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Immunohistochemical Evaluation of Neoepitope Cytokeratin 18 Expression in Relation to p53 Protein in Ovarian Carcinoma*

Immunohistochemiczna ocena występowania neoepitopu cytokeratyny 18 z uwzględnieniem ekspresji białka p53 w rakach jajnika

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Abstract

Background. One of the major reasons for lack of response to therapy (both chemotherapy and radiotherapy) and the development of progression may be defects in the apoptotic cell death mechanism.
Objectives. Assessing the expression of caspase-cleaved cytokeratin 18 (CK18) (M30) in ovarian carcinoma and the relationship between cytokeratin 18 expression, clinicopathological parameters, and overexpression of p53 protein in tumors.
Material and Methods. The expressions of neoepitope cytokeratin 18 (clone M30) and p53 protein were evaluated by immunohistochemistry in frozen tissue sections from one hundred nine patients with primary ovarian carcinoma.
Results. Positive cytoplasmic staining for cytokeratin 18 (M30) was detected in 52 (47.7%) of the 109 ovarian carcinomas. The distribution of CK 18 positivity in ovarian carcinoma showed inter- and intra-tumoral heterogeneity. No association was observed between CK18 expression and clinicopathological parameters. Moreover, tumors showing CK18 staining were observed more frequently in FIGO stages III/IV. Nuclear accumulation of p53 protein was found in 58 (53.2%) of the ovarian carcinomas. The range of p53 positivity varied between 10–90% cells of ovarian carcinomas. Marginally significant differences between FIGO stage and p53 overexpression were found ($p = 0.07$). The association between different degrees of cytokeratin 18 or p53 expression and clinicopathological parameters was not statistically significant. Moreover, the expression of CK18 in over 30% of carcinoma cells was observed in well-differentiated ovarian carcinomas, whereas p53 expression above or below 30% of cells was independent of FIGO and comparable in moderately and poorly differentiated carcinomas. No relationship between cytokeratin 18 (M30) and p53 overexpression in the subgroups was observed.
Conclusions. These results suggest that the expression of caspase-cleaved cytokeratin 18 on the surface of ovarian carcinoma cells is independent of p53 protein expression. The neoepitope of cytokeratin 18 may be used as an additional marker for detecting apoptosis in ovarian carcinoma (**Adv Clin Exp Med 2007, 16, 2, 197–204**).

Key words: immunohistochemistry, ovarian carcinoma, p53, neoepitope cytokeratin 18, M30 CytoDEATH.

Streszczenie

Wprowadzenie. Jedną z przyczyn rozwoju procesu nowotworowego jest brak odpowiedzi na chemioterapię i radioterapię będącą następstwem uszkodzeń w mechanizmach warunkujących apoptozę komórek nowotworowych.
Cel pracy. Ocena występowania neoepitopu cytokeratyny 18 (CK18) (M30) w rakach jajnika z uwzględnieniem wskaźników klinicznych i patologicznych raka jajnika oraz obecności białka p53.
Materiał i metody. Występowanie cytokeratyny 18 (M30) oraz białka p53 oceniano metodą immunoperoksydazową na materiale tkankowym pochodzącym z 109 pierwotnych raków jajnika.
Wyniki. Cytoplazmatyczną obecność cytokeratyny 18 (M30) stwierdzono w 52/109 (47,7%) raków jajnika. Obserwowano heterogenne występowanie cytokeratyny 18 zarówno w całej badanej grupie raków, jak i w indywidu-

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alnych przypadkach. Nie stwierdzono zależności między występowaniem cytokeratyny 18 a typem histologicznym, stopniem zróżnicowania raka jajnika oraz stopniem zaawansowania choroby. W przypadkach III/IV° wg FIGO obecność cytokeratyny 18 stwierdzono jednak częściej. Jądrową akumulację białka p53 wykazano w 58/109 (53,2%) raków jajnika, przy czym dodatnia reakcja immunohistochemiczna dotyczyła 10–90% powierzchni tkanki nowotworowej. Obecność białka p53 częściej stwierdzano w III/IV° aniżeli w I/II° wg FIGO, wykazane różnice były na granicy statystycznej istotności ($p = 0,07$). Nie stwierdzono wzajemnych zależności między zakresem immunoreaktywności cytokeratyny 18 (> 30% lub < 30% dodatniej tkanki) a białkiem p53 oraz wskaźnikami klinicznym i patologicznymi raków jajnika.

