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From Cell Biology to Tissue Engineering

Expression of aurora kinases: Predictor of tumor dissemination in uterine carcinosarcoma

Kyung Hee Han[1], Min A. Kim[2] and Noh Hyun Park[3]

[1]Department of Obstetrics and Gynecology, Seoul National University Hospital Healthcare System Gangnam Center, [2]Department of Pathology, Seoul National University College of Medicine and [3]Department of Obstetrics and Gynecology, Seoul National University College of Medicine, Seoul, Republic of Korea

Summary. Uterine carcinosarcoma is a rare, aggressive, and biphasic tumor. It comprises carcinomatous and sarcomatous components, and mitosis-associated factors are thought to discriminate these two lesions. Aurora kinases are mitotic enzymes that are highly expressed in uterine malignancies. To identify the clinical significance of aurora kinase expression, we performed immunohistochemistry on tissue microarrays using cores selected from areas with typical carcinomatous and sarcomatous characteristics. A total of 24 samples were included, from patients at Seoul National University Hospital diagnosed with uterine carcinosarcoma, and who undergone a staging operation between 1997 and 2012. Patients' clinical and pathological data were analyzed, and expression patterns of aurora kinases were investigated. Aurora kinases A and B were dominantly expressed in the cytoplasm, and phospho-aurora kinases A and B were expressed in the nuclei. Phospho-aurora kinase A and aurora kinase B showed significantly higher expression in the carcinomatous component (P=0.012 and 0.008). High expression of phospho-aurora kinase A was associated with lymphatic metastasis such as positive pelvic lymph node and omental involvement (P=0.012 and 0.037). Overexpression of aurora kinase B was related to vascular invasion (P=0.011). High expression of both phospho-aurora kinase A and aurora kinase B was a prognostic factor for progression-free survival in uterine carcinosarcoma (P=0.049). In conclusion, expression of aurora kinases is associated with bidirectional tumor dissemination into the lymphatic and hematogenous pathways. In addition, high expression of phospho-aurora kinase A and aurora kinase B is a predictor of progression-free survival. Therefore, inhibitors of aurora kinases might be a prospective therapeutic options for uterine carcinosarcoma.

Key words: Uterine neoplasms, Carcinosarcoma, Aurora kinase, Immunohistochemistry

Offprint requests to: Noh Hyun Park, M.D., Ph.D., Department of Obstetrics and Gynecology, Seoul National University College of Medicine, Seoul 110-744, Republic of Korea. e-mail: pnhkhr@snu.ac.kr

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Introduction

Uterine carcinosarcoma (UCS) is a rare and highly aggressive gynecologic cancer. UCS affects 2 in 100,000 women annually. The 5-year disease-specific survival was reported to be less than 60%, even for patients in the early stages of the disease (Gonzalez Bosquet et al., 2010). The prognosis for UCS is worse than that of other epithelial tumors without a sarcoma component (Zhang et al., 2015). However, the cause of this aggressiveness is not yet understood. Known prognostic factors for UCS include the extent of tumor and vascular invasion at diagnosis (Macasaet et al., 1985). UCS has biphasic pathology and is composed of sarcomatous components (SC) of mesenchymal origin and carcinomatous components (CC) of epithelial origin. Two characteristics differ between SC and CC: mitotic count and DNA ploidy. Whereas CCs have increased mitotic indices, SCs display aneuploidy (Nicotina et al., 1997). Because an increased mitotic index and aneuploidy are

the result of cell proliferation, a mitosis-associated factor is expected to be involved in UCS.

Aurora kinase is a serine-threonine kinase that is acutely involved in mitosis. Many previous studies have documented the role of aurora kinase A (AURKA) and aurora kinase B (AURKB) in malignant tumors. Aurora kinase inhibitors have been shown to decrease the mitotic index of carcinoma (Hegyi et al., 2012). Aurora kinases induce cellular aneuploidy through centrosome amplification, multi-polar mitosis, and incomplete cytokinesis (Zhou et al., 1998). Auto-phosphorylated aurora kinases are known as an activated form of aurora kinases. Phospho-aurora kinase A (p-AURKA) demonstrates increased kinase activity when compared to the activity of AURKA (Ohashi et al., 2006). Phospho-aurora kinase B (p-AURKB) has enhanced enzymatic activity and associated with cytokinesis (Carmena and Earnshaw, 2003).

