Immunohistochemical analysis of hMLH1 and hMSH2 proteins in serous ovarian tumours

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Inactivation of DNA mismatch repair (MMR) genes is involved in carcinogenesis of sporadic and inherited human cancers. Mutations in MMR genes are associated with the loss of immunoexpression of hMLH1 and hMSH2 and may play a role in the mechanism of carcinogenesis of ovarian tumours. The aim of the study was to evaluate the immunoexpression of hMLH1 and hMSH2 in serous ovarian tumours. Sixty cases of benign, borderline, G1, G2 and G3 serous ovarian tumours were retrieved from the archival material. The immunoexpression of hMLH1 and hMSH2 was detected using an immunohistochemical method and a percentage of immunopositive cells in a 1000 tumour cells for each slide was measured. Loss of immunoexpression of hMLH1 and hMSH2 was infrequent in ovarian tumours, including both serous cystadenomas and carcinomas. Lack of immunoexpression of hMLH1 was observed only in 4 of 8 and hMSH2 in 5 of 8 of borderline cases. Moreover, the percentage of hMLH1 positive cells was significantly lower in borderline tumours as compared to G2 cancers. The percentage of hMSH2+ cells was in the borderline group significantly lower in comparison with all other groups investigated. Our results suggest that hMLH1 and hMSH2 proteins may be involved in ovarian carcinogenesis.

Key words: hMLH1, hMSH2, serous ovarian tumours.

Introduction

Ovarian cancer takes a first place as a cause of death from gynaecological malignancies in Poland and it is the fifth leading cause of cancer deaths among women [1]. The poor ratio of survival characteristic of ovarian cancers results from the high percentage of cases diagnosed at an advanced stage. Ovarian cancer in its early stages (I/II) is difficult to diagnose until it spreads and advances to later stages (III/IV). The early detections of ovarian cancer does not exist because of the lack of well-defined prognostic markers for prescreening. However, there is significant heterogenity within the epithelial ovarian cancer group. Most primary ovarian carcinomas are of four morphological types: serous, endometrioid, mucinous and clear cell. Histologically defined subtypes and low- and high-grade malignancies all have variable clinical manifestation and underlying molecular signatures. Relatively little is known about the molecular pathogenesis of ovarian cancer. The best recognized causes of ovarian cancer susceptibility are germline mutation in the BRCA1 and BRCA2 tumours suppressor genes [2, 3]. The role of the DNA mismatch repair (MMR) genes in this malignancy is not clearly understood.

The human DNA mismatch repair family is a highly conserved group of proteins that function in genome stabilization and mutation avoidance. The DNA mismatch repair system consists of many genes, including: hMSH2, hMLH1, hMSH3, hPMS1, hPMS2 and hMSH6. These genes are very important in distinguishing and repairing misparing and slippage errors in DNA synthesis. MMR inactivation leads to the occurrence of unrepaired deletions in mono- and dinucleotide repeats resulting in variable lenghts of these repeats. This is called microsatellite instability

(MSI). MSI can be caused by genetic or epigenetic inactivation of several genes involved in MMR and is used as a marker for MMR deficiency [4, 5]. In most studies MSI is evaluated using different kinds of microsatellite markers. An MSI criterion was standardized with the use of two mononucleotide repeats (BAT26 and BAT25), and three dinucleotide repeats (D5S346, D2S123 and D17S250) known as the National Cancer Institute panel [6].

Defective DNA mismatch repair gene function is thought to promote tumourigenesis by accelerating mutations in oncogenes and tumour suppressor genes. The lack of functional hMLH1 and hMSH2 is associated with the presence of microsatellite instability found in some human tumours. Germline mutations in MMR genes are associated with colorectal, endometrial, or gastric carcinomas that develop in individuals with hereditary non-polyposis colon carcinoma (HNPCC). Some patients with HNPCC also have increased risk of other cancers, including ovarian cancer [7]. There is evidence that MSI may play a role in the mechanism of carcinogenesis.

The use of PCR-based methods to detect MSI is relatively expensive and time-consuming. Immunohistochemical analysis offers an alternative method for estimation of MSI status as a result of the inactivation of the hMLH1 and hMSH2 genes [8]. Tumours showing high frequency of microsatellite instability are immunohistochemically characterized by lack of hMLH1 and hMSH2 protein expression. Many researchers have demonstrated that immunohistochemistry can be used to identify MSI [9-11]. The sensitivity and the specificity of this test were 97% and 100%, respectively [9, 11]. The use of immunohistochemistry offers a relatively rapid method for prescreening tumours for defects in the expression of MMR genes therefore, the aim of the present study was to evaluate the immunoexpression of hMLH1 and hMSH2 proteins in serous ovarian tumours.

