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Prediction of Survival in Serous Ovarian Carcinoma on the Basis of Ki-67 Index Analysis

Analiza rokownicza w rakach surowiczych jajnika na podstawie oceny indeksu proliferacyjnego Ki-67

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Abstract

Background. A comparative analysis of proliferation activity of cells was carried out and Ki-67 antigen expression level was determined in serous ovarian carcinoma as well as in benign serous ovarian adenomas. At the same time, the possibility of a practical application of Ki-67 index estimation as a prognostic marker in serous ovarian carcinomas was investigated on the basis of analysis of 24-month survival of the patients. Ovarian carcinoma accounts for 85% to 90% of ovarian malignancies and in 42% of cases it is serous cystadenocarcinoma. The search for factors increasing the efficiency of treatment in patients with ovarian carcinoma, which is manifested by prolonged remission and survival, is of utmost importance as it enables proper planning and effective implementation of the treatment process if necessary.

Objectives. The aim of the study was the evaluation of clinical parameters compared with histological and laboratory findings in serous carcinomas. The estimation of proliferation activity of tissues in the form of Ki-67 index is obtained by calculating the percentage of cells which react with this antibody in relation to the total amount of cells in the specimen with ovarian cancers and in control group.

Material and Methods. The material used consisted of 41 cases of serous ovarian cancer. The control group for Ki-67 levels were 15 patients with benign serous ovarian adenomas. For clinical examinations the authors chose only patients who underwent primary surgical operation. To show Ki-67 reacting antigen in paraffin samples of ovarian cancer the authors used DAKO Serum (Rabbit Anti Human Ki-67 Antigen N 1574 LSAB). Tissue proliferation activity called proliferation index – IP Ki-67 was measured as a proportion of numbers of cells reacting with antigen to the total number of cells in the sample.

Results. An average level of Ki-67 index in the study group was 29.01% in relation to 5.84% in the control group and it was statistically significant ($p < 0.05$). The results obtained in Ki-67 positive groups ranged 9.36–70.35% in carcinoma cases and 1.72% to 14.69% in benign tumours. In the study group Ki-67 (+) activity was confirmed in 29 (70.74%) women with serous ovarian carcinoma. Also in the control group Ki-67 expression was found only in 11 (73.33%) specimens. Among patients with a positive Ki-67, women with lower proliferation index revealed longer survival.

Conclusions. 1. The differences in Ki-67 proliferation activity in patients with benign serous adenoma of the ovary comparing to patients with serous ovarian carcinoma are statistically significant. 2. Among patients with a positive Ki-67 index, life prognosis in a period of 24 month is better for women with lower cells proliferation index. 3. The first year of treatment is characterized by a higher mortality in patients with lower values of IP Ki-67. 4. There was no correlation between mean values of proliferation index and patients survival period (*Adv Clin Exp Med* 2005, 14, 2, 231–236).

Key words: Ki-67, positive proliferation index, serous ovarian cancer.

Streszczenie

Wprowadzenie. W pracy została przeprowadzona analiza porównawcza aktywności proliferacyjnej komórek w rakach surowiczych i łagodnych gruczolakach jajnika na podstawie oceny indeksu proliferacyjnego Ki-67. Poszukiwano również możliwości praktycznego wykorzystania oceny indeksu proliferacyjnego Ki-67 jako markera pro-

gnostycznego w raku surowiczym jajnika, na podstawie przeżycia pacjentek w badanym okresie 24 miesięcy. Raki jajnika stanowią 85–90% spośród wszystkich nowotworów złośliwych jajnika, przy czym w tej grupie aż 42% to raki surowicze. Poszukiwane są czynniki, które pomogą podnieść skuteczność prowadzonej terapii i wydłużyć okresy remisji oraz przeżycia pacjentek dzięki szybkiemu rozpoznaniu i prawidłowo dobranemu leczeniu.

Cel pracy. Ocena wskaźników klinicznych, laboratoryjnych i histologicznych w rakach surowiczych jajnika w odniesieniu do indeksu proliferacyjnego Ki-67 oraz porównanie wyników z łagodnymi gruczolakami surowiczymi, które stanowiły grupą kontrolną.

