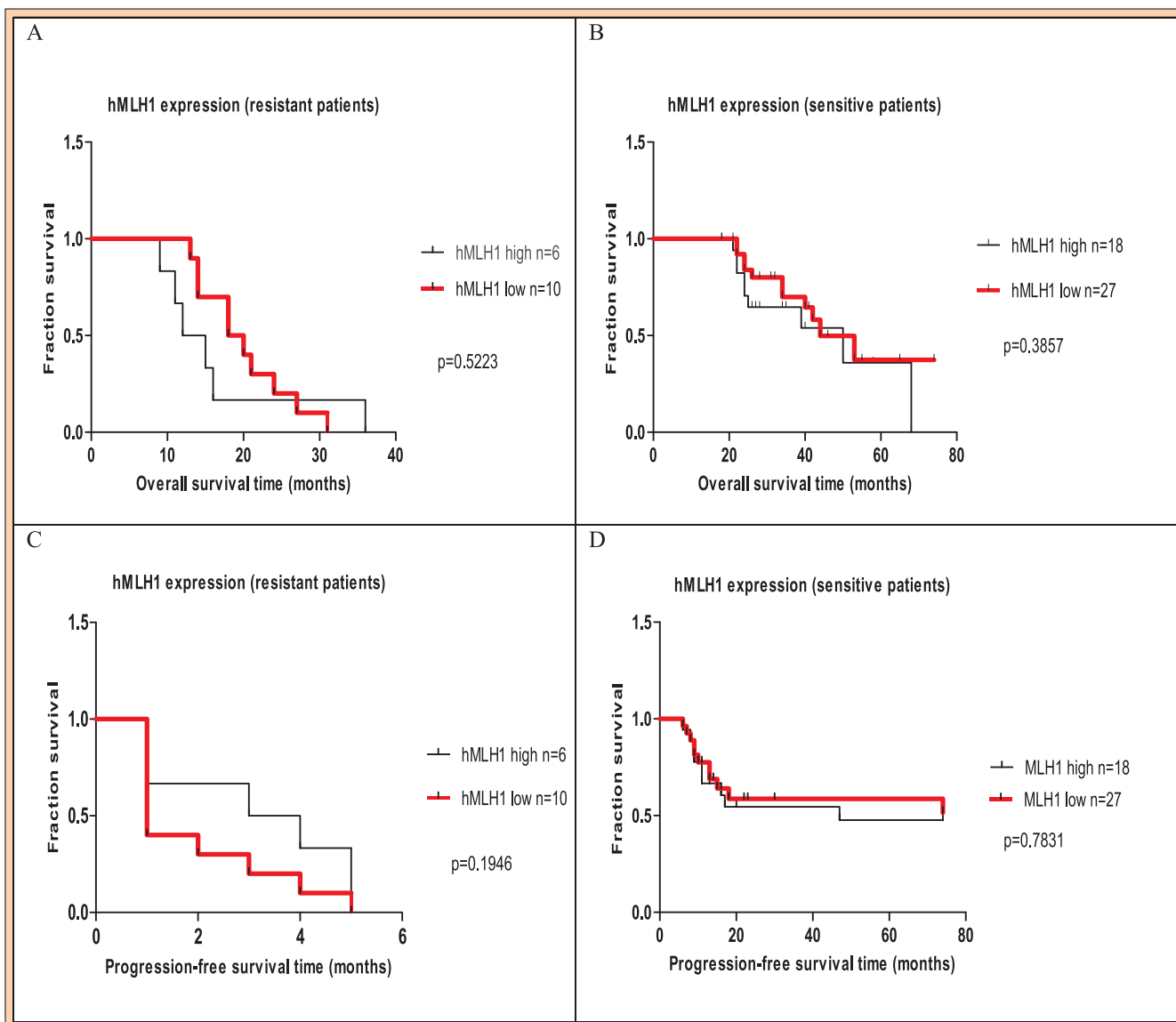
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**Figure 2.** Kaplan-Meier curves for survival and expression of hMLH1 in the group of patients resistant to cisplatin (n=16) and in the sensitive to cisplatin one (n=45).

