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ORIGINAL RESEARCH

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Cell cycle regulatory markers as potential prognostic biomarkers in uterine carcinosarcoma

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Abstract

The relevance of cell cycle regulatory markers with uterine carcinosarcoma was investigated. The immunohistochemical expression of p16, p53, and cyclin D1 were assessed using tissue microarray of 55 eligible patients. p16 and p53 showed a high rate of strong (+3) immune reaction in carcinomatous/sarcomatous components (61.8%/70.9% and 52.7%/56.4%, respectively). Cyclin D1 showed a 14.5%/7.3% of strong immune reaction in the carcinomatous/sarcomatous components. Strong expression of p16 was related to a higher rate of recurrence, lymph node metastasis, and bigger tumor size. Strong expression of cyclin D1 was related to the lower International Federation of Gynecology and Obstetrics (FIGO) stage and recurrence rate. In univariate regression analysis, FIGO stage, lymph node metastasis, p16, and cyclin D1 were prognostic factors for disease-free survival. FIGO stage, p16, p53, and cyclin D1 were prognostic factors for disease specific survival. In a multivariate regression analysis, FIGO stage and p16 in carcinomatous component were independent factors for disease-free survival (odds ratio (OR), 95% confidence interval (CI); 3.2 (1.1–9.6) and 3.5 (1.3–9.7); $p = 0.035$ and 0.017). p16 was a predictor of lymph node metastasis, tumor size, and prognostic outcome in uterine carcinosarcoma.

Keywords

Uterine neoplasm; Carcinosarcoma; p16; Cell cycle; Immunohistochemistry

1. Introduction

Uterine cancer is one of the most common gynecologic malignancies worldwide [1]. Also, its incidence has more than doubled in the recent 10 years in Korea [2, 3]. Uterine carcinosarcoma, also known as "malignant mixed Mullerian tumor" is a rare and unique malignancy that harbors both carcinomatous and sarcomatous components [4]. Initially, it was categorized as one of the uterine sarcomas with leiomyosarcoma, endometrial stromal sarcoma, and undifferentiated sarcoma, but was later reassigned to the metaplastic form of type II endometrial cancer [5].

The majority of the patients with uterine carcinosarcoma experience recurrence within 1 year and overall 5-year survival is less than 30% despite aggressive adjuvant treatment [4]. Therefore, the incorporation of targeted agents into traditional management to improve prognosis is needed like other gynecologic malignancies. But little is known about this rare disease, and understanding the characteristics of the tumor and molecular profile in more variable aspects is a prerequisite [1, 4].

One of the most important processes of investigating the nature of human malignancy is understanding the cell cycle because almost every case of tumor mechanism is controlled under the action of cyclin D kinase (CDK) and its inhibitor (CDKI) [6]. There are few studies about its relevance to the prognosis and characteristics of uterine carcinosarcoma even though numerous studies report frequent overexpression, chromosomal instability, and molecular alteration of cell cycle-related proteins such as p53, phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), and KRAS proto-oncogene, GTPase (KRAS) [7]. Knowing the significance of certain biomarkers in tumors with their disease characteristics and prognostic impact will not only help in planning reasonable treatment but also will aid in identifying an adequate cohort for prospective clinical trials which is difficult due to its rarity.

Abnormal expression and function of p16 (cyclin inhibitor) and its associated proteins including D-type cyclin is a common feature in every human cancer [8, 9]. Aberration of p16 and cyclin D1 is associated with a common pathway of tumorigenesis [8, 9]. These findings are also observable in sarcoma and gene amplification with increased CDK complex activity [8]. Therefore, we investigated the relevance of p53, p16, and cyclin D1 with disease characteristics and survival outcomes of uterine carcinosarcoma.

2. Materials and methods

2.1 Patients

Fifty-five patients who were diagnosed with uterine carcinosarcoma and treated at the Asan Medical Center (AMC) from January 2001 to October 2014 were found. Clinicopathological data were analyzed by reviewing electronic medical records and tissue microarray (TMA) was manufactured by using paraffin blocks of each patient.

