Inter-component immunohistochemical assessment of proliferative markers in uterine carcinosarcoma

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Abstract. In the scientific literature, a selected number of reports have investigated the impact of proliferative activity on the development and progression of uterine carcinosarcomas (UC). The aim of the present retrospective study was to compare the immunohistochemical proliferation markers [Ki67, proliferating cell nuclear antigen (PCNA), minichromosome maintenance complex component 3 (MCM3), and topoisomerase IIα (topoIIα)] assessment in both components of UC. A total of 30 paraffin-embedded slides of UCs, obtained from patients who underwent surgery between January 1, 2006, and December 31, 2020, were analyzed. Medical records and clinicopathological data of patients were reviewed. Formalin-fixed, paraffin-embedded tissue sections were immunostained with monoclonal antibodies against Ki67, PCNA, MCM3 and topoIIα. Ki67-positive nuclear immunoreactivity was reported in 20 (67%) and 16 (53%) UC carcinomatous and sarcomatous components, respectively. In the epithelial component, Ki67 positive staining was related to the International Federation of Gynecology and Obstetrics (FIGO) stage (P=0.025), and histological grade (G1 vs. G2/G3, P=0.031). Nuclear PCNA reactivity was observed in 18 (60%) and 16 (53%) carcinomatous and sarcomatous components, respectively. Notably, all four cases with omental metastases were PCNA-positive, and a relationship between staining pattern and the existence of metastases was of significant value (P=0.018). MCM3-positive nuclear staining was found nearly twice as high in the carcinomatous (n=19; 63%), compared with the sarcomatous (n=11; 37%) component, respectively, and MCM3 expression in the epithelial component was related to clinical stage (P=0.030), and the existence of omental metastasis (P=0.012). In addition, out of the 30 UCs, 17 (57%) and 13 (43%) showed topoIIα positivity in the carcinomatous and sarcomatous UC components, respectively. A significant relationship between protein immunoreactivity and FIGO stage (P=0.049), and omental metastasis (P=0.026) was revealed to exist. However, no significant differences between expression of proliferation markers and clinicopathological features in the sarcomatous UC component were identified. Finally, a significant correlation between each protein immunohistochemical staining was demonstrated, particularly in the sarcomatous UC component. Collectively, a combined analysis of Ki67, PCNA, MCM3, and topoIIα may provide more detailed information of cell-cycle alterations determining the heterogeneity of uterine carcinosarcomas.

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Introduction

Carcinosarcomas are relatively uncommon but highly malignant tumors originated from female genital tract organs, including the uterus (1-4). Recently, uterine carcinosarcomas (UCs) were incorporated into the 'high-risk' endometrial cancer (EC) group by the European Society of Gynecological Oncology/European Society of Radiation Oncology/European Society for Pathology Consortium (5). They are composed of two different components, carcinomatous and sarcomatous, and both of them are malignant. Although controversies still exist over their origin, it is generally accepted that UCs are monoclonal, in general (6-9). Four theories of their histogenesis have been presented in numerous studies up to now, although the composition theory (the stromal component is not truly neoplastic, but acts as a reactive response to the existence of a malignant epithelial component) has been abandoned (8-10). A 'milestone' genetic/immunohistochemical (IHC) study by Wada *et al* (11) argued that although most UCs are combination tumors, some may develop as collision neoplasms as well. Recently, somatic DNA mutational analysis was undertaken, and gene expression and allelic imbalance of several genes were separately analyzed in the sarcomatous and carcinomatous components of 10 UC patients (12). The researchers reported that both components of UCs exhibited similar molecular profiling, suggesting that 'the carcinomatous and sarcomatous components may rise from a common precursor or perhaps one of the components rises from the other at a late stage' (12).

The 5-year overall survival of patients affected by UCs is poor, and is significantly decreased when the clinical stage of the disease increases (for example, the figures are nearly 50% for stage I, whereas they are below 10% for stage IV) (13,14). In general, patients affected by UCs are usually >50 years old, with the median age being 62 years-old (2,8,15). Risk factors for the development of UCs are similar to those of ECs: Nulliparity, advanced age of patients, obesity, exposure to estrogens and SERMs, as well as exposure to radiation therapy (16-18).

