

## ORIGINAL PAPER

**MOLECULAR PROGNOSTIC FACTORS IN EARLY-STAGE CERVICAL ADENOCARCINOMA**

MACIEJ BODZEK<sup>1</sup>, PAWEŁ BLECHARZ<sup>2</sup>, JANUSZ RYŚ<sup>3</sup>, WIKTOR SZATKOWSKI<sup>2</sup>, MAREK JASIÓWKA<sup>2</sup>, PAWEŁ MAJCHRZAK<sup>3</sup>, KRZYSZTOF HALASZKA<sup>3</sup>, ANNA KRUCZAK<sup>3</sup>, BOŻENA LACKOWSKA<sup>3</sup>

<sup>1</sup>Department of Obstetrics and Gynecology, Provincial Hospital in Chrzanow, Poland

<sup>2</sup>Gynecologic Oncology Department, Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Krakow Branch, Krakow, Poland

<sup>3</sup>Department of Tumour Pathology, Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Krakow Branch, Krakow, Poland

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Within the past years the proportion of cervical adenocarcinomas has increased, however, there is a shortage of data regarding immunohistochemical and molecular features and their prognostic relevance in early-stage cervical adenocarcinoma (esCAC). Aim of the present study was to evaluate molecular prognostic factors in esCAC patients treated with primary surgery.

Analyses of surgical specimens in 59 patients with esCAC were performed on fixed paraffin-embedded sections of tumour tissue. Tumour tissue sections were routinely stained with hematoxylin and eosin followed by microscopic examination. Immunohistochemical analyses (IHC) were performed on paraffin-embedded section. Flow cytometry (FCM) analysis of paraffin-embedded tumor tissue was performed using flow cytometer FACSCalibur equipped with argon laser. DNA histogram analysis was performed with ModFit application. Treatment effectiveness was evaluated using overall 5-year survival. Survival probability was estimated using the Kaplan-Meier method.

Overall survival rate estimated using Kaplan-Meier method was 74.6%. Among the IHC and FCM features univariate analysis showed statistical significance of *nm23-H1* gene expression and total S-phase fraction  $\leq 11.9\%$  (S-TOT). In multivariate analysis LVSI and parametrial involvement had significant, negative impact on survival (HR = 8.04,  $p < 0.003$  and HR = 4.03,  $p < 0.017$ , respectively). However, none of the tested IHC and FCM features had any influence on overall 5-year survival.

**Key words:** cervical adenocarcinoma, flow cytometry, prognosis.

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**Introduction**

According to the literature reports adenocarcinoma of the uterine cervix accounts for 15-25% of all the cervical cancer (CC) cases [1]. Unlike squamous cell carcinoma, in which case lower incidence rate in the last decades is accompanied by decrease in its

mortality, the mortality of cervical adenocarcinoma (CAC) remains unaltered and may indicate more aggressive character of the disease [2].

Within the past 24 years in the USA, the proportion of CAC has increased to around 29% [3]. It is probably due to more and more frequent detection of precursor lesions and related decrease in the rate of

squamous cell carcinomas. Cervical adenocarcinoma, compared with primary cervical cancer (PCC), has more histological subtypes of different lymph node metastases potential or tendency to relapse. It seems that the proportion of adenocarcinomas of the CC in the world has been increasing over the last decades, particularly in younger women [4, 5, 6, 7, 8, 9].

There is a shortage of data regarding immunohistochemical and molecular features and their prognostic relevance in CAC. The most of clinical results discuss clinicopathological factors only [10, 11, 12, 13, 14, 15, 16, 17, 18]. Aim of the present study was to evaluate molecular prognostic factors in early-stage cervical adenocarcinoma (esCAC) patients treated with primary surgery.

## Material and methods

Between 1985 and 2000, 195 patients with CAC confirmed by biopsy, uterine cervix scrapings examination or resection tissue material examination were treated at the Gynaecology Department of Centre of Oncology, Maria Skłodowska-Curie Memorial Institute, Krakow Branch, Poland. In that number, 120 patients with esCAC were treated with primary surgery.

Results of combined treatment (surgery only or surgery + radiotherapy) in term of progression-free and overall survival were evaluated during follow-up visits. Disease progression or recurrence was defined as the presence of measurable lesions observed in medical examination (physical examination, particularly gynaecological) or imaging (computer tomography of abdomen and pelvis, X-ray examination of chest, US examination of abdomen and pelvis).