2.2 Tissue microarray (TMA)

Hematoxylin & Eosin (H&E) slides from the paraffin blocks were reviewed by a gynecologic oncology subspecialty pathologist (YSP) who was blinded to the clinical data of the patients. Blocks were built with tumor tissues obtained at the time of surgery for routine pathologic diagnosis. The most tumor-dense part was obtained by a 2 mm needle puncture and was sent to the Bio-Resource Center (BRC) in AMC for TMA construction. Antibodies were immunohistochemically stained to formalin-fixed paraffin-embedded tissue sections by using a BenchMark ULTRA automatic immunostaining device (Ventana Medical Systems, Tucson, Arizona (AZ), United States of America (USA)) with an OptiView DAB IHC Detection Kit (Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's manual. By using a microtome, 4-micrometer-thick sections were obtained. These sections were moved to silanized charged slides and were dried at room temperature for 10 min, followed by 65 °C incubators for 20 min. Heat-induced epitope retrieval (HIER) method using Cell Conditioning 1 buffer for 32 min and incubation for 16 min was performed on sections with anti-p16 (mouse monoclonal, clone E6H4, 1:6, cat.805-7413, VENTANA, TUSAN, AZ, USA), anti-p53 (mouse monoclonal, clone DO-7, 1:1500, cat.M7001, DAKO, GLOSTRUP, DENMARK), and anti-CyclinD1 (mouse monoclonal, clone SP4, 1:100, cat.241R-15, CELL MARQUE, Rocklin, CA, USA) in the auto-immunostainer. Ventana OptiView DAB IHC Detection Kit (OptiView HQ Linker 8 min, OptiView HRP Multimer 8 min, OptiView H_2O_2/DAB 8 min, and OptiView Copper 4 min) was used for visualization of antigen-antibody reactions. Ventana Hematoxylin II and bluing reagent for 32 min and 4 min were used for performing counterstaining. For further interpretation, all slides were removed from the stainer, dehydrated, and coverslipped. Negative controls for each antibody were produced by omitting the primary antibodies. Positive control for p16 was produced by using cervical carcinoma, pancreas, and tonsil tissue samples. p53 was produced by using lung, breast, ovary, prostate, and colon carcinoma tissue samples. Cyclin D1 was produced using tonsil, placenta, brain, cervix, mantle cell lymphoma, and breast carcinoma tissue samples. The immune reaction of p16 and cyclin D1 was evaluated by observing the expression pattern of the nucleus. The immune reaction of p53 was assessed by analyzing both the nucleus and cytoplasm. A semi-quantitative scoring system was adopted for the assessment of the intensity of the immune reaction in our study. The score was categorized as 0 (0–20 points), 1+ (21–80 points), 2+ (81–180 points), and 3+ (181–300 points) by multiplying stain intensity and the percentage of the total stained area (Fig. 1) [10].

2.3 Statistics

Median and mean variables were analyzed by using Student's *t*-test and Mann-Whitney U test, respectively. Statistical differences between frequencies of a pattern of recurrence, lymph node metastasis, tumor size, myometrial invasion, and International Federation of Gynecology and Obstetrics (FIGO) stage with p16, p53, and cyclin D1 were analyzed by using Chi-square- and Fisher's exact test. Disease-free survival and disease specific survival were defined as from the date of surgery to the date of tumor recurrence or death from any cause, or last follow-up, and the date of surgery to the date of death due to cancer or last follow-up, respectively. To assess the statistical difference in survival outcome, the Kaplan-Meier method with a log-rank test and Cox's proportional regression analysis were used. Statistical analyses were performed by using Statistical Package for the Social Sciences (SPSS) software version 22.0 (International Business Machines (IBM) Corp., Armonk, NY, USA). $p < 0.05$ was defined as a cut-off value for statistical significance.