In the scientific literature, a selected number of reports have investigated the impact of proliferative activity on the development and progression of UCs (19-29). For example, Ki67 expression pattern was found to be higher in UCs, as compared with uterine adenosarcomas (P=0.03) (19). Furthermore, Lee *et al* (23) reported an elevated expression of topoI and Ki67 in 20 UCs, although no correlation between the two IHC proliferative markers existed (P=0.817). A previous study from the authors also showed a significant correlation of Ki67 immunoreactivity between two malignant components of UCs (R=0.676, P<0.001) (26). However, there are no studies investigating the relationship between proliferative markers immunoreactivity independently in both components of UCs.

The aim of the present study was to ascertain IHC proliferative markers [Ki67, proliferating cell nuclear antigen (PCNA), minichromosome maintenance complex component 3 (MCM3), and topoisomerase IIα (topoIIα)] expression in UCs, by analyzing immunostaining reactivity independently in the two malignant components. Moreover, the relationship of staining results to clinicopathological variables of the neoplasm was examined, and the correlation between proliferative markers was analyzed.

Materials and methods

Patients and tissue samples. A total of 30 paraffin-embedded slides of UCs were collected from patients who underwent radical surgery at the Second Department of Gynecology of Lublin Medical University (Lublin, Poland) between January 1, 2006, and December 31, 2020. The dilatation and curettage procedure was performed in all patients pre-operatively, and the diagnosis of UC was conducted. Although primarily 34 cases were included, there was not enough material to perform all IHC experiments in three cases, and, in another, the coexistence of two synchronous, independent neoplasms (UC and cervical adenocarcinoma) was surprisingly discovered during the reassessment of the slides for the experiments. Collectively, 26 endometrioid-type endometrial carcinomas and 4 non-endometrioid carcinomas (2 clear-cell carcinomas, 1 papillary-serous carcinoma and 1 undifferentiated carcinoma). Moreover, there were 21 homologous-type tumors (stromal sarcoma, n=14; leiomyosarcoma, n=4; and not otherwise specified, n=3), and 9 heterologous-type (rhabdomyosarcoma, n=5; chondrosarcoma, n=3; and osteosarcoma, 1) tumors (Table I). The mean age of patients was 67 years (from 42-84 years of age; median: 68 years). No chemotherapy, radiotherapy or hormonotherapy were applied before the surgery. Post-operative material was selected following pathological review at the Department of Clinical Pathology, Lublin Medical University, Lublin, Poland, by a highly-experienced pathologist (DL). Histopathological assessment was performed based on revised classification of World Health Organization (30), whereas clinical stage of the disease was classified according to the revised the International Federation of Gynecology and Obstetrics (FIGO) staging system for ECs (31). The present study was approved (approval no. 0254/144/2018) by the Independent Ethics Committee of the Lublin Medical University (Lublin, Poland). Signed informed consent was provided by all women prior to surgery, that they agreed to use of paraffin-embedded slides in future scientific research. Clinical and pathological characteristics of the study group are presented in Table I.

Table I. Clinicopathological features of 30 women affected by uterine carcinosarcomas.

Parameters	n (%)
Age, years	
<50	2 (7)
50-60	5 (17)
>60	23 (76)
Carcinomatous component	
Endometrioid	26 (87)
Non-endometrioid	4 (13)
Sarcomatous component	
Homologous	21 (70)
Heterologous	9 (30)
Myometrial invasion	
Yes	16 (53)
No	14 (47)
Lymphovascular space invasion	
Yes	18 (60)
No	12 (40)
Stage (FIGO)	
I	11 (37)
II	6 (20)
III	7 (23)
IV	6 (20)
Presence of tumor in the oviduct	
Yes	7 (23)
No	23 (77)
Grade	
G1	5 (17)
G2	7 (23)
G3	18 (60)
Metastasis	
Yes	12 (40)
No	18 (60)
Omental metastasis	
Yes	4 (13)
No	26 (87

Immunohistochemistry (IHC). Tissue material collected at the operation theatre was immediately fixed in 10% buffered formalin (pH 7.4) at room temperature overnight, and the paraffin blocks were prepared according to standard