Materiał i metody. Materiał początkowy stanowiło 41 preparatów raka surowiczego jajnika i 15 preparatów łagodnych gruczolaków surowiczych. Natomiast po zawężeniu przypadków tylko do grupy ze stwierdzoną aktywnością proliferacyjną Ki-67 odpowiednio 29 i 11 przypadków. Wszystkie wybrane preparaty pochodziły od pacjentek poddanych pierwotnemu leczeniu operacyjnemu. W celu wykazania reakcji antygenu Ki-67 w badanych tkankach zastosowano surowicę firmy DAKO (Rabbit Anti Human Ki-67 Anigen N 1574 LSAB). Tkankowa aktywność proliferacyjna była przedstawiona jako procent komórek reagujących z surowicą w stosunku do całkowitej liczby komórek.

Wyniki. Średni poziom indeksu proliferacyjnego Ki-67 w badanej grupie wynosił 29,01% w porównaniu do 5,84% w grupie kontrolnej ($p < 0,05$). Zakres uzyskanych wyników zawierał się między 9,36–70,35% w rakach surowiczych jajnika i 1,72–14,69% w łagodnych gruczolakach surowiczych. W grupie badanej aktywność proliferacyjną uzyskano w 29 przypadkach (70,74%), przy 11 przypadkach (73,33%) w grupie kontrolnej. Wśród badanych pacjentek, chore z niższymi wartościami indeksu proliferacyjnego Ki-67 cechowały się dłuższym przeżyciem.

Wnioski. 1. Stwierdzono różnice istotne statystycznie między poziomem proliferacji komórkowej w rakach surowiczych i łagodnych gruczolakach surowiczych jajnika. 2. W grupie pacjentek z dodatnim indeksem proliferacyjnym, w okresie 24-miesięcznym, dłuższe przeżycie korelowało z niższym poziomem proliferacji komórkowej. 3. Pierwszy rok leczenia charakteryzowała większa śmiertelność pacjentek z niższymi wartościami IP Ki-67. 4. Nie stwierdzono zależności między średnią wartością indeksu proliferacyjnego a długością przeżycia (*Adv Clin Exp Med* 2005, 14, 2, 231–236).

Słowa kluczowe: Ki-67, dodatni indeks proliferacyjny, rak surowiczy jajnika.

Ovarian carcinoma constitutes for 23% of all gynaecologic cancers and accounts for 47% of deaths due to female reproductive system malignancy. According to recent statistics, the incidence of ovarian carcinoma is increasing. Poland is among the countries in which the incidence of ovarian carcinoma is high. Standardized morbidity index in 1996 was 12.2 and it showed a significant increase in comparison with that from 1963. Ovarian carcinoma accounts for 85% to 90% of ovarian malignancies and in 42% of cases it is serous cystadenocarcinoma. The search for factors increasing the efficacy of treatment of ovarian carcinoma patients, which is manifested by prolonged remission and survival, is of utmost importance as it enables proper planning and effective implementation of the treatment process [1, 2]. Ki-67 monoclonal antibody was first described in 1983 by Gerdes et al. in experimental studies on mice who had been previously immunized with Hodgkin's disease lymphocytes and monocytes [3]. Ki-67 is used to detect specific antigen, which is a non-histonic protein of the cell nucleus, which in the Western blotting method stains as a double band with the molecular weight of 345 and 395 kilodaltons.