3. Results

3.1 Patient characteristics

Approximately, half of the patients had a deep myometrial invasion of the tumor. Twenty-two (40.0%), 5 (9.1%), 20 (36.4%), and 8 (14.5%) patients were FIGO stage I, II, III, and IV, respectively. Lymph node dissection was performed in most of the patients (92.7%), and 18 patients (35.3%) had lymph node metastasis. In total, 46 (83.6%) patients underwent adjuvant treatment and 36 (65.5%) received chemotherapy (ifosfamide + platinum = 17, taxane + platinum = 9, taxane + ifosfamide = 6, vincristine + ifosfamide + cisplatin = 2, taxane + doxorubicin = 1, and ifosfamide + doxorubicin = 1, respectively). There were 30 cases (54.5%) of recurrence and 28 cases (50.9%) of death (Table 1).

3.2 Expression of cell cycle regulatory markers

The immune reaction of p53, p16, and cyclin D1 was observed in 35 (63.6%)/39 (70.9%), 48 (87.3%)/49 (89.1%), and 34 (61.8%)/25 (45.5%) of 55 patients in carcinomatous/sarcomatous component of uterine carcinosarcoma, respectively (Table 2). Of these patients, strong semi-quantitative score immune-reaction (3+) to p53, p16, and cyclin D1 was seen in 29 (52.7%)/31 (56.4%), 38 (61.8%)/39 (70.9%), and 8 (14.5%)/4 (7.3%), respectively.

3.3 Survival outcome

The median follow-up period was 18.7 months (0.33–181.9 months). Immune-reaction of each biomarker was categorized as 0, 1+, 2+ *vs.* 3+ to analyze its relevance with survival outcome according to the semi-quantitative scoring system. In univariate analysis, FIGO stage, lymph node metastasis, strong semi-quantitative score immune-reaction (3+) of p16, and cyclin D1 in the carcinomatous component of cancer were statistically significant factors for disease-free survival. FIGO stage and strong semi-quantitative score immune-reaction (3+)

TABLE 1. Clinicopathological characteristics of patients with uterine carcinosarcoma (n = 55).

Variable	No (%)
Age	
Median (range)	60.0 (39.0–77.0)
Parity	
0	3 (5.5)
1	3 (5.5)
≥2	49 (89.0)
BMI (kg/m^2)	
Median (range)	24.01 (17.14–33.42)
Menopause	51 (92.7)
Myometrial invasion	
<1/2	22 (40.0)
≥1/2	28 (50.9)
Not available	5 (9.1)
FIGO stage	
I	22 (40.0)
II	5 (9.1)
III	20 (36.4)
IV	8 (14.5)
Lymphadenectomy	51 (92.7)
Lymph-node metastasis	
Positive	18 (35.3)
Negative	33 (64.7)
Adjuvant treatment	
None	9 (16.4)
Chemotherapy	36 (65.5)
Radiation	2 (3.6)
Both	8 (14.5)
Recur	30 (54.5)
Pattern of recurrence	
Loco-regional	6 (10.9)
Distant	19 (34.5)
Both	5 (9.1)
Death	28 (50.9)

BMI, body mass index; BSO, bilateral salpingo-oophorectomy; FIGO, international federation of gynecology and obstetrics.

TABLE 2. Expression of each biomarker categorized by the semiquantitative scoring system in patients with uterine carcinosarcoma (n = 55).

		Positive		
Biomarkers	Negative, No (%)	Weak, No (%)	Moderate, No (%)	Strong, No (%)
	Carcinoma/Sarcoma	Carcinoma/Sarcoma	Carcinoma/Sarcoma	Carcinoma/Sarcoma
p53	20 (36.4)/16 (29.1)	3 (5.5)/5 (9.1)	3 (5.5)/3 (5.5)	29 (52.7)/31 (56.4)
p16	7 (12.7)/6 (10.9)	10 (18.2)/4 (7.3)	4 (7.3)/6 (10.9)	34 (61.8)/39 (70.9)
Cyclin D1	21 (38.2)/30 (54.5)	20 (36.4)/13 (23.6)	6 (10.9)/8 (14.5)	8 (14.5)/4 (7.3)