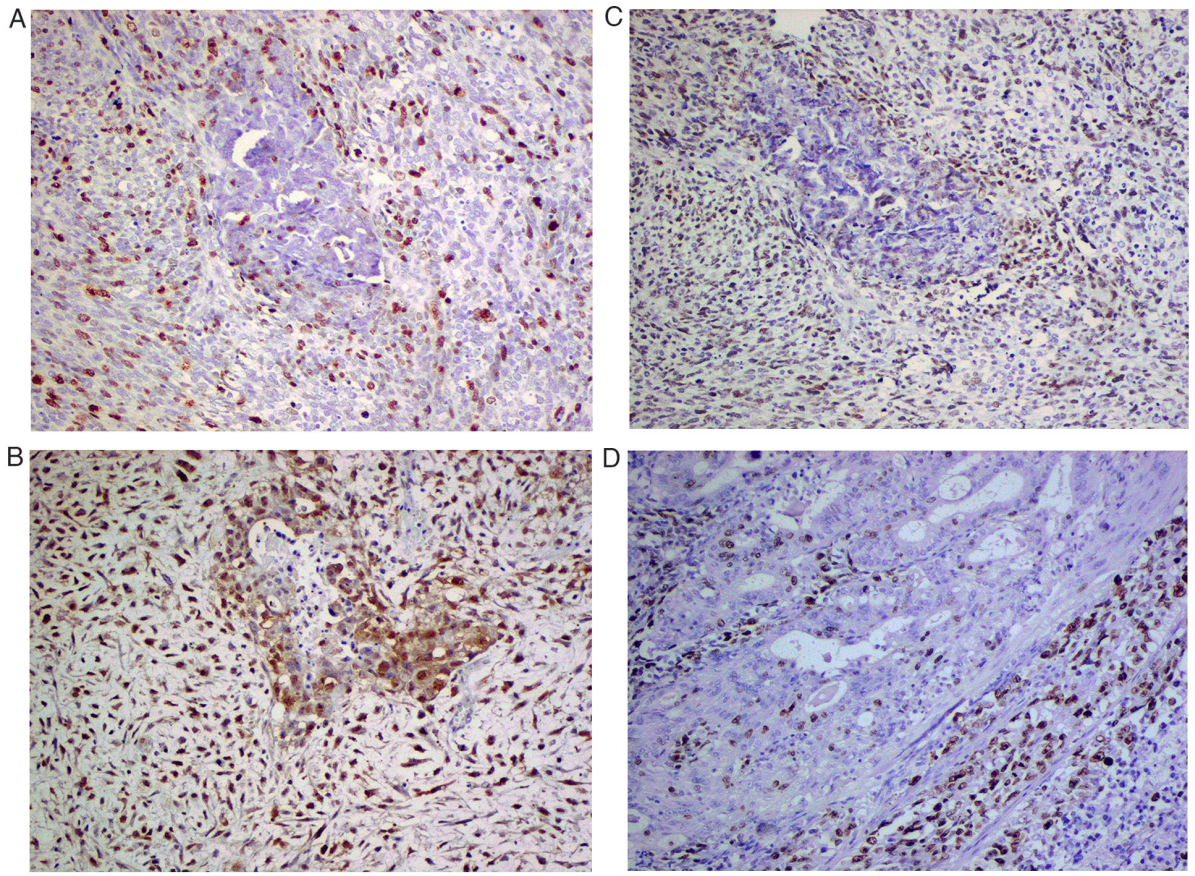

Figure 1. Immunohistochemical nuclear staining for (A) Ki67, (B) proliferating cell nuclear antigen, (C) MCM3 and (D) topoisomerase IIα in both components of UCs (magnification, x200).

laboratory technique. Paraffin blocks were cut on 3-μm slides, and put on silanized slides (Sigma-Aldrich; Merck KGaA). The IHC technique was performed using the DAKO REAL™ EnVision™/HRT kit (Dako; Agilent Technologies, Inc.) according to the manufacturer's protocol. DAB (3,3'-di-aminobenzidine tetrahydrochloride) was exploited as a chromogen. The following primary antibodies (Dako; Agilent Technologies, Inc.) were applied: Monoclonal mouse anti-human antibody against Ki67 (cat. no. M7240; clone MIB-1; 1:50); monoclonal mouse anti-human antibody against PCNA (cat. no. M0879; clone PC10; 1:1,000); monoclonal mouse anti-human antibody against MCM3 (cat. no. M7263; clone 101; 1:25); and monoclonal mouse anti-human antibody against topoIIα (cat. no. M7186; clone Ki-S1; 1:50). All antibodies were incubated for 30 min at room temperature. Afterwards, the detection system was employed, and the visualization was performed by 0.1% DAB solution for 5 min at room temperature. The sections were finally counterstained with Mayer's hematoxylin for 1 min at room temperature, dehydrated and cover-slipped after being embedded in mounting medium. The slides were stored at room temperature.