Wnioski. Wyniki badań wykazały, że ekspresja neopepitopu cytokeratyny 18 w komórkach raka jajnika jest niezależna od obecności białka p53. Stwierdzono, że neoepitop cytokeratyny 18 może być dodatkowym wartościowym markerem oceny procesu apoptozy w rakach jajnika (**Adv Clin Exp Med 2007, 16, 2, 197–204**).

Słowa kluczowe: immunohistochemia, rak jajnika**,** białko p53, neoepitop cytokeratyny 18, M30 – CytoDEATH.

Epithelial ovarian cancer is the leading cause of death among the gynecological cancers. The majority of patients present an advanced stage of disease at the time of diagnosis. Despite cytoreductive surgery and chemotherapy, most patients with advanced stage die of progressive disease [1]. Traditional clinicopathological parameters are the most important factors for distinguishing between patients who will have favorable or unfavorable outcomes, but not for defining biological features of tumors. In patients with malignant tumors, the aim of therapeutic strategies is to damage the carcinoma cells by inhibiting cell proliferation and increasing the rate of cell death [2, 3]. The effect of such treatment depends on several different factors, such as the type of drugs and the chemosensitivity of the tumor cells. Generally, the cell damage caused by chemiotherapeutic drugs is first of all arrest of the cell cycle in the G1-S or G2-M phases and repair of DNA damage. Secondly, in cases of insufficient repair mechanisms, the induction of apoptosis occurs [3].

One of the major reasons for lack of response to therapy (chemotherapy and radiotherapy) and the development of progression may be defects in the apoptotic cell death mechanism. This mechanism is regulated by a cascade of proteins which are encoded by several genes (apoptosis-regulating genes), including *Bcl-2* family genes and *p53* gene [4, 5]. Certain members of the family promote apoptosis (e.g. Bax, Bad, Bcl-Xs), while others have an antiapoptotic function (Bcl-2, Bcl-XL) [4]. The biochemical mechanisms underlying p53-dependent apoptotic responses are not fully characterized. It is well documented that p53 is involved in both the extrinsic and intrinsic pathways of apoptosis by initiating apoptosis through mitochondrial depolarization and sensitizing cells to inducers of apoptosis [4–6].

A lot of data suggest that mutations in the *p53* gene in different human cancers are generally missense, map the DNA-binding domain of the protein, and inhibit the transactivation of genes that are involved in the regulation of apoptosis [5]. p53 can also promote apoptosis through a transcription-independent mechanism [6]. Mitochondria play a central role in apoptotic events through the release of apoptogenic mitochondrial proteins, e.g. cytochrom c, an apoptosis-inducing factor which triggers the formation of the apoptosome in the cytoplasm [7]. This complex activates several proteolytic enzymes, among which caspases play a particular role in intracellular apoptotic signaling [5, 7]. Recently it has been shown [7, 8] that activated p53 can directly or indirectly modulate the expressions of proteins that control mitochondrial membrane permeability by forming complexes with proteins leading to the release of cytochrome c.

During apoptosis, many products of cell death are located in the cytoskeleton and the nuclear membrane, whereby they can be measured. One of these is cytokeratin, an intracellular structural protein specific to normal epithelial cells and carcinoma cells that are cleaved and released during apoptosis [2]. Some data show that cytokeratins, in particular cytokeratin 18, are affected in early events of apoptosis [9]. This neoepitope is derived from the cleavage of cytokeratin 18 by caspase 8, which is recognized by the specific monoclonal antibody M30 CytoDEATH and is not detectable in non-apoptotic cells [9]. Single studies have demonstrated reactivity of monoclonal antibody M30 (MAb M30) in human salivary glands and in cervical, endometrial, and ovarian carcinomas [10–13]. The results suggest that immunohistochemical staining with MAb M30 may be useful in detecting the apoptosis-inducing activities of various chemical compounds. To our knowledge, the immunohistochemical detection of caspase-cleaved cytokeratin 18 (M30) has been hardly evaluated in ovarian carcinomas [13]. In addition there are no data evaluating the association between cytokeratin 18 (M30) and clinical variables or p53 status in ovarian carcinomas. The purpose of this study was to investigate the expression of the neoepitope of cytokeratin 18 in ovarian carcinomas and the relationships between CK18 (M30), clinicopathological parameters, and overexpression of p53 protein.