Materials and methods

Patients

The analysis comprised 60 women diagnosed and treated for epithelial ovarian tumours at the Gynaecology and Obstetrics Institute of Medical University of Lodz between 1997 and 2002. Women were aged from 19 to 80 years (mean $\pm$SD = 55.6 $\pm$12.8). Twenty one women aged from 38 to 80 years (mean $\pm$SD = 57.2 $\pm$11.6) were treated for G3 serous cancer. Ten women aged from 40 to 73 years (mean $\pm$SD = 57.9 $\pm$10.1) had G2 serous cancer. Six women aged from 44 to 73 years (mean $\pm$SD = 53.8 $\pm$10.6) were treated for G1 serous cancer. Eight women aged from 36 to 68 years (mean $\pm$SD = 55.4 $\pm$10.5) had borderline serous ovarian tumours. Fifteen women aged from 19 to 75 years (mean $\pm$SD = 52.5 $\pm$17.7) suffered from benign tumours (cystadenomas) of the ovary.

Light microscopy

Paraffin-embedded tissue sections taken from postoperative material were diagnosed using standard haematoxylin and eosin staining.

Immunohistochemistry

Paraffin-embedded tissue sections were mounted onto SuperFrost slides, deparaffinized, then treated in a microwave oven in a solution of TRS (Target Retrieval Solution, pH 6.0, Dako) for 30 minutes (2×6 minutes 360 W, 2×5 180 W, 2×4 minutes 90 W) and transferred to distilled water. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide in distilled water for 30 minutes, and then sections were rinsed with Tris-buffered saline (TBS, Dako, Denmark) and incubated all night with mouse monoclonal anti-human antibodies: hMLH1 (PharMingen International, San Diego, USA; dilution 1 : 200) and hMSH2 (abcam Inc, Cambridge, UK; dilution 1 : 250). Immunoreactive proteins were visualized using EnVision-horseradish peroxidase kit (Dako, Carpinteria, CA, USA) according to the instructions of the manufacturer. Visualisation was performed by incubating the sections in a solution of 3,3'-diaminobenzidine (DakoCytomation, Denmark). After washing, the sections were counter-stained with haematoxylin and coverslipped. For each antibody and for each sample a negative control was processed. Negative controls were carried out by incubation in the absence of the primary antibody and always yielded negative results.

Morphometry

The hMLH1 and hMSH2 immunoexpression was assessed by means of an image analysis system consisting of a personal computer equipped with a Pentagram graphical tablet, Indeo Fast card (frame grabber, true-color, real-time), produced by Indeo (Taiwan), and Panasonic colour TV camera (Japan) coupled with a Carl Zeiss microscope (Germany). This system was controlled by MultiScan 8.08 software, produced by Computer Scanning Systems, Poland, working under macroinstructions written specially for this analysis. The percentage of immunopositive cells in a 1000 tumour cells for each slide was measured.

Statistical analysis

Differences between groups were tested using one-way ANOVA with post-hoc RIR Tukey test, preceded

Table I. A morphometric comparison of immunoexpression of hMLH1and hMSH2 in ovarian cancer, borderline tumours and cystadenomas

Tumours	G3 (n = 21)	G2 (n = 10)	G1 (n = 6)	Borderline (n = 8)	Benign (n = 15)	P value
hMLH1+ cells (%)	63.52 ±24.44	72.74 ±26.09[a]	71.14 ±10.57	35.16 ±39.56	58.19 ±24.95	[a]Between borderline and G2 cancers < 0.04
hMSH2+ cells (%)	61.62 ±12.65[c]	57.64 ±7.32[b]	68.14 ±13.08[a]	26.20 ±37.32	60.02 ±27.8[d]	[a]Between borderline and G1 cancers < 0.01 [b]Between borderline and G2 cancers < 0.037 [c]Between borderline and G3 cancers < 0.014 [d]Between borderline and benign tumours < 0.02

Data are expressed as mean ± standard deviation

by evaluation of normality. The Kruskall-Wallis test was used where appropriate. Results were considered statistically significant if $p < 0.05$.

Results

hMLH1 and hMSH2 protein expression was exclusively nuclear. Loss of expression of hMLH1 was detected in 3 of 37 (8.1%) cases of ovarian cancer (one case in G2 and two cases in G3), and in 4 of 8 (50%) cases of borderline tumour. hMSH2 immunoreactivity was observed in all examined cases of ovarian cancer. Lack of hMSH2 immunoexpression was observed in 5 of 8 (62.5%) borderline tumours. Positive staining of hMLH1 and hMSH2 was detected in all cases of ovarian cystadenomas.