Immunohistochemical controls. Positive and negative controls were included in each experiment. Positive control was an EC showing enhanced staining for each antibody applied. Negative control was a section in which the primary antibody was replaced by Tris-buffered saline.

Immunohistochemical assessment. The representative areas (500 cells) were selected on a light microscope (Nikon Corporation) and counted by 2 independent researchers (DL and AS) who were aware of clinicopathological variables. A full agreement of nearly 90% was reported. However, when the consensus was not reached, both researchers cooperatively analyzed region by region until the full agreement was achieved. Nuclear Ki67 expression was considered positive if >30% of the tumor cells showed positive immunostaining as previously described (23). For nuclear PCNA, MCM3 and topoIIα immunoreactivity, the scores >90, >25, and >5% were deemed positive, respectively (32-34).

Statistical analyses. Statistical analyses were performed with Kołmogorow-Smirnow, Shapiro-Wilk, χ^2-Pearson's and Fisher's exact tests. Spearman's rank correlation coefficient was applied to determine correlations between proteins and patients' age. Statistical analysis was carried out using Statistica 9.0 software (StatSoft, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of Ki67 in both components of UC. Ki67-positive nuclear staining was observed in 20 (67%) and 16 (53%) of the UC carcinomatous and sarcomatous components, respectively (Fig. 1A). In the epithelial component, Ki67-positive reactivity was related to FIGO stage (P=0.025), and histological grade

Table II. Expression of Ki67 in relation to clinicopathological features within the carcinomatous component of uterine carcinosarcomas.

Parameter	n	Expression level of Ki67		P-value
		Yes, n (%)	No, n (%)	
Age, years				0.385[a]
<50	2	2 (100)	0	
50-60	5	3 (60)	2 (40)	
>60	23	14 (61)	9 (39)	
Carcinomatous component				1.00[b]
Endometrioid	26	9 (35)	17 (65)	
Non-endometrioid	4	1 (25)	3 (75)	
Myometrial invasion				0.122[b]
Yes	16	3 (19)	13 (81)	
No	14	7 (50)	7 (50)	
Lymphovascular space invasion				0.139[b]
Yes	18	4 (22)	14 (78)	
No	12	6 (50)	6 (50)	
Stage (FIGO)				0.025[a]
I	11	6 (55)	5 (45)	
II	6	1(17)	5 (83)	
III	7	0	7 (100)	
IV	6	3 (50)	3 (50)	
Presence of tumor in the oviduct				0.657[b]
Yes	7	3 (43)	4 (57)	
No	23	7 (30)	16 (70)	
Grade				0.057[a]
G1	5	4 (80)	1 (20)	
G2	7	2 (29)	5 (71)	
G3	18	4 (22)	14 (78)	
Metastasis				0.694[b]
Yes	12	3 (25)	9 (75)	
No	18	7 (39)	11 (61)	
Omental metastasis				0.095[b]
Yes	4	3 (75)	1 (25)	
No	26	7 (27)	19 (73)	

[a]X^2-Pearson's test; [b]Fisher's exact test.

(G1 vs. G2/G3; P=0.031) (Table II). However, only a trend was reported when histological grading was analyzed separately (G1 vs. G2 vs. G3; P=0.057). In the sarcomatous component, no significant relationship between Ki67 expression and clinicopathological variables was identified.

Expression of PCNA in both components of UC. Nuclear PCNA reactivity was detected in 18 (60%) and 16 (53%) of the UC carcinomatous and sarcomatous components, respectively (Fig. 1B). Notably, all four cases with omental metastases were PCNA-positive, and a relationship between staining pattern and the existence of metastases was of significant value (P=0.018; Table SI). None of the clinicopathological variables in the mesenchymal component were related to PCNA staining.