Material and Methods

Patients

One hundred and nine patients with primary epithelial ovarian carcinoma before chemotherapy were entered in this study between April 1996 and April 2005. Tumor tissue sections were obtained from the initial surgery at the First and Second Departments of Gynecology, Silesian Piasts University of Medicine, Wrocław, Poland. After surgery the patients were treated using standard chemotherapy: CP (cisplatin-cyclophosphamide or carboplatin-cyclophosphamide) or CAP/CP (with addition of doxorubicin). The patients were evaluated according to the staging system of the International Federation of Gynecology and Obstetrics (FIGO). Histological typing and grading of each tumor was assessed on paraffin-embedded tissue specimens according to the classification of the World Health Organization (WHO) (Jerzy Rabczyński, Department of Pathology, Wrocław). The study group of 109 ovarian carcinomas comprised 57 serous (47 tumors in stage III/IV, 10 tumors in stage I/II), 25 endometrioid (12 in stage III/IV, 13 in stage I/II), 8 mucinous (2 in stage III/IV, 6 in stage I/II), and 19 undifferentiated (16 in stage III/IV, 3 in stage I/II) ovarian carcinomas. The tumors were graded as well (G1), moderately (G2), or poorly (G3) differentiated. Twenty-six tumors were well, 38 moderately, and 26 poorly differentiated.

Immunohistochemical Staining

The indirect peroxidase–antiperoxidase test was performed on 4-μm-thick cryostat (Reichert) acetone-fixed tissue specimens. The endogenous peroxidase activity was blocked by periodic acid (2.28%) and sodium borohydride (0.02%). After the inhibition of endogenous peroxidase, the tissue sections were treated with primary monoclonal antibodies against p53 protein (clone DO-7) (Dako, Copenhagen, Denmark) and caspase cleaved of cytokeratin 18 (clone M30) (M30 CytoDEATH, Boehringer Mannheim, Germany). Dilutions of 1:25 and 1:50 of the stock were used for p53 and M30, respectively. Replacement of the primary antibody with 0.1 M Tris buffer, pH 7.4, served as a negative control. After 60 min of incubation with the primary antibody, peroxidase-conjugated rabbit anti-mouse IgG (Dako) was applied for 30 min. Following washing in 0.1 M Tris buffer, pH 7.4, for 2 × 5 min, the preparations were treated with peroxidase-conjugated swine anti-rabbit IgG (Dako) followed by 3,3'diaminobenzidine (Sigma, St. Louis, MO, USA) as a chromogen. For microscopic evaluation the preparations were counterstained with hematoxylin and mounted. The preparations were evaluated under a BH-2 Olympus light microscope. The localization, distribution, and intensity of immunostaining were evaluated in the tissue sections. For mAb M30 only a cytoplasmic immunostaining pattern and for p53 only a nuclear immunostaining pattern was considered as a positive result. For p53 and M30 monoclonal antibodies, the reaction was considered positive when at least 10% of cells were stained. The intensity of staining was scored as 0 for negative, + weak, ++ moderate, and +++ strong. Appropriate positive and negative controls were used for all markers. The immunohistochemical analyses were interpreted without prior knowledge of the clinical information.

Statistical Analysis

Correlation between cytokeratin 18 (M30) and clinicopathological parameters and p53 was evaluated by the chi squared test. Spearman rank correlation was calculated to investigate the association between CK18 (M30) and p53 expression. For all correlation analyses, cytokeratin 18 (M30) and p53 immunoreactivity were divided into the following groups: negative vs. positive or ≤ 30% positive tumor cells vs. > 30% positive tumor cells.