The function of the detected antigen has not been determined so far, however, a very characteristic feature is that it is present only in cells that undergo division. The antigen expression was noted only in the late G1 phase and in the phases

of DNA replication, G2 and mitosis of the cellular cycle. On the other hand, Ki-67 antibody was found not to react with finally specialized cells which have already completed their mitotic activity. They are, among others, the lymphocytes, monocytes, hepatocytes, nerve cells and sperm cells [4]. Due to the lack of antigen-antibody (Ag-Ab) reaction in the resting phase G0, Ki-67 was regarded as a specific marker of proliferation [3]. Thus, it was used to assess cell proliferation, first of all, in quickly proliferating cancer tissues. The estimation of proliferation activity of tissues in the form of Ki-67 index is obtained by calculating the percentage of cells which react with this antibody in relation to the total amount of cells in the specimen. In order to obtain reliable results, it is necessary to examine at least several microscopic fields with highest possible expression of Ki-67. The assessment of cell proliferation index and interchangeably used synonyms (Ki-67 index, proliferation index, cell proliferation fraction) can be used in predicting the clinical course and prognosis in many kinds of cancer [5–8].

Ki-67 index was used to compare the mitotic activity in serous carcinoma and benign serous adenoma of the ovary. Moreover, a relationship between the level of Ki-67 antigen expression in serous ovarian carcinoma and the survival rate of patients on 24-month follow-up period was investigated.

Material and Methods

The clinical material (41 cases) involved 34 women treated from January 1995 to June 2000 at the 1st Department and Clinic of Gynaecology and Obstetrics, Wrocław Medical University and 7 patients hospitalized from January to September 2000 at the Department of Oncology and Clinic of Oncological Gynaecology, Wrocław Medical University for serous carcinoma of the ovary. The age of the patients in the study group ranged from 31 to 74 years. The mean age of the patients was 50 years. The clinical staging and grading of the disease was as follows: FIGO I – 7 cases, II – 2 cases, III – 28, IV – 4 cases and G1 – 8, G2 – 13, G3 – 20 cases. Only patients who underwent primary operation were qualified for the study. Cases with second-look operation, recurrence of the disease, metastases and previous chemotherapy were excluded. Moreover, patients who had received oncological treatment for other neoplastic diseases in the past were also excluded. The control group for Ki-67 index assessment comprised 15 patients with benign serous adenoma of the ovary. The investigations were carried out at the Immunocytological Laboratory of the Department of Pathological Anatomy, Wrocław Medical University.

All the histochemical investigations were performed on 4 nm paraffin sections after placing them on gelatinous glass (2 g $\text{KCr}(\text{SO}_4)_2 \times 12\text{H}_2\text{O}$ + 2.5 g of gelatin). Next they were cleansed of the admixture of gelatin by placing in xylene and passing through a number of alcohol solutions with concentrations decreasing to pure water. Antigens of fixed in formalin tissues were determined by boiling the section specimens in a 700 W microwave oven in 0.01 M citrate buffer at pH 6.0 for 30 minutes. After cooling, the specimens were washed in TBS (0.05 M hydroxymethylaminomethane – TRIS pH 7.6 + 0.15 M NaCl + 1% bovine albumin). The activity of endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide solution for 5 minutes. Rabbit antibody against human Ki-67 antigen manufactured by DAKO (Rabbit Anti Human K67 Antigen N 15 74 LSAB) was used to demonstrate Ki-67 expression in the malignant tissue. Every sample was incubated for 20 minutes at room temperature and next washed in TBS (2 × 5 minutes). The second antibody and marker complex was obtained from Universal LSAB 2 KITS HRP, Rabbit/Mouse (liquid DAB) K 0675 manufactured by DAKO. In order to demonstrate the reaction, DAB (3,3'-diaminebenzidine tetrahydrochloride) was used again as a substrate. Next the Ki-67 preparations were stained with

Mayer's hematoxylin, dehydrated and covered with a cover glass and preserved with Canada balsam.