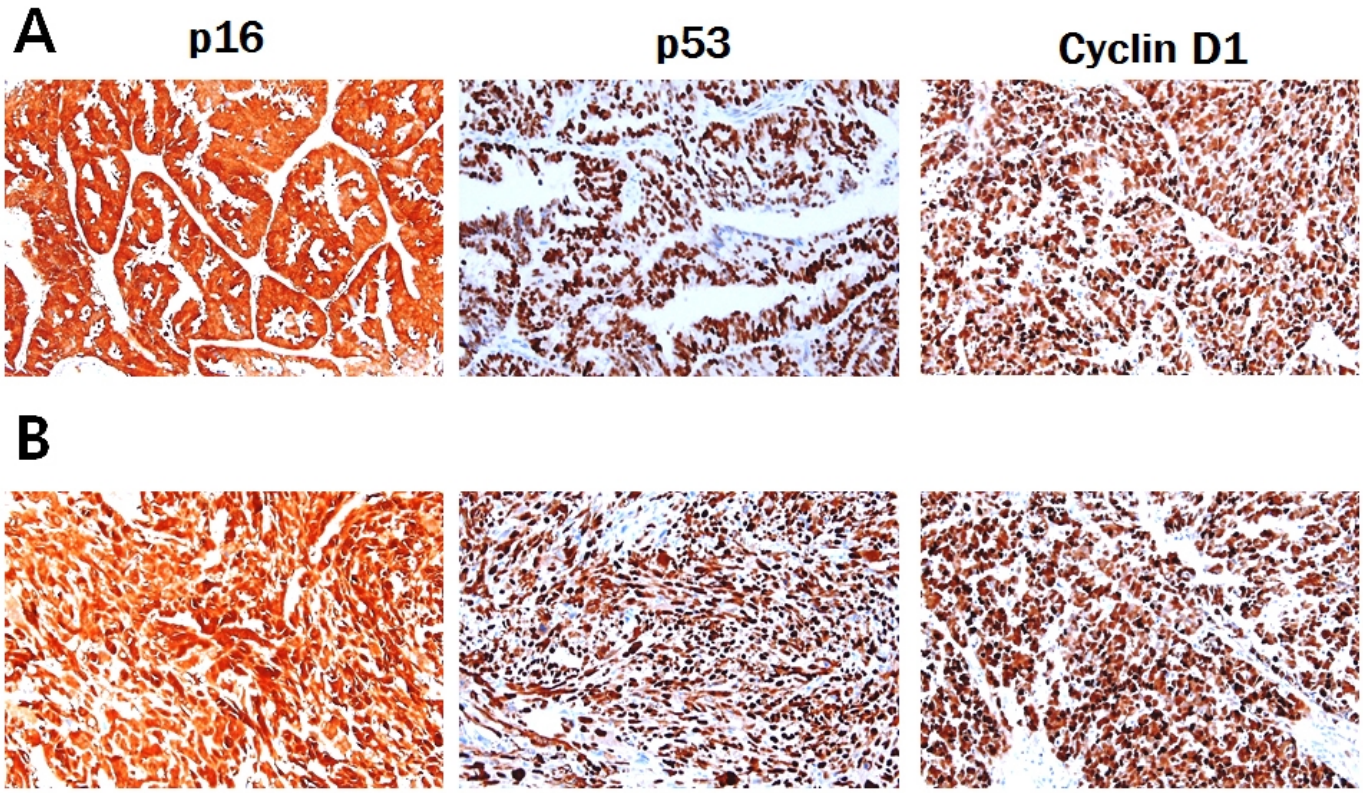

FIGURE 1. Immune-expression of biomarkers. (A) Strong immune-expression (3+) of p16, p53, and cyclin D1 in carcinomatous, (B) and sarcomatous components of uterine carcinosarcoma. Images were provided by the Department of Pathology, Asan Medical Center (magnification ×200).