Results

Cytokeratin 18 (M30) Immunostaining

Positive cytoplasmic staining of the caspase cleaved product of cytokeratin 18 recognized by MAb (M30) was detected in 52 of the 109 (47.7%) ovarian carcinomas, with the percentage of positive cells ranging from 10 to 90%. Some tissue sections showed immunostaining of the vast majority of carcinoma cells forming glandular structures, whereas in other samples the CK18 (M30)-positive reaction was restricted to small areas of the tumor tissue. A focal nature of immunostaining was the dominant feature of CK18 (M30)-positive tumors (Fig. 1). The distribution of CK18 (M30) positivity in individual cases showed high heterogeneity. In most cases, mAb M30 reactivity was limited to 40% of the tissue section (Fig. 2).

The association between cytokeratin CK18 (M30) immunoreactivity and histological type of ovarian carcinoma is shown in Table 1. The highest proportion of cytokeratin 18 (M30)-positive cells was observed in undifferentiated (mean: 14.7%, *SD*: 20.1 $n = 19$) and serous car-

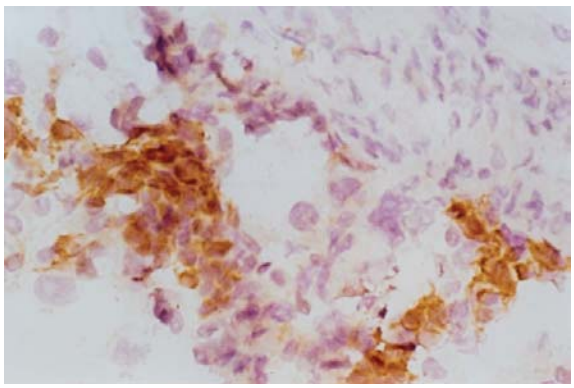

Fig. 1. The expression of the neoepitope cytokeratin 18 (M30) in ovarian carcinoma (immunoperoxidase staining ×400)

Ryc. 1. Ekspresja neoepitopu cytokeratyny 18 (M30) w raku jajnika (metoda immunoperoksydazowa ×400)

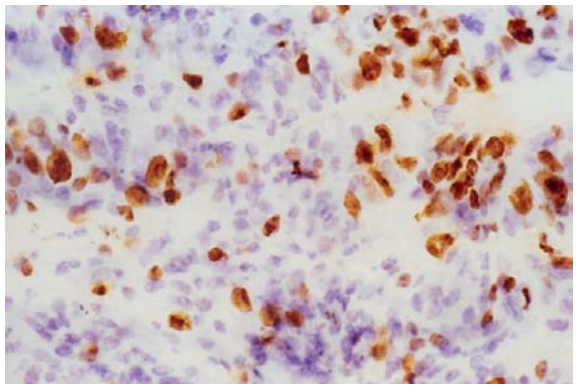

Fig. 3. Expression of p53 in ovarian carcinoma (immunoperoxidase staining ×400)

Ryc. 3. Ekspresja białka p53 w raku jajnika (metoda immunoperoksydazowa ×400)

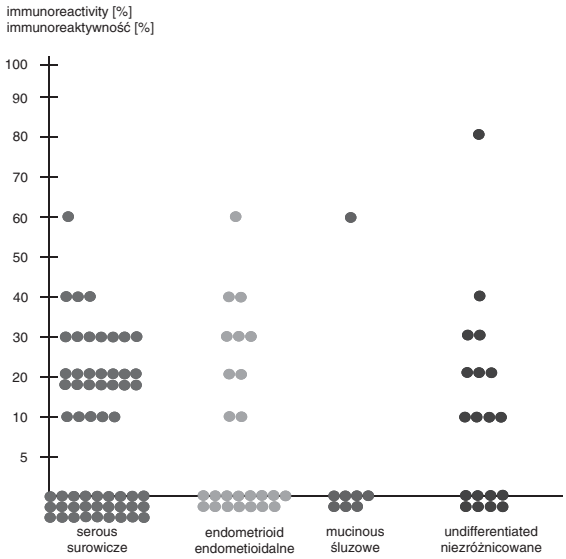

Fig. 2. Neoepitope cytokeratin 18 (M30) expression in different histological subtypes of ovarian carcinoma