Expression of MCM3 in both components of UC. MCM3 expression was found nearly twice as high in the carcinomatous (n=19, 63%), as the sarcomatous (n=11, 37%) UC component, respectively. Only nuclear MCM3 reactivity was considered positive (Fig. 1C). MCM3 staining in the epithelial component was related to clinical stage (P=0.03), and to the existence of omental metastasis (P=0.012) (Table SII). None of the clinicopathologic features showed significant relationship with MCM3 staining in the sarcomatous UC component.

Expression of topoIIα in both components of UC. Out of the 30 cases, 17 (57%) and 13 (43%) showed nuclear positivity in the carcinomatous and sarcomatous component, respectively (Fig. 1D). A significant relationship between

Table III. Correlational analysis of different proliferative markers in the carcinomatous component of uterine carcinosarcomas (Spearman rank correlation test).

	Ki67	PCNA	MCM3	topoIIα
Ki67		R=0.2886 P=0.1218	R=0.3423 P=0.064	R=0.6659 P=0.000059
PCNA	R=0.2886 P=0.1218		R=0.3671 P=0.04597	R=0.3844 P=0.0359
MCM3	R=0.3423 P=0.064	R=0.3671 P=0.04597		R=0.3403 P=0.06577
topoIIα	R=0.6659 P=0.000059	R=0.3844 P=0.0359	R=0.3403 P=0.06577	

PCNA, proliferating cell nuclear antigen; topoIIα, topoisomerase IIα.

Table IV. Correlational analysis of different proliferative markers in the sarcomatous component of uterine carcinosarcomas (Spearman rank correlation test).

	Ki67	PCNA	MCM3	topoIIα
Ki67		R=0.7321 P=0.000004	R=0.4193 P=0.02108	R=0.5483 P=0.0017
PCNA	R=0.7321 P=0.000004		R=0.4193 P=0.0211	R=0.5483 P=0.0017
MCM3	R=0.4193 P=0.02108	R=0.4193 P=0.0211		R=0.3429 P=0.0635
topoIIα	R=0.5483 P=0.0017	R=0.5483 P=0.0017	R=0.3429 P=0.0635	

PCNA, proliferating cell nuclear antigen; topoIIα, topoisomerase IIα.

protein immunoreactivity with clinical stage (P=0.049), and omental metastasis (P=0.026) was found to exist (Table SIII). Moreover, there was a trend towards increasing topoIIα expression pattern with the advancement of myometrial invasion (P=0.063). No significant differences between topoIIα expression and clinicopathological features in the sarcomatous UC component were indicated.

Correlation between protein expression patterns in different components of UC. Statistical analyses of the correlation between selected IHC markers in different UC components are presented in Tables III and IV. For example, in the cancerous UC component, Ki67 staining was correlated with expression of MCM3 and topoIIα, and PCNA with MCM3 and topoIIα (Table III). Finally, it is worth pointing out that proliferative markers revealed a significant correlation with each other in the sarcomatous component of UC (Table IV). There was no correlation between protein reactivity and patient age (P>0.05; Spearman rank correlation test; data not shown).

Discussion

Assessment of cell proliferation activity by antigen retrieval techniques, using formalin-fixed and paraffin-embedded slides, has been extensively applied as prognosticator in various gynecologic malignancies (35-37). In certain circumstances, IHC cell proliferation assessment has also been incorporated into routine clinical practice (35,38). For example, Mittal *et al* (35) recommended the application of a panel of antibodies, including cytokeratin, desmin, S100 and myoD1, for distinguishing between undifferentiated EC, undifferentiated uterine sarcoma and UC. However, quantification of epithelial cell proliferation in biphasic female genital tract neoplasms is a scientific challenge due to the lack of reported investigations. Based on the fact that their histogenesis remains a matter of controversy, the assessment of cell proliferation is an innovative and interesting task.

The present study is an extension of our broad scientific interest in investigating the impact of various IHC/genetic alterations on the development and progression of UCs and their corresponding metastases (26,39-43). In the present study, an enhanced proliferative expression pattern of all markers was demonstrated in the carcinomatous component of UC, as compared with the sarcomatous one. Moreover, IHC markers immunostaining (Ki67, MCM3 and topoIIα) were found to be related to clinical stage [the most important prognostic factor of patients with UC (8)], and to the development of omental metastasis, but only in the carcinomatous component.