Evaluation of the Specimens

Obtained in such a way stained microscopic specimens of Ki-67 were characterized by distinct brown staining of the investigated cells. The evaluation was carried out by means of a computer program for picture analysis MultiScan 98. Areas with the highest density of active structures were searched for in the whole specimen using Olympus BX 50 light microscope at 200-fold magnification of the image. For the purpose of analysis of Ki-67 index in selected visual fields with the highest expression of active cells, the magnification of the microscope was increased from ×200 to ×400. After the image was transferred to the computer screen, the amount of Ki-67 positive cells was calculated on the basis of the overall amount of cells. At least 500 cells were evaluated in every examination and next, their active percentage was calculated according to the following mathematical formula:

$$\text{Ki-67 index} = \frac{\text{the amount of active cells}}{\text{total amount of cells}} \times 100\%.$$

Clinical data elicited from the case history as well as the obtained findings were ranked using data base from a computer program Microsoft Access 97. The statistical evaluation was carried out using *t*-Student test (for the comparison of mean values in population divided into two parts), unifactoral variance analysis (for unequal population divided into three or more subgroups) and, where necessary from the statistical viewpoint, non-parametric tests where used (χ^2).

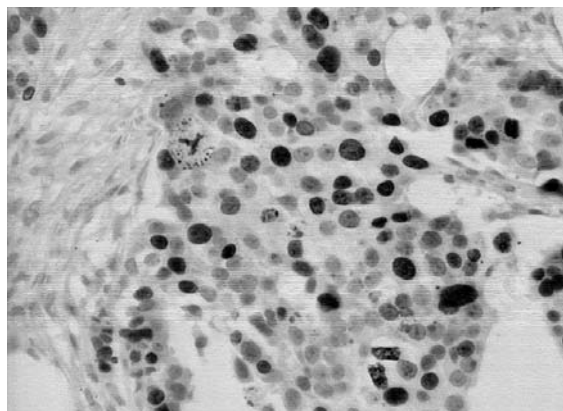


Fig. 1. Microscopic picture of a specimen treated with the above-described method

Ryc. 1. Zdjęcie mikroskopowe tkanki nowotworowej uzyskanej po zastosowaniu opisanej metody

Results

Ki-67 index was defined for 41 patients suffering from serous ovarian carcinoma and for 15 patients with benign serous adenoma of the ovary in order to determine the mitotic activity in the studied tissues. Next the patients were divided into groups depending on the presence or absence of Ki-67 antigen expression in the investigated tissue samples. In the study group Ki-67 (+) activity was confirmed in 29 (70.74%) women with serous ovarian carcinoma while 12 (29.26%) women from the group did not reveal Ki-67 activity. The borderline for the division was set at Ki-67 index < 1%. Also in the control group Ki-67 expression was not defined for all the investigated cases. Out of 15 cases of benign serous adenoma of the ovary, antigen Ki-67 expression was confirmed in 11 specimens (73.33%) and it was absent in 4 cases (26.66%). The results obtained in Ki-67 positive groups ranged from 9.36% to 70.35% in carcinoma cases (percentage of reactive cells in the specimen) and 1.72% to 14.69% in benign tumours. In the first stage of the study, the average levels of Ki-67 index in the group of patients with Ki-67 expression were compared to the control group. An average level of Ki-67 index in the study group was 29.01% comparing to 5.84% in the control group and it was statistically significant ($p < 0.05$). Standard deviations were 13.55 and 5.44 respectively.

In the first phase of the clinical analysis the authors concentrated on finding a relationship between the degree of clinical stage by FIGO and Ki-67, but unfortunately the authors could not reveal any statistical correlation – $p > 0.05$. The results were as follows (FIGO I – 21.88%, II – 30.37%, III – 30.85% and IV – 28.41%). It also did not reveal the dependence between cell malignancy – grading and IP Ki-67 (G1 = 30.71%, G2 = 26.39%, G3 = 30.18%).

Next, a multistage analysis of the patients' survival rate was carried out in relation to the level of Ki-67 antigen expression. In the first stage, the differences in survival were evaluated in relation to the presence or absence of Ki-67 expression. The results are presented in Table 1.

The differences were found to be statistically insignificant, $p > 0.05$.

Subsequent Tables (2, 3 and 4) present prognostic evaluation in the patients with Ki-67 antigen expression (29 cases with a positive Ki-67 index > 1%).

Table 2 presents the differences in Ki-67 expression in women who died and in those who survived 24 months after the surgery.

Obtained mean indexes revealed statistically significant differences ($p = 0.02$).