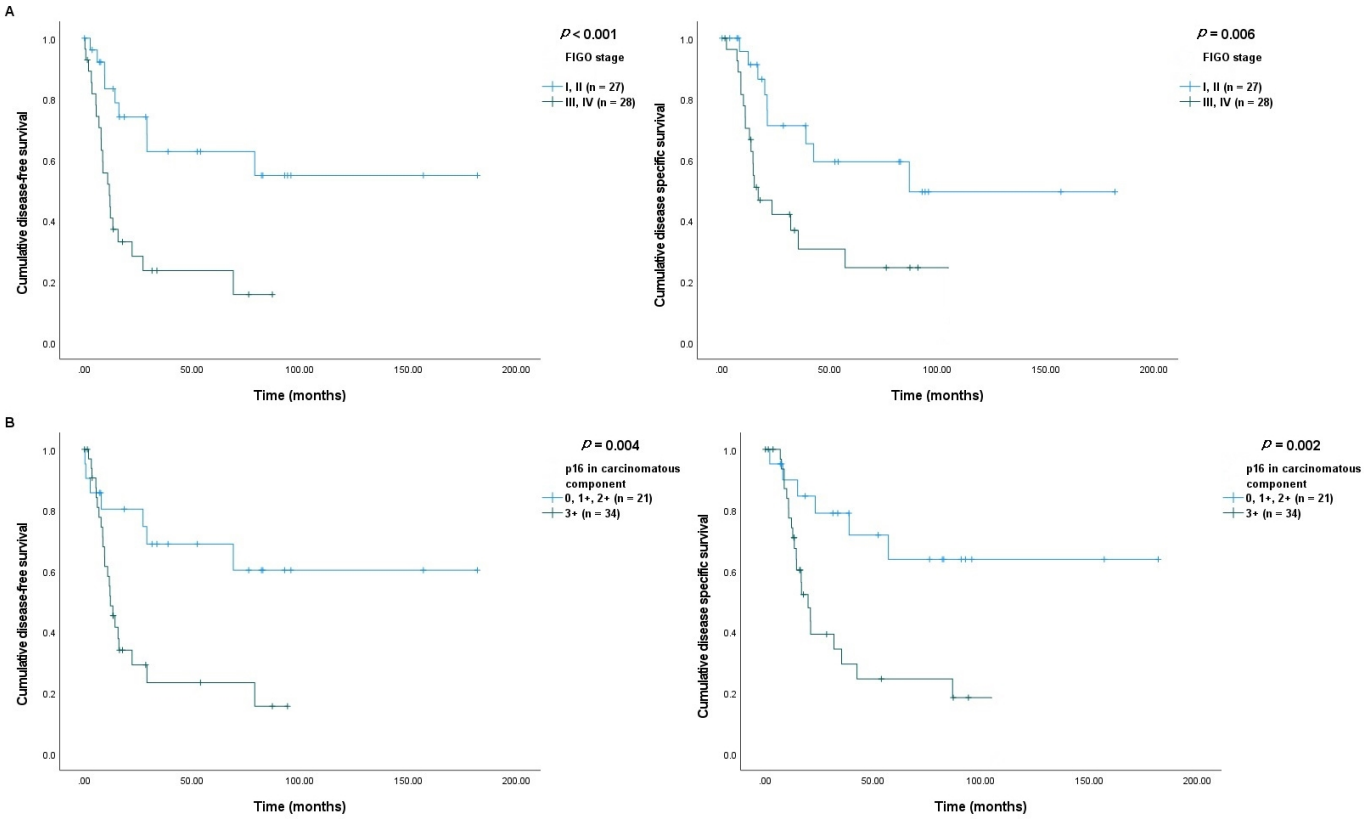

FIGURE 2. Disease-free and disease specific survival curves. (A) Disease-free and disease specific survival based on FIGO stage and (B) p16 in uterine carcinosarcoma. FIGO, international federation of gynecology and obstetrics.