By contrast, no proliferative markers expression pattern was demonstrated to be related to clinico-prognostical features in the sarcomatous component. Based on the present observations, there are two possible explanations of this phenomenon.

Firstly, the carcinomatous UC component may be more important in the malignant transformation of UCs, as previously described (21,44,45). The present data is also in line with that of Ikeda *et al* (22), who reported significantly higher Ki67 in the carcinomatous, compared with the sarcomatous component (P=0.0173). Similar observations have been presented previously by Nicotina *et al* (20), who suggested that mitotic index and MIB-1 labeling index may be used as complementary indices to assess the outcome of UCs. It is thus of interest to cite the study by Yoshida *et al* (21), who reported that carcinomatous component 'may play an important role in aggressive biologic behavior in UCs'.

Upon investigating molecular markers and clinicopathological features of UCs, de Jong *et al* (45) also put forward that the carcinomatous component is 'truly' a major factor determining the prognostic impact of UC patients, causing the majority of metastases, as well as vascular infiltration. By contrast, however, there are also data reporting no significant difference of Ki67 immunostaining between carcinomatous and sarcomatous UC components (46).

Secondly, the present observations indicated via indirect evidence that the carcinomatous component may be a 'driving force' in potential tumor spread, being responsible for the development of distant (omental) metastases. The most common sites of UC metastasis are the lung (49%), peritoneum (44%), pelvic/paraoartic lymph nodes (35%), adrenal gland or bone (19%), heart or pericardium (9%) and/or brain (7%) (8). Notably, most of these metastases are clinically asymptomatic and generally spread throughout the lymphatic system. Although only ten cases were investigated by Yoshida *et al* (21), regional lymph node metastases (3 out of 10) were found to be only fold ups of the carcinomatous component. These data are in line with the 'core' study by Sreenan and Hart (47), which demonstrated that most metastatic UCs consist primarily of a pure carcinomatous component, although carcinomatous/sarcomatous, and a pure sarcomatous component were also incidentally detected.

In the present study, although only four UC (all poorly-differentiated disseminated neoplasms) cases showed omental metastasis, all of them revealed proliferative markers (PCNA, MCM3 and topoIIα) positive immunostaining in the carcinomatous component. It is worth to mention that investigations of UC omental metastasis are of utmost uncommon based on pertinent literature review. However, to make a final conclusion, a cohort of primary UC tumors corresponding with metastases should be simultaneously assessed using IHC in an international cooperative research study.

In the literature, there is only one study reporting the simultaneous correlational analysis of different proliferative markers in both UC components. While no significant difference between Ki67 expression pattern and elevated topoI (not topoIIα) staining was presented in 20 UCs by Korean researchers (23), on the contrary, however, significant correlation between each different proliferative marker in UCs has been currently documented, particularly in the sarcomatous component. It was assumed that certain of the proliferative markers (Ki67 and PCNA) may be potentially applied to assess the proliferative potential in selected histological subtypes of uterine sarcomas. According to the opinion of the authors, positive correlation between proliferative markers may be explain by the enhanced antigen activity in selected phases of the cell-cycle (48). Limited number of cases investigated (although one of the largest series reported so far) may be also responsible for the lack of correlation in different IHC UC components, particularly in the carcinomatous one. Finally, the primary size UC assessment was not performed due to the retrospective nature of the present study.

In conclusion, the assessment of proliferative activity may be associated with tumor aggressiveness during the process of development and widespread of UCs. A combined analysis of Ki67, PCNA, MCM3 and topoIIα may, therefore, provide detailed data of cell-cycle regulation mechanisms determining the inter-component heterogeneity of UCs. Finally, pertinent literature review, being in preparation from the authors, may support the role of selected proliferative markers assessment in differential diagnosis of uterine carcinoma, uterine sarcoma and UC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AP, DL, BB and MC conceived and designed the study. AAG, ASS and AS analyzed the data and wrote the manuscript. AS supervized the final preparation and submission of the manuscript. All authors read and approved the final version of the manuscript. AP and AS confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved (approval no. 0254/144/2018) by the Independent Ethics Committee of the Lublin Medical University (Lublin, Poland). Signed informed consent was provided by all patients prior to surgery, who agreed to the use of paraffin-embedded slides in future scientific research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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