Available histological specimens from 82 CAC patients treated with primary surgery were reviewed by an experienced pathologist prior to immunohistochemistry (IHC) and flow cytometry (FCM) analysis for prognostic factors. Retrospective morphological assessment of tissue material was performed on sections prepared from paraffin-embedded histological specimens from the archives of the Department of Tumour Pathology, Centre of Oncology, Maria Skłodowska-Curie Memorial Institute, Krakow Branch, or borrowed from other pathology departments (hospitals of the Małopolska province). Due to technical issues, 23 of the specimens were not suitable for the planned testing and were excluded from detailed analysis. For the remaining 59, IHC and FCM analyses were performed on fixed paraffin-embedded sections of tumour tissue. Tumour tissue sections were routinely stained with hematoxylin and eosin followed by microscopic examination. Based on the microscopic evaluation, all tumours were classified according to the current World Health Organization (WHO) guidelines

published in 2014 [19]. In all cases, tumour histology and grade were established as well as nuclear atypia grade of tumour cells.

All immunohistochemical analyses were performed on paraffin-embedded section of  $5\mu$  thickness. Diagnostic techniques applied are presented in Table I.

Flow cytometry (FCM) analysis of paraffin-embedded tumor tissue was performed using flow cytometer FACSCalibur equipped with argon laser (15 mW, 488 nm). DNA histogram analysis was performed with ModFit application. The analysis included at least 20 000 collected parameters.

DNA histograms were classified according to the criteria established by Cytometry Consensus Conference in 1992 [20]. DNA ploidy was described by DNA index (DI). Histogram of  $DI = 1.00$  was classified as diploid, and of  $DI \neq 1.00$  as aneuploid. Tumour proliferation rate was expressed in S-phase fraction or proliferation index (S+G2M-phase fraction) [21].

Significant demographic, microscopic and clinical characteristics of 59 patients, whose specimens were analysed, were compared with the whole group of consecutive 120 patients with esCAC. Those included age, menopausal status, comorbidities, FIGO stage, surgical procedures, grading of the tumour, surgical margin, parametrial involvement, lymphovascular space invasion (LVSI), regional lymph node status. The analysis confirmed no differences in the distribution of statistically significant variables. Hence, the prognostic value of analysed factors can be translated into the general population of esCAC patients.

Treatment effectiveness was evaluated using overall 5-year survival. Survival probability was estimated using the Kaplan-Meier method [22].

The Peto log-rank test was used to evaluate statistical significance of the observed result differences [23]. Statistical significance level was set at  $p \leq 0.05$ . Continuous variables, such as age or overall survival time, as well as discrete variables were tested, both original and adjusted, to establish any variability range significantly affecting the survival.

Influence of selected factors on patient survival times was assessed using Cox's proportional hazard model [24].

## Results

Comparison of demographic, microscopic and clinical characteristics of 59 esCAC patients included in the detailed IHC and FCM with the whole group of 120 patients with esCAC showed no differences in distribution of following: age, menopausal status, concomitant diseases, FIGO stage, surgical margin, parametrial and uterine infiltration, LVSI presence

**Table I.** Antibodies used in IHC diagnostics of tissue specimens from esCAC patients

ANTYBODY	CLONE	PRODUCER	DILUTION	INCUBATION TIME	DETECTION SYSTEM	ANTIGEN DEMASKING
p16	–	CINtec HISTOLOGY	1 : 1	30 min	Visualization Reagent	Epitop Retrieval Solution
PGR	SP2	Thermo Scientific	1 : 100	30 min	UltraVision LP Value Detection System Large Volume HRP Polymer (RTU)	citrate buffer pH = 6.0 water bath 97°C 20 min
ER	SP1	Thermo Scientific	1 : 250	24 h	UltraVision LP Value Detection System Large Volume HRP Polymer (RTU)	citrate buffer pH = 6.0 microwave oven
Vimentin	V9	Cell Marque	1 : 250	24 h	UltraVision LP Value Detection System Large Volume HRP Polymer (RTU)	2 × 10 min 600 W
MIB	MIB-1	DAKO	1 : 100	24 h	UltraVision Detection System Large Volume Anti-Polyvalent, HRP	
c-erbB2	SP3	Thermo Scientific	1 : 100	24 h	UltraVision Detection System Large Volume Anti-Polyvalent, HRP	
EGFR	2-18C9	DAKO	1 : 1	30 min	Labelled Polymer HRP	proteinase K 15 min
S-100	–	Novocastra	1 : 600	24 h	UltraVision Detection System Large Volume Anti-Polyvalent, HRP	trypsin 15 min
EBAG9	22-1-1	Acris Antibodies	1 : 1000	24 h	Immunologic BrightVision, Poly HRP-Anti Ms/Rb/Ra IgG	
nm23-H1	37.6	Novocastra	1 : 50	24 h	Immunologic BrightVision, Poly HRP-Anti Ms/Rb/Ra IgG	
CEA	B01-94-11M-P	Biogenex	1 : 400	24 h	Immunologic BrightVision, Poly HRP-Anti Ms/Rb/Ra IgG	