of p53 (carcinomatous/sarcomatous component), p16 (carcinomatous/sarcomatous component), and cyclin D1 (carcinomatous component) of cancer were statistically significant factors for disease specific survival. Variables that were statistically significant in univariate analysis were put into multivariate analysis. In a multivariate regression analysis, FIGO stage and p16 in carcinomatous component were independent factors for disease-free survival (odds ratio (OR), 95% confidence interval (CI); 3.2 (1.1–9.6) and 3.5 (1.3–9.7); p = 0.035 and 0.017). (Fig. 2 and Table 3).

3.4 Association of expression of each biomarker with clinic-pathologic characteristics

Strong expression of p16 according to the semi-quantitative scoring system in carcinomatous components showed a significantly higher rate of recurrence and lymph node metastasis. Also, strong expression of p16 according to the semi-quantitative scoring system in both carcinomatous and sarcomatous components was associated with larger tumor size. Strong expression according to the semi-quantitative scoring system of cyclin D1 in carcinomatous components was associated with lower FIGO stage and recurrence rate (**Supplementary Table 2**).

4. Discussion

Recent categorization of uterine carcinosarcoma as dedifferentiated endometrial cancer rather than biologically sarcoma enabled more focused clinical trials and established adequate management [1]. But, still, no consensus on treatment and guidelines exists, and there is no improvement in survival outcomes for several decades [11]. Like previous reports, tumor extension beyond the pelvis according to the FIGO stage was an independent factor for survival outcomes in our series. But little is known about the biological background of uterine carcinosarcoma, and an additional prognosticator based on biomarkers is needed in addition to the traditional staging system to identify a suitable group of patients for further clinical trials and to properly incorporate the right targeted agents in near future. The rarity of this tumor is an obstacle not only to knowing the mechanism of tumorigenesis, prognosticator, and disease characteristics but also to performing prospective clinical trials that include proper targeted therapy. Considering the special situations in uterine carcinosarcoma, immunohistochemical analysis can be an efficient method to analyze the expression of biomarkers and their relevance to the disease in relatively small patient numbers with less expense and time.

In our series, a high frequency of p16 expression was observed and strong expression of p16 at the carcinomatous component was an independent prognostic factor for disease-free survival with the FIGO stage. Also, a higher rate of recurrence, lymph node metastasis, and larger tumor size was observed in cases showing strong expression of p16 in both carcinomatous and sarcomatous components which was a strong predictive factor for disease extent. In a previous study, the majority of the metastatic tumors and tumors spread beyond the uterus displayed carcinomatous components and were related to more aggressive disease characteristics. We think this may explain the result of our series [4]. p53 was not an independent prognosticator, but a strong immune reaction was related to lower overall survival in univariate analysis. In contrast to p16 and p53, cyclin D1 was associated with better survival outcomes and earlier FIGO stages.

p16 is a tumor suppressor protein (CDKI) that is related to the retinoblastoma (Rb) gene-mediated pathway by combining with the CDK4/6-cyclin D complex and negatively regulating the G1-S cell cycle [12]. Almost all Rb/E2F transcription cell cycle regulatory pathways are disrupted in human malignancies and are a universal target for the incorporation and investigation of anticancer drugs [13]. Rb phosphorylation state which has tumor suppressor activity is regulated by CDK. Therefore, loss or mutation of p16 leading to overexpression of CDK promotes constitutive Rb phosphorylation and tumor growth [14]. p16 is significantly overexpressed in uterine leiomyosarcoma and undifferentiated endometrial sarcoma compared to indolent endometrial stromal sarcoma and benign leiomyoma with p53 [15], and chromosomal instability with these markers is assumed as pathogenesis of tumor [16]. Moreover, strong and diffuse p16 expression can be a predictive factor for recurrence [17]. Recently, one study reported the expression of p16 in both components of uterine carcinosarcoma [18], but no studies have looked into its prognostic value and association with clinicopathologic character in uterine carcinosarcoma although it shares the same histologic component with other sarcomas. To our knowledge, this is the first study to date.