Table 1. The influence of Ki-67 index on survival in serous ovarian carcinoma in relation to presence or absence of Ki-67 antigen expression

Tabela 1. Wpływ indeksu Ki-67 na przeżycie w rakach surowiczych jajnika w odniesieniu do obecności lub braku ekspresji antygenu Ki-67

Survival (Przeżycie)	Ki-67 index < 1% (Indeks Ki-67 < 1%)	Ki-67 index > 1% (Indeks Ki-67 > 1%)	Patients (Chorzy)
< 24 months (< 24 miesiące)	4	14	18
> 24 months (> 24 miesiące)	8	15	23
Patients (Chorzy)	12	29	41

Table 2. Analysis of survival rate in relation to the level of Ki-67 expression in 24-month period

Tabela 2. Analiza przeżycia pacjentek w okresie 24 miesięcy w zależności od poziomu indeksu Ki-67

24-month survival, Ki-67 index (+) > 1%.			
History (Historia choroby)	Number of patients (Liczba chorych)	Ki-67 (%)	Standard deviation (Błąd standardowy)
Died (Zgon)	14	34.80%	15.42
Survived (Przeżycie)	15	23.60%	9.05

Table 3. Analysis of the number of deaths in relation to the level of Ki-67 index on 24-month follow-up period

Tabela 3. Analiza śmiertelności pacjentek w okresie 24 miesięcy w zależności od poziomu indeksu Ki-67

Death within 12/24 months Ki-67 (+) > 1%.			
History (Historia choroby)	Number of patients (Liczba chorych)	Ki-67 (%)	Standard deviation (Błąd standardowy)
Death < 12 month (Zgon < 12 miesięcy)	6	24.95	8.71
Death 12–24 month (Zgon 12–24 miesięcy)	8	42.18	15.5

The results prove that Ki-67 index can be used as a predictive factor for patients' survival in the investigated 24-month period, however, it is of a prognostic value only in the cases in which Ki-67 index was > 1%. No prognostic value of the parameter was found when its level in the tissue

Table 4. Analysis of survival rate in relation to mean level of Ki-67 index**Tabela 4.** Analiza przeżywalności pacjentek w zależności od średniego poziomu indeksu proliferacyjnego Ki-67

24-month survival/mean Ki-67 level in the group (+)			
Survival (Przeżycie)	Ki-67 index < 29% (Indeks Ki-67 < 29%)	Ki-67 index > 29% (Indeks Ki-67 > 29%)	Patients (Chorzy)
< 24 months (< 24 miesiące)	6	8	14
> 24 months (> 24 miesiące)	10	5	15
Patients (Chorzy)	16	13	29

material obtained during the surgery was < 1%. Table 3 presents differences in the level of Ki-67 antigen expression in women who died during the investigation period of 24 months in relation to the time of their death.

Mean levels of the index proved to be statistically significant ($p = 0.03$).

Although the results were found to be statistically significant, their interpretation is difficult as the group with longer survival displayed increased tendency to cell proliferation. This indicates that the survival rate is affected by other factors, complementary treatment being among the most significant ones, however this problem was beyond the scope of presented analysis.

The last Table (4) illustrates the dependence of the predicted survival on the mean level of Ki-67 index (29.01%).

The results are statistically insignificant ($p > 0.05$). No relationship was found between 24-month survival and mean level of Ki-67 index.

Discussion

The process of mitosis in healthy tissue is a subject to numerous mechanisms regulating its rate and normal course. Ki-67 index is used to determine the percentage of cells undergoing mitotic proliferation in the investigated tissue. The prognostic value of Ki-67 index in oncology is associated with the possibility of predicting the course of the neoplastic process by determination of the dynamics of cell proliferation. Also in ovarian carcinoma, increased Ki-67 index may indicate higher aggressivity and poorer prognosis for the patients.