and lymph node status. There were a minor differences in distribution of surgery extent (patients with specimen analysed were more often treated with pelvic lymphadenectomy than the whole group of 120 patients – 57.6% vs. 45.8%, respectively;  $p = 0.027$ ) and the grade of the CAC (analyzed group was more likely to have G2/G3 tumour than the whole group – 77% vs. 63.3%, respectively;  $p = 0.003$ )

Detailed results of IHC and FCM analyses performed for the 59 esCAC patient subgroup are given in Table II.

Mean DNA index was 1.2 (SD  $\pm 0.32$ ), mean percentage of cells in S-phase (SPF) was 1.07 (SD  $\pm 10.54$ ) and mean SG2M index was 14.58 (SD  $\pm 12.95$ ).

Overall survival rate estimated using Kaplan-Meier method was 74.6% in the investigated

group of 59 CAC patients and 73.3% in the entire group of 120 patients with esCAC. Kaplan-Meier chart for overall 5-year survival both groups is presented in Fig. 1.

Among the IHC and FCM features univariate analysis showed statistical significance of *nm23-H1* gene expression and total S-phase fraction  $\leq 11.9\%$  (index S-TOT). Differences in treatment outcome in the 59 CAC patient group depending on S-TOT value are provided in Table III.

In multivariate analysis LVSI and parametrial involvement had significant, negative impact on survival (HR = 8.04,  $p < 0.003$  and HR = 4.03,  $p < 0.017$ , respectively). However, none of the tested IHC and FCM features had any influence on overall 5-year survival.

**Table II.** Results of IHC and FCM analyses in the group of 59 esCAC patients

IMMUNOHISTOCHEMICAL AND CYTOFLUOROMETRIC FEATURES	NUMBER OF PATIENTS		IMMUNOHISTOCHEMICAL AND CYTOFLUOROMETRIC FEATURES	NUMBER OF PATIENTS	
	N = 59	%		N = 59	%
Nuclear atypia grade			nm23-H1 intensity		
1	33	55.9	0	2	3.4
2	16	27.2	1+	8	13.6
3	10	16.9	2+	20	33.9
p16			3+	29	49.1
expression	43	72.9	S-100		
no expression	16	27.1	positive reaction	7	11.9
Estrogen receptor			negative reaction	52	88.1
> 50%	3	5.1	MIB-1		
10-50%	3	5.1	≤ 35.9%	29	49.2
< 10%	3	5.1	> 35.9%	30	50.8
no expression	50	84.7	S-TOT		
Progesterone receptor			≤ 11.9%	52	88.1
< 20%	4	6.8	> 11.9%	7	11.9
no expression	55	93.2	DNA ploidy		
CEA			diploid	39	66.1
expression	46	78.0	aneuploid	20	33.9
no expression	13	22.0	Total	120	100
Vimentin					
expression	0	0			
no expression	59	100			
RCAS1/ag22-1-1 in squamous cells – reaction type					
membranous	15	25.4			
membranous (focal)	6	10.2			
cytoplasmic	6	10.2			
cytoplasmic (focal)	4	6.8			
no reaction	14	23.7			
squamous cells not present in the specimen	14	23.7			
RCAS1/ag22-1-1 in glandular component – reaction type					
membranous	3	5.1			
membranous (focal)	3	5.1			
cytoplasmic	2	3.4			
cytoplasmic (focal)	1	1.7			
no reaction	15	25.4			
no normal glandular cells in the specimen	35	59.3			
EGFR					
expression	4	6.8			
no expression	55	93.2			

## Discussion

Group of 59 patients, with pathological specimens available, was subject to thorough microscopic evaluation and IHC and FCM analyses described above. In total, 18 different factors were studied using both methods. As mentioned above this group of patients were compared with the whole group of consecutive 120 patients with esCAC to confirmed no differences in the distribution of statistically significant, clinical characteristics.