p53 is a tumor suppressor protein and promotes apoptosis. Mutation of p53 makes its cells challenging to stay at the G1 cell cycle for gene repair and is thought as one of the causes of tumorigenesis by accelerating the cell cycle [19]. The half-life of mutated p53 is longer than wild-type p53 which makes it easier to detect by immunohistochemical staining and is related to more aggressive clinical behavior and lower survival [19]. Numerous human malignant tumors occur because of overexpression of p53 which is related to tumor protein (TP) 53 alteration. Like other malignancies, studies show that p53 expression is also observable in both components of uterine carcinosarcoma during the early stage of tumorigenesis. In our study, more than half of the carcinomatous and sarcomatous components showed p53 expression which was similar to previous findings [20, 21].

A negative p53 immunostain with positive internal control can also be observed in cases with mutated p53, also known as null mutant [22]. Hence, it is possible that some of the negative p53 cases might carry mutated p53. Therefore, completely negative and the 3+ cases were additionally analyzed to obtain a more comprehensive understanding of p53 expression patterns. There was no difference in survival outcome and clinicopathologic characteristics between the two groups (**Supplementary Table 1** and **Supplementary Fig. 1**).

TABLE 3. Differences in survival outcome by treatment type and expression of biomarkers in patients with uterine carcinosarcoma (n = 55).

	Cox's proportional regression analysis							
Variables	Univariate		Multivariate		Univariate		Multivariate	
	Disease-free survival (%)	*p*	OR	*p*	Disease specific survival (%)	*p*	OR	*p*
Clinicopathological characteristics								
Age (<60 yr *vs.* ≥60 yr)	44.0 *vs.* 46.7	0.766			44.0 *vs.* 53.3	0.955		
Parity (≤2 *vs.* >2)	52.0 *vs.* 40.0	0.608			56.0 *vs.* 43.3	0.512		
Menopause (No *vs.* Yes)	25.0 *vs.* 47.1	0.678			25.0 *vs.* 51.0	0.976		
BMI (kg/m^2) (<24 *vs.* ≥24)	48.1 *vs.* 42.9	0.420			51.9 *vs.* 46.4	0.461		
Myometrial invasion (<1/2 *vs.* ≥1/2)	59.1 *vs.* 42.9	0.090			63.6 *vs.* 46.4	0.156		
FIGO stage (I, II *vs.* III, IV)	66.7 *vs.* 25.0	0.001	3.2 (1.10–9.60)	0.035	66.7 *vs.* 32.1	0.006	2.2 (1.00–5.20)	0.060
Lymphadenectomy (No *vs.* Yes)	50.0 *vs.* 45.1	0.460			50.0 *vs.* 49.0	0.457		
Lymph node metastasis (No *vs.* Yes)	54.5 *vs.* 27.8	0.014	0.8 (0.30–2.20)	0.679	54.5 *vs.* 38.9	0.082		
Adjuvant therapy	77.8 *vs.* 39.1	0.252			88.9 *vs.* 41.3	0.153		
No	77.8	0.710			88.9	0.548		
Chemotherapy	41.7				44.4			
Radiation	50.0				50.0			
Both	25.0				25.0			
Biomarkers								
p53 (0, 1+, 2+ *vs.* 3+)								
Carcinomatous component	57.7 *vs.* 34.5	0.052			61.5 *vs.* 37.9	0.033	0.4 (0.04–3.70)	0.407
Sarcomatous component	58.3 *vs.* 35.5	0.057			62.5 *vs.* 38.7	0.031	3.6 (0.40–34.30)	0.264
p16 (0, 1+, 2+ *vs.* 3+)								
Carcinomatous component	66.7 *vs.* 32.4	0.004	3.5 (1.30–9.70)	0.017	71.4 *vs.* 35.3	0.002	3.6 (0.60–20.60)	0.158
Sarcomatous component	62.5 *vs.* 38.5	0.080			68.8 *vs.* 41.0	0.040	0.7 (0.10–4.70)	0.734
Cyclin D1 (0, 1+, 2+ *vs.* 3+)								
Carcinomatous component	38.3 *vs.* 87.5	0.048	0.6 (0.10–5.30)	0.658	42.6 *vs.* 87.5	0.042	0.4 (0.04–4.40)	0.464
Sarcomatous component	43.1 *vs.* 75.0	0.238			47.1 *vs.* 75.0	0.304		