Harłodzińska et al. assessed Ki-67 antigen expression in a group of 45 cases of malignant and 15 cases of benign ovarian cancer [9]. In the group of patients with adenocarcinoma, the average per-

centage of positive cells amounted to 16% and ranged from 2 to 80%, the highest levels being found in 28 patients with serous ovarian carcinoma in which the level of Ki-67 (positive) cells in the malignant tissue reached 20.1%. However, the Ki-67 antigen expression in the benign tumours was found to be very low in relation to cancer, the difference being statistically significant. Darai et al. investigated the level of proliferation activity in benign and malignant tumours of the ovary [10]. They found Ki-67 activity in 12 and 70% of the investigated cases respectively. The level of proliferation index was as follows: $1\% \pm 8$ in benign tumours and $44.5 \pm 31\%$ in carcinoma. Thus, the differences were statistically significant, however, such a relationship has not been found neither comparing Ki-67 index in various histological types of the tumour nor analyzing the cancer-specific survival in relation to Ki-67 index.

The aim of the studies carried out by Frigerio et al. was similar [11]. Analyzing 10 cases of benign serous adenoma of the ovary and 9 cases of serous ovarian carcinoma, they defined the following levels of Ki-67 index: 0–2.1% and 4.7–20.3% respectively. The differences were also statistically significant. In presented studies the authors obtained a positive reaction with Ki-67 antibody in 70.74% of the investigated cases of serous ovarian carcinoma and 63.63% in benign serous adenomas. The values in the groups with positive Ki-67 proliferation index, i.e. the percentage of positive cells ranged from 9.36 to 70.35% for ovarian carcinoma and from 1.72 % to 14.69% for adenoma. Mean values of Ki-67 expression were 29.01% for malignant and 5.84% for benign tumours ($p < 0.05$). Presented findings are consistent with data from literature – in ovarian carcinoma Ki-67 index assumes higher values. Moreover, mean values of the proliferation index for malignant tumours were similar to those reported by other authors while in benign adenomas they were slightly higher.

Jordan et al. analyzed Ki-67 cell proliferation index in a group of 50 women with advanced malignancy (grade III and IV according to FIGO) [12]. In 35 grade III cases and 15 grade IV cases of ovarian carcinoma the authors analyzed the patients' survival in relation to the degree Ki-67 index surpassed 7.5%. Average survival in this group was 16.8 months vs. 31.5 months in patients with lower proliferation activity ($p < 0.01$). Moreover, the authors reported mean levels of the proliferation index in grade III and IV of the clinical advancement, which were 8.9% and 17.7% respectively at significance level of $p = 0.06$. They did not find any correlation between Ki-67 index and the degree of malignancy. On the other hand,

Altavilla et al. found such a correlation for G3 grade [13]. Having analyzed 120 cases of malignant carcinoma of the ovary, Viale et al. confirmed a correlation between Ki-67 index and patients' survival rate [14]. The authors reported significantly poorer prognosis in cases in which the values of the proliferation index were higher than 30%.

The analysis of correlation between expected survival and cell proliferation index in our study involved the assessment of prognosis in the whole investigated group. Moreover, additional calculations were carried out for patients with Ki-67 > 1%. The findings revealed lack of a significant correlation between the proliferation index and the expected length of survival in the investigated group of 41 patients, but at the same time the results indicated such correlation in the group of Ki-67 positive patients. During the 24-month follow up period 14 patients died (mean Ki-67 =

= 34.8%) and 15 patients who survived had mean Ki-67 index of 23.6%, $p = 0.02$. Next, the authors were looking for associations between the lapse of time between death and the onset of treatment. Among 14 women who died, 6 deaths occurred in the first year of the follow up and 8 deaths in the second year of the study. The findings were surprising, as the patients who died in the first year of treatment had lower Ki-67 index (24.95% vs. 42.18%), $p = 0.03$. The authors suppose this might be due to a better response of fast cell division under influence of chemotherapy, a condition which gives a better short term prognosis.

The final comparison included the assessment of survival in relation to Ki-67 index with reference to the mean values in the group of Ki-67 positive women. No significant differences in expected survival rate were found among women with Ki-67 expression, both higher and lower than 29%.

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