Aurora kinases are expressed in malignant endometrial tissue, and predict poor prognosis for uterine carcinoma (Kurai et al., 2005). In addition, the effects of aurora kinases on the growth of uterine leiomyosarcomas, through controlling cellular mitosis, have been reported. However, it is unknown if aurora kinases are related to the aggressiveness of UCS. Therefore, we selected AURKA, AURKB, p-AURKA, and p-AURKB to evaluate the expression pattern of aurora kinases in UCS using immunohistochemistry. We then identified the clinical significance of aurora kinase expression in UCS.

Materials and methods

Patients and tumor samples

Among 39 patients diagnosed with UCS between 1997 and 2012 at Seoul National University Hospital, we enrolled 24 patients who had undergone a staging operation such as total hysterectomy, bilateral salpingo-oophorectomy, pelvic and paraaortic lymph node dissection, omentectomy, and peritoneal washing cytology. To evaluate expression patterns in components of UCS, we excluded endometrial adenocarcinoma without a sarcomatous component. Medical records and pathologic specimens from all patients with UCS were reviewed retrospectively to investigate clinical and pathologic data. The Institutional Review Board at Seoul National University Hospital approved this study.

All tissue sections were stained using the hematoxylin and eosin method, and two independent pathologists evaluated the components of UCS. Tissue microarray (TMA) was used and has been validated for correlating diagnosis, prognosis, and therapy with aurora kinase expression in uterine cancer (Hassan et al., 2008). To construct TMA cores, two representative areas that typically contained CCs and SCs in one UCS sample, were selected. Sections (4 μm thick) were cut from paraffin-embedded samples and mounted on slides.

Immunohistochemistry

Immunohistochemistry was performed with antibodies against AURKA, AURKB, p-AURKA, and p-AURKB. Sections were deparaffinized in xylene, and incubated in a dry oven at 60°C for 1 hour. After deparaffinization, samples were dewaxed and hydrated at 72°C for 3 minutes using alcohol, three times. Heat pretreatment was performed using epitomic retrieval solution at 100°C for 20 minutes. Solutions of pH 9.0 for AURKA and pH 6.0 for AURKB were applied. Upon incubation for 5 minutes, endogenous peroxidase was blocked. For immunostaining, samples were incubated with specific antibodies against AURKA (1:70, Santa-Cruz, SC-25425) and AURKB (1:100, Santa-Cruz, SC-25426) for 15 minutes, and post-primary incubation was performed for 8 minutes. The polymer was quenched by incubation for 8 minutes. DAB substrate was applied for 10 minutes and hematoxylin counterstaining was performed for 10 seconds. The bound antibody was detected using a Bond polymer detection kit (Leica, Wetzlar, Germany). Sections were counterstained in hematoxylin for 10 seconds, deparaffinized in xylene, and hydrated with phosphate buffered saline (PBS). Antigens such as p-AURKA and p-AURKB were retrieved at pH 9.0 using cell conditioning 1 solution at 100°C for 60 minutes. Immunostaining was performed using specific antibodies against p-AURKA (1:200, Sigma, SAB4300270) and p-AURKB (1:100, Gentex, GTX85607) at 37°C for 32 minutes. After primary antibody staining, the samples were ultrawashed with PBS. The bound antibody was detected using Ventana BenchMark XT Staining systems (Ventana, Basel, Switzerland). Samples were incubated with this reagent for 4 minutes, which was followed by hematoxylin counterstaining. Post-counterstain was performed by incubating with bluing reagent for 4 minutes.

Immunohistochemical staining intensity and cell proportion were analyzed for both the nuclear and cytoplasmic areas. All TMA cores were evaluated according to relative staining intensity (0, 1+, 2+, 3+) and proportion of stained cells (1, 0-5%; 2, 6-25%; 3, 26-50%; 4, 51-75%; 5, 76-100%). The expression score was calculated by multiplying the staining intensity by the cell proportion (Liang et al., 2012; Shan et al., 2012). All samples were divided into high and low groups based on the proportion of positive staining using the appropriate criteria of a previous study (Yen et al., 2012). Samples exhibiting scores of ≥2+ for staining intensity and ≥30% for stained cell proportion were assigned to the high expression groups for AURKA and AURKB (Tanaka et al., 2005). High expression groups of p-AURKA and p-AURKB were determined by expression scores ≥6 (Shan et al., 2012).

Statistical analysis

To compare expression between SCs and CCs, a

paired t-test or McNemar test was used. Comparisons of clinical parameters with immunoexpression of aurora kinases were performed using a student's t test, chi-square test, or Fisher's exact test. The survival function was calculated using the Kaplan-Meier method with a log-rank test. Statistical analyses were conducted using SPSS statistical software (version 18.0; SPSS Inc., Chicago, IL, USA).

Table 1. Patients' characteristics of uterine carcinosarcoma.