**Table IV.** The relationship between hMLH1 and hMSH2 expression and the clinicopathological factors (ANOVA rang Kruskal-Wallis test).

| | hMLH1 expression | hMSH2 expression |
|---|---|---|
| Histology (solid *vs.* serous *vs* mucinous *vs* endometrioid *vs* other) | P = 0.5730 | P = 0.7585 |
| Grade (G1 *vs* G2 *vs* G3) | P = 0.3007 | P = 0.2333 |
| FIGO staging ( I vs. II vs. III) | P = 0.6934 | P = 0.9223 |
| Clinical response (complete *vs* partial *vs* stable *vs* progression of disease) | P = 0.7537 | P = 0.7860 |
| Cytoreduction (primary optimal *vs* secondary optimal *vs* secondary suboptimal) | P = 0.4269 | P = 0.9245 |
| Cytoreduction (primary optimal *vs* primary suboptimal)* | P = 0.2185 | P = 0.9010 |
| Cytoreduction (primary optimal + secondary optimal *vs* secondary suboptimal)* | P = 0.7904 | P = 0.7045 |

* Mann-Whitney test

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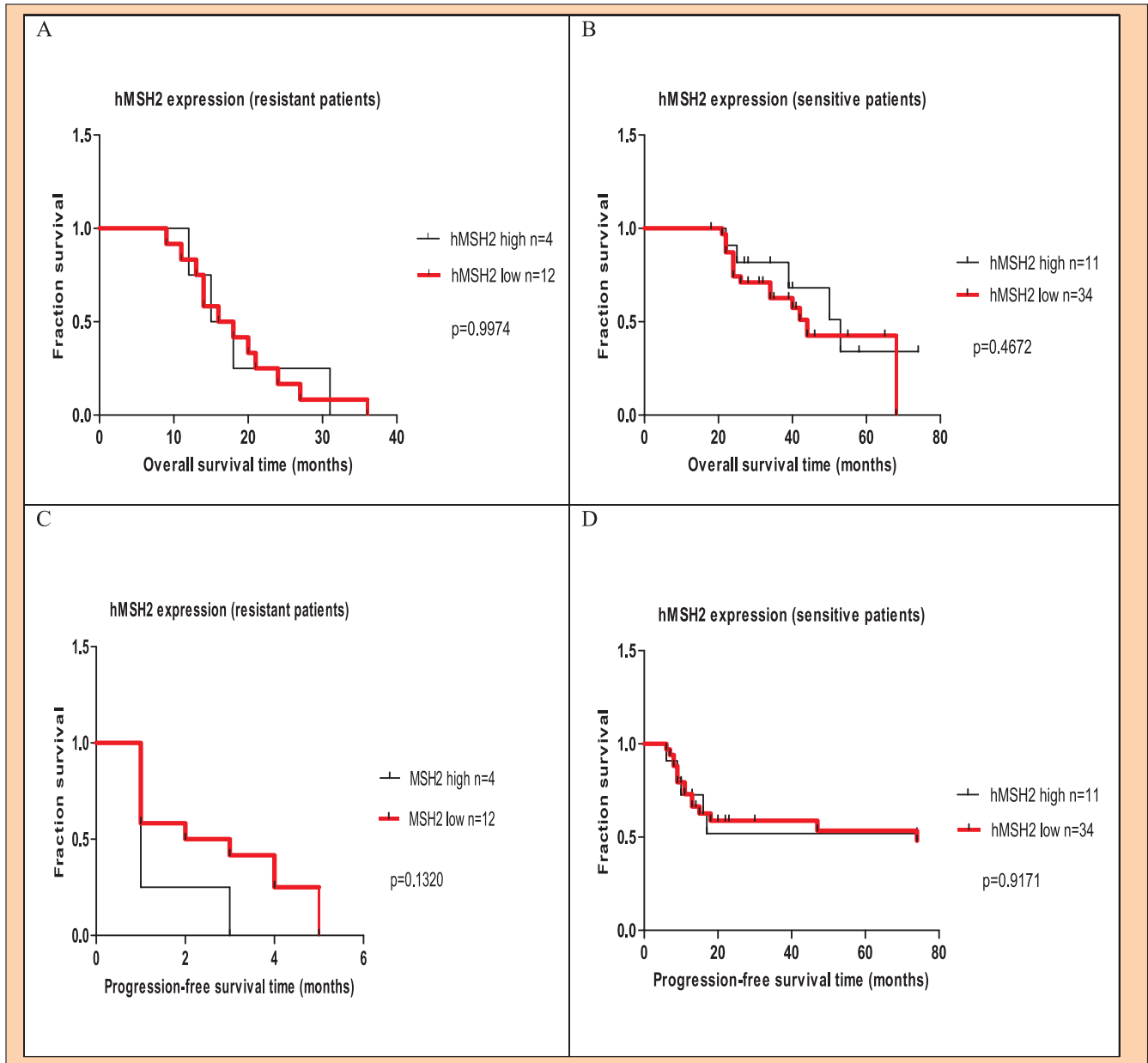


**Figure 3.** Kaplan-Meier curves for survival and expression of hMLH1 in the group of patients resistant to cisplatin (n=16) and in the sensitive to cisplatin one (n=45).