The significance of *nm23-H1* metastasis suppressor gene expression in CAC is not well determined. According to the literature reports, the gene expression is observed in 44-75% CAC patients. In the investigated group, as much as 96.6% of patients shows *nm23-H1* gene expression; however, it is moderate (2+) or strong (3+) in 83% of them. Reports on the prognostic value of *nm23-H1* gene expression are contradictory. Mandai *et al.* suggested already in 1995 that its expression is directly correlated with 10-year survival rate in univariate analysis, and when combined with *c-erbB-2/Her2-neu* gene expression the correlation is observed also in multivariate analysis. Similar conclusions were drawn by Huang *et al.* and Kristensen *et al.*, on the other hand, present opposite findings and suggest inverse correlation between the gene expression and survival times, however only in univariate

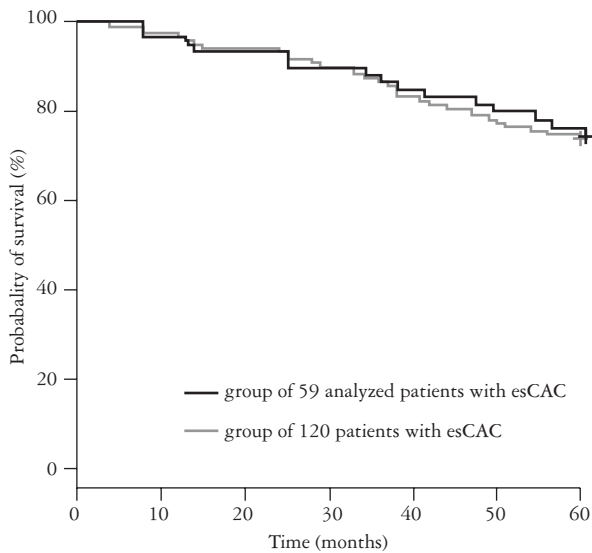


Fig. 1. Overall 5-year survival of esCAC patients

analysis. Moreover, against the suggested gene role, none of the authors did prove any suppressing effect of the gene on locoregional lymph node metastasis.

In the present study, *nm23-H1* expression inversely correlated with survival times and was not connected with the rate of lymph node metastasis. Patients of strong *nm23-H1* expression lived shorter than patients without the gene product expression and had 65.5% and 89.2% of 5-year survival rate, respectively [25, 26, 27].

S-phase fraction was the only FCM feature that in univariate analysis was distinctly related with survival. The patients with esCAC characterized by low S-TOT ( $\leq 11.9\%$ ) had over two times higher chances to survive 5 years than patients of S-TOT  $> 11.9\%$ . Five-year survival rate was 42.9% and 78.9%, respectively. In the available literature, there were no reports to be found concerning FCM data in CAC.