Ryc. 2. Ekspresja neoepitopu cytokeratyny 18 (M30) w raku jajnika z uwzględnieniem typu histologicznego raka jajnika

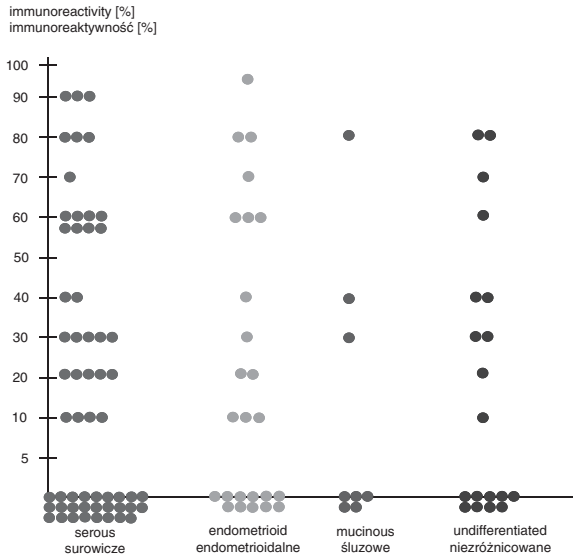

Fig. 4. Expression of p53 protein in different histological subtypes of ovarian carcinoma

Ryc. 4. Ekspresja białka p53 w raku jajnika z uwzględnieniem typu histologicznego raka jajnika

cinomas (mean: 12.5%, *SD*: 14.6, $n = 57$), lower in endometrioid (mean: 11.6%, *SD*: 17.2, $n = 25$), and the lowest in mucinous (mean: 7.5%, *SD*: 21.2%, $n = 8$) ovarian carcinomas. These differences were not statistically significant ($p > 0.05$). No association was observed between CK18 (M30) expression and clinicopathological parameters. Moreover, tumors showing CK18 (M30) staining tended to be higher in FIGO stage III/IV.

P53 Immunoreactivity

Nuclear accumulation of p53 protein was found in 58 of the 109 (53.2%) ovarian carcinomas. In the majority of cases, marked heterogeneity of p53 overexpression was observed. The pattern of staining in the malignant tumor cells was found to be nuclear (Fig. 3). The range of p53 positivity varied between 10–90% of ovarian carcinoma cells (Fig. 4). A high and comparable percentage of p53-positive cells was observed in endometrioid (mean: 25.8%, *SD*: 31.94, $n = 25$), serous (mean: 25.09%, *SD*: 30.3, $n = 54$), and undifferentiated (mean: 24.21%, *SD*: 29.5, $n = 19$), but lower in mucinous (mean: 18.75%, *SD*: 29.49, $n = 8$) ovarian carcinomas. These differences were not statistically significant ($p > 0.05$).

Overexpression of p53 protein was not associated with the grade of tumor differentiation. Similar percentages of p53-positive cases were

Table 1. Expressions of neoepitope cytokeratin 18 (M30) and p53 protein with clinicopathological variables in ovarian carcinomas

Tabela 1. Ekspresja neoepitopu cytokeratyny 18 (M30), białka p53 z uwzględnieniem parametrów kliniczno-patologicznych raka jajnika