The morphometric data of the immunoexpression of hMLH1and hMSH2 in ovarian cancer, borderline tumours and cystadenomas are shown in Table I.

The immunoexpression of hMLH1 was lower in borderline patients as compared to all other groups however, only the difference between borderline and G2 cases (Fig. 1, Fig. 2) was statistically significant ($p = 0.047$). The immunoexpression of hMSH2 protein (Fig. 3, Fig. 4) was significantly lower in borderline group than in G1, G2, G3 cancers ($p = 0.01$, $p = 0.037$, $p = 0.014$ respectively) and benign ovarian tumours ($p = 0.02$). No statistically significant differences were found among particular groups of cancers and ovarian cystadenomas for hMSH2 protein.

Discussion

Ovarian cancer is one of the most common forms of hereditary cancer in adults females and the most common gynaecological malignancy in perimenopausal and postmenopausal women. It has been recently suggested that ovarian serous cancer follows a dualistic pathway with low-grade carcinomas arising from borderline tumours and high-grade carcinomas originating *de novo* [12]. Numerous data suggest that microsatellite instability and loss of heterozygosity are implicated in ovarian carcinogenesis [13, 14].

Ovarian cancer is a heterogeneous disease characterized by various histological types which have

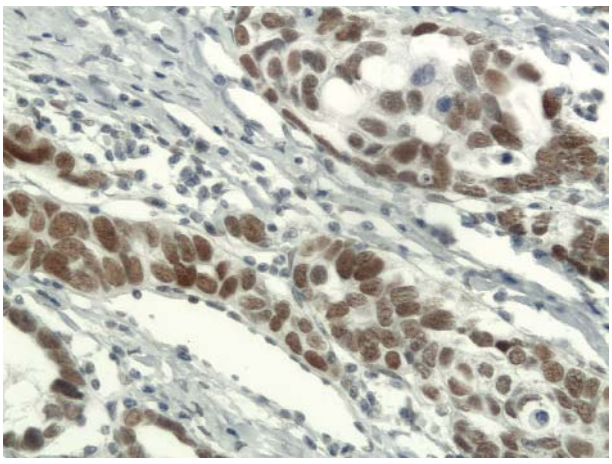

Fig. 1. Intensive immunoexpression of hMLH1 protein in G2 ovarian cancer. Immunohistochemistry. Magnification 400×

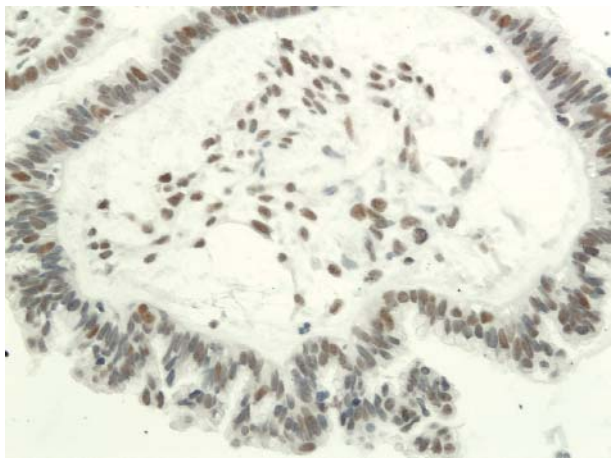

Fig. 2. Weak immunoexpression of hMLH1 in borderline tumour. Immunohistochemistry. Magnification 200×

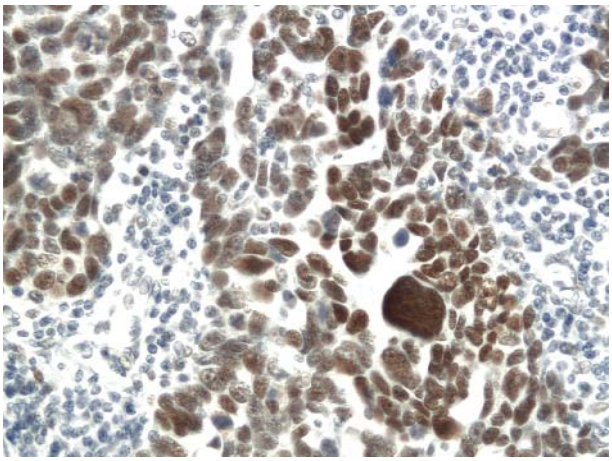

Fig. 3. Strong immunoexpression of hMSH2 in G3 ovarian cancer. Immunohistochemistry. Magnification 400×