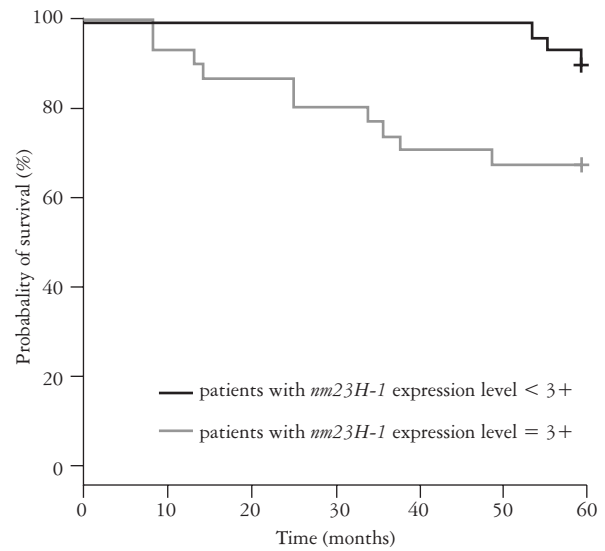


Fig. 2. Survival of patients, depending on *nm23-H1* intensity

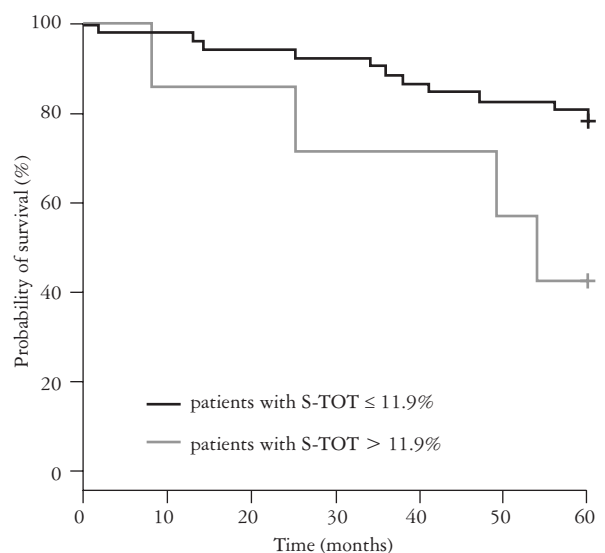


Fig. 3. Survival of patients depending on S-TOT value

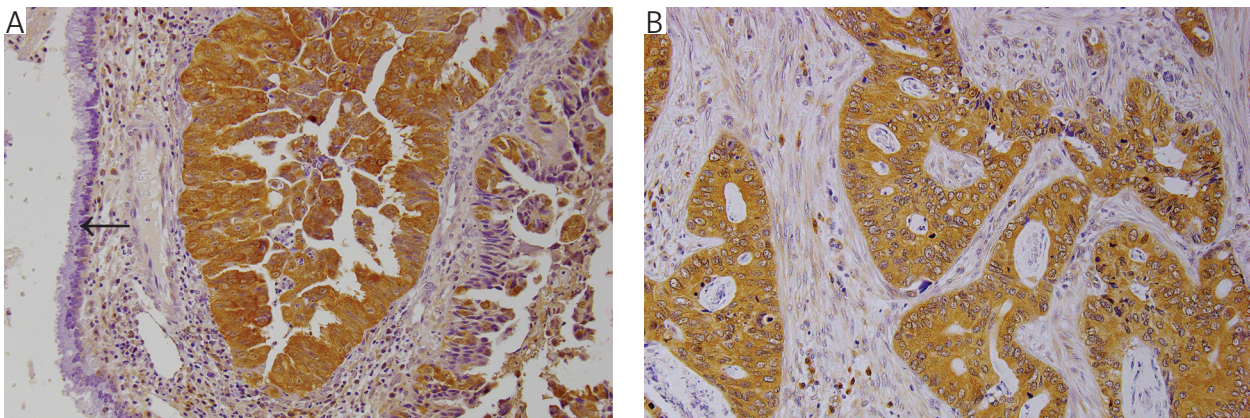


Fig. 4A, B. Positive cytoplasmatic reaction for *nm23-H1* antigen in cervical adenocarcinoma cells. Negative reaction in normal cervical glandular cells (A, arrow)

**Table III.** Treatment outcome in 59 early stage CAC patients and its relationship with *nm23-H1* gene expression and S-TOT index value

IMMUNOHISTOCHEMICAL AND CYTOFLUOROMETRIC FEATURES	NUMBER OF PATIENTS	5-YEAR SURVIVAL*		P
		NUMBER	OF PATIENTS SURVIVING (%)	
<i>nm23-H1</i> intensity				
0/1+/2+	30	25	83.3	0.022
3+	29	19	65.5	
S-TOT				
≤ 11.9%	52	41	78.9	0.028
> 11.9%	7	3	42.8	

\*5-survival rate estimated using Kaplan-Meier method

Other FCF features, that is DNA ploidy, DNA index and SG2M, had no prognostic value in the present study.

In the summary, it should be emphasized that, regardless of the univariate analysis results presented above, multivariate analysis of the 59-patient group did not show any statistical significance of IHC and FCM features for CAC patients prognosis.

The present analysis has several limitations that should be mentioned. Firstly, the investigated group of 59 patients, for which detailed IHC and FCM analyses were performed, had rather good prognosis and, thus, relatively low mortality in the studied period. Combined with relatively small size of the patient group, it lowered statistical impact of the results and made it more difficult to evaluate prognostic factors of distinctly lower influence on the patients' prognosis.

Secondly, due to variable quality and quantity of the available histological material, it was impossible to repeat or clarify some of the analyses. For example, many specimens lacked tissue components necessary to run analyses, as was in case of 22-1-1 antigen.

Regardless the limitations, the present study seems to assess comprehensively molecular prognostic factors in CAC patients. Available reports present no such extended analysis as this one that would evaluate in detail immunohistochemical and cytofluorometric characteristics of CAC and their influence on patient prognosis. Multivariate analysis allowed to conclude that IHC and FCM features have little impact on patient prognosis in esCAC, and the most important seem to be clinical and histopathological characteristics.

*The authors declare no conflict of interest.*

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### Address for correspondence

Wiktor Szatkowski  
Gynecologic Oncology Department  
Maria Skłodowska-Curie Memorial Cancer Center  
and Institute of Oncology  
Krakow Branch  
Garncarska 11  
31-115 Krakow, Poland  
e-mail: vigor27@wp.pl