Factors (Parametry)		Immunoreactivity – percentage of positive cells (Immunoreaktywność – odsetek dodatnich komórek)					
		neoepitope cytokeratin 18 (M30) neoepitop cytokeratyny 18 (M30)			p53 protein białko p53		
Histological types (Typy histologiczne)	N	positive (%)	≤ 30%	> 30%	positive (%)	≤ 30%	> 30%
Serous (Surowicze)	57	30 (52.6)	26 (86.7)	4 (13.3)	31 (54.4)	14 (45.2)	17 (54.8)
Endometrioid (Endomerioidalne)	25	10 (40.0)	7 (70.0)	3 (30.0)	14 (56.0)	6 (42.8)	8 (57.1)
Mucinous (Śluzowe)	8	1 (12.5)	0	1 (100)	3 (37.5)	1 (33.3)	2 (66.7)
Undifferentiated (Niezróżnicowane)	19	11 (57.9)	9 (81.8)	2 (18.2)	10 (52.6)	4 (40.0)	6 (60.0)
All (Wszystkie)	109	52 (47.7)	42 (38.5)	10 (9.2)	58 (53.2)	25 (22.9)	33 (30.2)
FIGO							
I/II	32	12 (37.5)	11 (91.7)	1 (8.3)	12 (37.5)*	5 (41.7)	7 (58.3)
III/IV	77	40 (51.9)	31 (77.5)	9 (22.5)	46 (59.7)	20 (43.5)	26 (56.5)
Grade (Stopień zróżnicowania)							
G_1	26	13 (50.0)	10 (76.9)	3 (23.1)	13 (50.0)	8 (61.5)	5 (38.5)
G_2	38	15 (39.5)	12 (80.0)	3 (20.0)	20 (52.6)	7 (35.0)	13 (65.0)
G_3	26	13 (50.0)	11 (84.6)	2 (15.4)	15 (57.7)	6 (40.0)	9 (60.0)

*p = 0.07.
*p = 0,07.

observed in histological subtypes of ovarian carcinoma. Differences between FIGO stage and p53 overexpression had only borderline significance (p = 0.07) (Fig. 5).

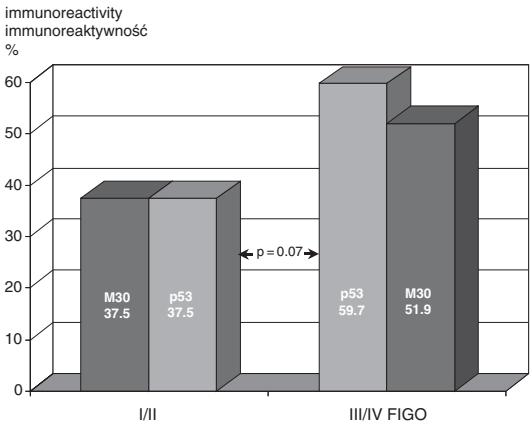

Fig. 5. Relationships between neoepitope cytokeratin 18 (M30), p53 protein expression, and clinical stage of ovarian carcinomas (FIGO)

Ryc. 5. Zależność między obecnością neoepitopu cytokeratyny 18 (M30), białka p53 a stopniem klinicznego zaawansowania choroby (FIGO)

Relationships Between the Level of Cytokeratin CK18 (M30), p53 Expression, and Clinicopathological Parameters

The association between different degrees of cytokeratin CK18 (M30) and p53 expression and clinicopathological parameters of ovarian carcinoma is shown in Table 1. There was no significant correlation between different percentages (≤30% vs. > 30%) of cytokeratin 18-positive cells and clinical parameters of ovarian carcinoma. However, higher expression of cytokeratin 18 (> 30% positive cells) was more frequently detected in FIGO stage III/IV than in stage I/II. The expression of cytokeratin 18 (M30) in > 30% of cells was observed mainly in well-differentiated (G1) tumors. p53 expression above or below 30% of carcinoma cells was independent of FIGO stage and comparable in moderately and poorly differentiated ovarian carcinomas. No relationship was observed between cytokeratin CK18

(M30) and p53 overexpression in the whole group of analyzed ovarian carcinomas or in the two subgroups.

Discussion

Ovarian cancer is the second most common and most fatal of the gynecologic malignancies; even when treated at an early stage it has a poor prognosis and only approximately 30% of all patients survive five years [1]. The prognosis of patients with ovarian carcinoma depends on several biological factors [14]. One is apoptosis, which may occur spontaneously or be induced by anticancer therapy [15]. Some of the biochemical features of apoptotic cells result from the selective proteolytic cleavage of a subset of cellular polypeptides [2]. During the apoptotic process, many different products of cell death are expressed on the cell surface or released into the blood circulation [2]. Cytokeratins (CKs) are intracellular structural proteins expressed by most types of epithelial cells that are cleaved and released during apoptosis. One of them is a neoepitope of cytokeratin 18 (CK18) cleaved by caspase 8 [9]. In the present study, immunohistochemistry was used to detect the expression of the caspase-cleaved product of cytokeratin 18 (CK18) using MAb (M30) and p53 protein overexpression in ovarian carcinoma. To the knowledge of the present authors this is the first study describing the expression of the caspase-cleaved neoepitope of cytokeratin 18 (CK18) in relation to clinicopathological variables and p53 protein overexpression in ovarian carcinoma.