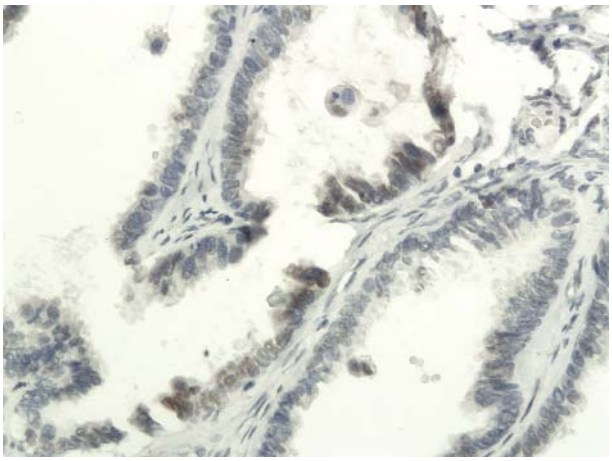

Fig. 4. Focal, weak immunoexpression of hMSH2 in borderline tumour. Immunohistochemistry. Magnification 200×

different MSI frequencies. Only a few reports have analyzed the immunoexpression of hMLH1 and hMSH2 proteins. Tumours are usually identified using molecular methods. The reported frequency of MSI in ovarian tumours varies, ranging from 0 to 50% [15-18]. MSI was found in 30-50% of cases of endometrioid ovarian cancer, in 38% of mucinous adenocarcinomas, in 14% of clear cell carcinomas and 0-13% of serous carcinoma [8, 16, 18-20]. Helleman *et al.* [21] observed that the frequency of MSI is higher in mucinous and endometrioid adenocarcinoma compared to clear cell and serous adenocarcinoma (the overall frequencies of MSI were 22%, 16%, 9% and 8%, respectively). The same authors hypothesize that histology of cancer might be correlated with different MSI frequency.

Serous carcinoma is the most common epithelial ovarian cancer, and MSI studies have focused mainly on this subtype. In our study, positive staining of hMSH2 was detected in all examined cases of serous cancer. We observed the loss of immunoexpression of hMLH1 in 8.1% of examined cases of serous cancer. Helleman *et al.* [21] using PCR methods, detected MSI in 8% of cases of serous adenocarcinoma. Our results are in concordance with previous findings reporting a relatively low frequency of MSI in ovarian serous carcinomas [16, 18, 22, 23].

Our study did not reveal statistically significant differences in hMLH1 and hMSH2 immunoexpression between G1, G2 and G3 cancer or between serous carcinomas and serous cystadenomas. According to Giarnieri *et al.* [24] in carcinoma of the uterine cervix no significant correlation of immunoexpression of hMLH1 and hMSH2 was observed among histopathological grades, but the number of cases for G1, G2 and G3 cancer was 8, 12 and 3, respectively. To our knowledge the data concerning the immunoexpression of hMLH1 and hMSH2 protein in histopathological grade of ovarian serous cancer are rather scanty. For this reason, verification of our results in a much larger number of cases is necessary.

The role of MSI in pathogenesis of borderline tumours is unclear. In our study the loss of immunoexpression of hMLH1 was observed in 50% and MSH2 in 62.5% of cases of borderline tumour. We also observed statistically significant differences of immunoexpression of hMSH2 between G1, G2, G3 serous carcinomas and borderline tumours, as well as hMLH1 immunoexpression between G1 serous carcinomas and borderline tumours. The mechanism underlying development of ovarian borderline tumours is still not well understood. Wolf *et al.* [25] in microdissected samples from serous borderline tumours, using comparative genomic hybridization and/or fluorescence *in situ* hybridization, showed that 3 of 13 ovarian borderline tumours had detectable numerical abnormalities. Yoon *et al.* [23], using the PCR method and 5 conventional MSI markers, described MSI in 4 of 46 (8.6%) ovarian tumours, including 2 of 21 (9.5%) borderline ovarian tumours and 2 of 25 (8%) malignant ovarian tumours. On the other hand, Wolf *et al.* [26] suggest that some borderline tumours may develop through mechanisms other than chromosomal imbalances or microsatellite instability.

In conclusion, the low immunoexpression of hMSH2 and hMLH1 in borderline tumours revealed in our study suggests that the examined proteins may be involved in the development of early stages of ovarian carcinogenesis.

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