Another survey carried out in isogenic pairs of cell lines, proficient and deficient in hMLH1 and hMSH2 proteins, proved the role of mismatch repair system in cisplatin resistance. It showed that a human colon cancer cell line HCT116+ch2 deficient in hMLH1 function was two times more resistant to cisplatin when compared to the proficient counterpart. Similarly, the human endometrial cancer cell line HEC59 without hMSH2 expression was two times more resistant to cisplatin when compared to a subline complemented with hMSH2 expression [12].

Since the loss of hMLH1 and hMSH2 expression is associated with cisplatin resistance *in vitro*, we might expect that low expression of these proteins would correlate with poor clinical outcome *in vivo*.

In our study we analyzed the hMLH1 and hMSH2 protein expression in ovarian cancer patients, both resistant and sensitive to cisplatin therapy. Thanks to the comparison of MMR proteins staining in these two groups of patients, we wanted to check if the loss of hMLH1 and hMSH2 expression may be a prognostic marker of cisplatin resistance.

Unfortunately, we did not find any significant relationship between the expression of mentioned proteins and overall survival rate and progression-free survival time, either in the group of cisplatin-resistant patients or the sensitive one.

Another Polish study, conducted on 223 ovarian carcinoma tissues, also did not confirm the influence of hMLH1 and hMSH2 expression on cisplatin resistance. Microsatellite genomic instability was not observed either [13].
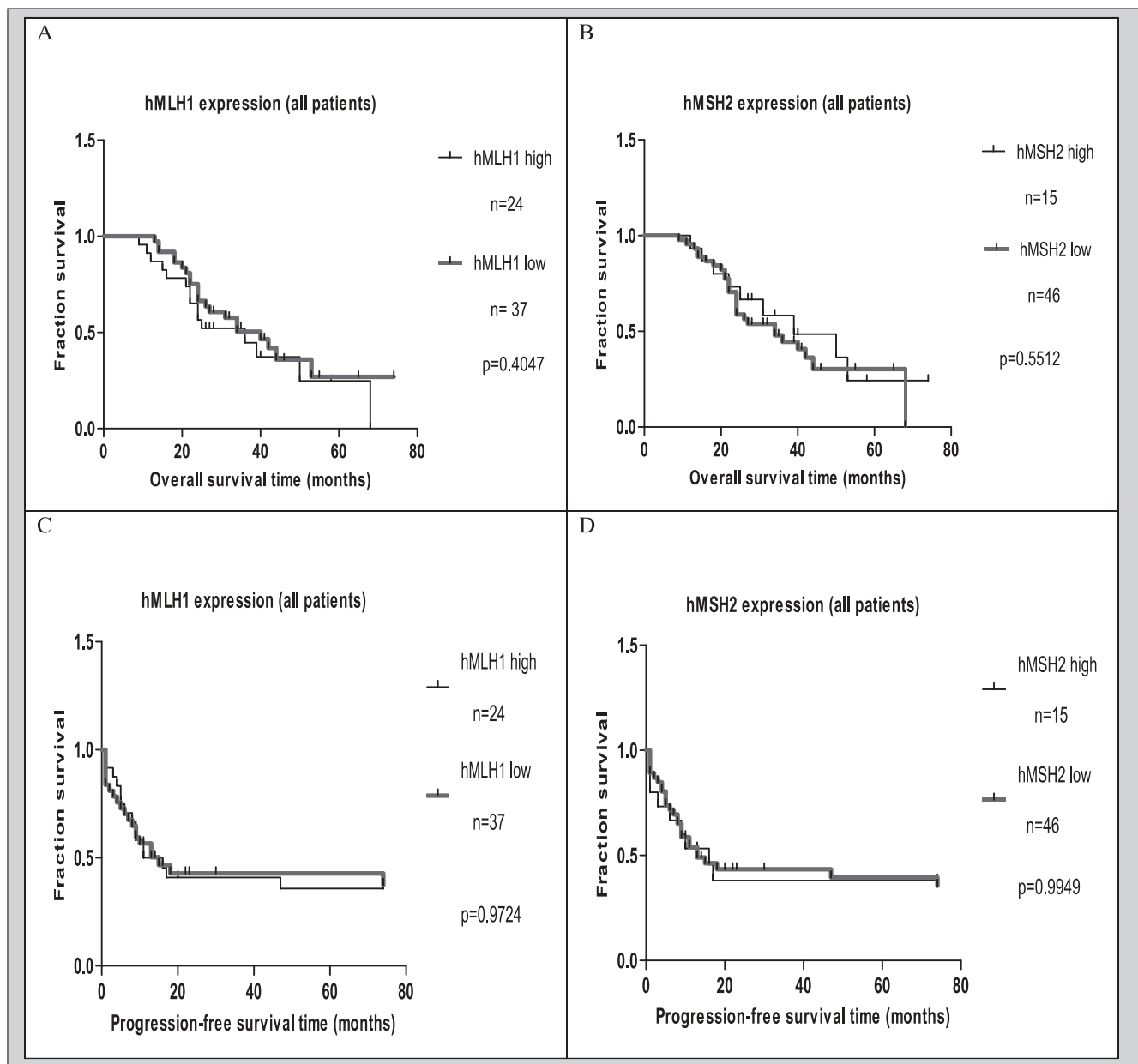
**Figure 4.** Kaplan-Meier curves for survival and expression of hMLH1 and hMSH2 in the group of all patients.