BMI, body mass index; FIGO, International Federation of Gynecology and Obstetrics; OR, odds ratio.

The association between the overexpression of cyclin D1 and the prognostic outcome of human malignancy is unclear and controversy still remains. Its expression was linked to worse survival outcomes in colorectal, B-lymphocyte, hepatocellular, esophageal, and cervical cancer [23]. Also, laryngeal and head & neck cancer showed more loco-regional spread and lymph node metastasis when there was cyclin D1 expression [24]. In contrast, the cyclin D1 mRNA expression group showed better survival outcomes in breast cancer [8, 25]. Cyclin D1 was more commonly observed in the proliferative phase or hyperplasia of endometrium than in the secretory phase, and it seems to stimulate the growth of endometrial glands and thereby be involved in the carcinogenesis of this region [23]. There is a controversy about cyclin D1 in endometrial cancer because some studies showed its relevance with tumor grade and stage while others found no association with clinical variables [23].

In our series, cyclin D1 showed no association with survival outcomes in uterine carcinosarcoma, but its expression was related to an earlier stage. Its finding was in concordance with breast cancer that expression of cyclin D1 is associated with a favorable outcome. The reason for different results and showing paradoxical behavior of cyclin D1 is unknown. It is thought that it mainly has a negative function than a positive one in certain conditions [25]. Excessive accumulation of cyclin D1 can cause a cytotoxic effect, and overexpression may decrease the viability of tumor cells [26]. Overexpression of cyclin D1 and/or CDK4 through loss of p16 function enhances cell division by phosphorylation of Rb [27]. But we do not know whether the abnormality of one or a part of these proteins cause-specific tumors and affect clinical outcome. We assume that change in gene copy number and steady-state level causes events such as functional loss of Rb and directly affects the clinical course of the tumor [8].

There are limitations to date in our series. The main limitation of this series is its retrospective study design. Due to the rarity of uterine carcinosarcoma, we included patients who were diagnosed more than 10 years ago. Possible changes in diagnosis policy and treatment strategy during the time may affect the analysis as a bias. All of the management, pathological diagnosis, and review for this study were performed by gynecologic oncologists and pathologists sub specializing in this field is the strength of this study. We hope that our research can provide fundamental knowledge for further clinical trials such as incorporating new targeted agents like CDK 4/6 inhibitors (Trilaciclib, Ribociclib, Palbociclib, and Abemaciclib) and understanding the characteristics of this rare aggressive tumor.

5. Conclusions

In conclusion, p16 showed a high rate of strong (+3) immune reaction in uterine carcinosarcoma. It was a predictor of lymph node metastasis, bigger tumor size, and prognostic outcome for disease-free survival in uterine carcinosarcoma.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article.

AUTHOR CONTRIBUTIONS

MHB and JYP—designed the research study; wrote the manuscript. YSP—performed the research. MHB—analyzed the data.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Research was performed under the approval of the Institutional Review Board of AMC (2009-0450). Informed consent was not needed and obtained because this study was a retrospective study.

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CONFLICT OF INTEREST

The authors declare no conflict of interest. JYP is serving as one of the Editorial Board members/Guest editors of this journal. We declare that (JYP) had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to (EH).

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found, in the online version, at https://oss.ejgo.net/files/article/1669258331839971328/attachment/Supplementary%20material.docx.

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