Data on the expression of the caspase-cleaved product of cytokeratin 18 in human cancers are scarce [10, 11, 16]. In the present study, granular reaction products for MAb M30 were observed in the cytoplasm in 47.7% of the ovarian carcinomas. Similarly to earlier sudies [11, 13, 16], a low percentage of cytokeratin CK 18-positive cells was observed in ovarian carcinoma. These observations suggest that spontaneous apoptosis of tumor cells in ovarian carcinomas is limited to a small area of tumor tissue. Based on earlier data [11, 12] and the present observation, one can speculate that tumors reflecting spontaneous apoptosis may be more sensitive to cancer therapy. On the other hand, the low level of MAb M30 positivity in ovarian carcinomas may explain its aggressive biological behavior [13].

No association was found between the amount of caspase-cleaved cytokeratin 18 and clinicopathological parameters. These results are in agreement with other data observed in cervical cancer [11], but contrary to results showing correlation between MAb M30 positivity and high grade of differentiation of endometrial cancer [12, 17]. Kramer et al. [12] revealed that caspase-cleaved CK 18 fragment released to the serum of patients with endometrial cancer can be measured and the level of the serum form of CK18 can demonstrate treatment efficiency during the patients' clinical course. The data of the present study indicate that the detection of the caspase-cleaved form of CK18 using M30 antibody is useful for the investigation of apoptosis in ovarian carcinoma tissue before the treatment of patients.

The p53 tumor suppressor protein has multiple functions. It can bind to DNA and transactive genes involved in cell cycle control and apoptosis [4]. Several studies on p53 concentrate on its ability to control apoptosis [4, 5]. It has been revealed that tumors with nuclear accumulation of p53 protein showed a disruption in the apoptotic process and often behaved more aggressively [5]. In the present study, p53 overexpression was found in 53.2% of the ovarian carcinomas. These results are in agreement with most published data [18–21]. The association between p53 status and the histological structure of the tumor, the grade of tumor differentiation, and the clinical stage of disease is still controversial [18, 20]. In the present study, no significant relationship between histological type of tumor and grade of differentiation and p53 expression was found. However, the higher incidence of p53 positivity in serous ovarian carcinomas and poorly differentiated tumors was reported by some authors [21], whereas in other studies [20, 22], no significant correlation between histological subtype, tumor grade, and p53 expression was observed. Similarly to our observation, some data have been able to show an association between p53 presence and stage of disease [21–23]. However, a few reports did not find such differences [18, 20]. Some authors suggest that the increase in the rate of p53 expression with advanced stage of disease may indicate that p53 overexpression is associated with a late event in ovarian carcinogenesis. It also may be a characteristic feature of progression and metastasis in ovarian carcinomas [23].

The present study fails to show a correlation between overexpression of p53 protein and caspase-cleaved cytokeratin 18 expression. These results could be explained by the different apoptotic pathways involved in the degradation of cell death substrates, such as cytokreatins. One of these is associated with receptor activating formation of the death-inducing signaling complex (DISC). In other pathway which includes the mito-

chondrial release of cytochrom c and activation of caspase-9 in the apoptosome, additional mechanisms such as the release of apoptosis-inducing factor (AIF) are involved. Both pathways merge in the degradation of cell death substrates such as cytokeratins, but they are not connected directly with p53 protein [2]. Taking into account the data mentioned above and the results of the present study, it can be suggested that the expression of caspase-cleaved cytokeratin 18 on ovarian carcinoma cells surface is independent of p53 expression. This finding also has clinical implications for the use of caspase-cleaved cytokeratin 18 as an additional apoptotic marker in ovarian cancer.

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