It seems that the loss of mismatch repair system may lead to drug resistance before cisplatin administration, as well as during chemotherapy. It may depend on an accidental mutagenesis caused by cisplatin. Loss of MMR expression was noted after consecutive series of therapies based on cisplatin [14]. Western blot analysis of ovarian carcinoma specimens revealed 10% lack of hMLH1 expression before initial chemotherapy, while after cisplatin administration tumor samples were in 36% hMLH1 deficient [15].

Opposite findings were reported by other researchers [16]. They investigated hMLH1 and hMSH2 expression in paired ovarian tumor sections from 54 ovarian carcinoma patients before and after platinum-based therapy. Although the expression of these proteins decreased significantly after treatment, the analysis has shown no correlation between the expression of either protein and overall survival, suggesting that hMSH2 and hMLH1 are not predictive factors of clinical response. In our previous study, we also did not find any influence of hMLH1 expression on patient's survival.

However, patients with negative hMSH2 staining have displayed significant difference in overall survival and progression-free survival, but only in the material from the first look laparotomy, before the application of platinum-based drugs. The tissue obtained after chemotherapy was not predictive [17]. In the light of this observation, platinum-based drugs appear to be a damaging tool for its own sensitivity.

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In the present study, we have examined specimens from the first look laparotomy exclusively, that is before chemotherapy was administered. Our goal was to determine if the intrinsic resistance to cisplatin can be predicted at the beginning of the treatment. Identifying prognostic markers would contribute to the improvement of ovarian cancer therapy.

Unexpectedly, the results have shown no differences between platinum-resistant and platinum-sensitive patients. It is difficult to draw conclusions based on the analysis of the resistant group of patients due to their heterogeneity.

The following criteria were used to define resistance: relapse within 6 months of therapy completion (acquired resistance), no response to the first-line therapy (intrinsic resistance) and persistent disease. The fact that only 4/16 resistant patients in our study were refractory (did not respond to cisplatin therapy) is worth mentioning. These patients probably developed resistance in other mechanism. To obtain more reliable data, we should compare a more numerous group of platinum-refractory and platinum-sensitive patients.

Another aspect of this study is the precision of immuno-histochemical staining.

The study by Marcus et al. proved immunohistochemistry to be a useful and reliable screening tool for mismatch repair-deficient neoplasms [6]. However, others question the role of this method in predicting the value of hMLH1 and hMSH2, because it does not measure direct MMR proteins activity in ovarian cancer samples [16].

## Conclusions

The immunohistochemical expression of hMLH1 and hMSH2 proteins in ovarian cancer has no predictive value in resistance to cisplatin.

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