	N (%)
All patients	24
Age (years)	
Median (range)	65 (46-81)
BMI (kg/m2)	
Median (range)	24.9 (18.5-31.0)
FIGO stage	
I-II	16 (66.7)
III-IV	8 (33.3)
Menopause	
Pre-menopause	3 (12.5)
Post-menopause	21 (87.5)
Adjuvant therapy	
None	9 (37.5)
Chemotherapy	7 (29.2)
Radiation	4 (16.7)
Concurrent chemoradiation	3 (12.5)
Intraperitoneal chemotherapy	1 (4.2)

FIGO, International Federation of Gynecology and Obstetrics.

Results

Patient characteristics and pathologic data

Table 1 shows the characteristics of patients with UCS. Twenty-four patients diagnosed with UCS who underwent surgical treatment were included in this study. Adjuvant therapy was performed in high-risk patients with advanced stage, deep myometrial invasion, positive lymphovascular space invasion, or positive lymph node metastasis. Sixteen patients underwent adjuvant therapy such as chemotherapy, radiotherapy, or concurrent chemoradiation. Various types of chemotherapeutic agents were used including ifosfamide, paclitaxel, cisplatin, or doxorubicin. Most of the included patients were old and postmenopausal. Two patients had previously been diagnosed with cervical cancer and thus had a history of irradiation of the pelvic cavity.

In each sample, the proportions of histologic components were analyzed. The median proportion of SC was 6.5-fold higher than that of CC (85%; range, 3-90 versus 15%; range, 1-50). The median mitotic count per 10 high-power fields was 12 for SCs and 18 for CCs (P=0.253). The histology of SCs was classified as heterologous (8/24, 33.3%), homologous (7/24, 29.2%), and undifferentiated sarcoma (9/24, 37.5%). The types of heterologous sarcomas among the specimens included

Table 2. Clinicopathological correlations for the expression of p-AURKA and AURKB.

	p-AURKA			AURKB		
	Low n (%)	High n (%)	P	Low n (%)	High n (%)	P
FIGO stage			0.027			0.390
I and II	11 (91.7)	5 (41.7)		10 (79.6)	6 (54.5)	
III and IV	1 (8.3)	7 (58.3)		3 (23.1)	5 (45.5)	
Myometrial invasion			0.680			1.000
Less than 1/2	6 (50.0)	4 (33.3)		4 (30.8)	4 (36.4)	
More than 1/2	6 (50.0)	8 (66.7)		8 (61.5)	6 (54.5)	
Lymphatic invasion			1.000			1.000
Negative	7 (58.3)	6 (50.0)		7 (53.8)	6 (54.5)	
Positive	5 (41.7)	6 (50.0)		6 (46.2)	5 (45.5)	
Vascular invasion			0.317			0.011
Negative	11 (91.7)	8 (66.7)		13 (100.0)	6 (54.5)	
Positive	1 (8.3)	4 (33.3)		0	5 (45.5)	
Adnexal involvement			0.155			0.127
Negative	11 (91.7)	7 (63.6)		12 (92.3)	6 (60.0)	
Positive	1 (8.3)	4 (36.1)		1 (7.7)	4 (40.0)	
LN metastasis			0.012			0.582
Negative	11 (100.0)	5 (50.0)		9 (90.0)	7 (70.0)	
Positive	0	5 (50.0)		1 (10.0)	3 (30.0)	
Omentum involvement			0.037			0.630
Negative	12 (100.0)	7 (58.3)		11 (84.6)	8 (72.7)	
Positive	0	5 (41.7)		2 (15.4)	3 (27.3)	

FIGO, International Federation of Gynecology and Obstetrics; p-AURKA, phospho-aurora kinase A; AURKB, aurora kinase B.

chondrosarcoma (4/8, 50%), rhabdomyosarcoma (2/8, 25%), and osteosarcoma (2/8, 25%). The histology of CCs included endometrioid (8/24, 33.3%), serous (5/24, 20.8%), adenosquamous (5/24, 20.8%), mucinous (1/24, 4.2%), and undifferentiated carcinoma (5/24, 20.8%).

Expression of aurora kinases

Aurora kinases were expressed differentially according to histologic components and phosphorylation. Aurora kinases presented diverse expression patterns in CCs and SCs, even in the same specimen from one case. Whereas AURKA and AURKB were mainly localized to the cytoplasm, p-AURKA and p-AURKB were present in the nucleus. Fig. 1 shows examples of overexpression of AURKA and p-AURKA in a CC and SC. Overexpression of p-AURKA was more frequently observed in CCs (P=0.012). It was determined that 12 cases had high expression of p-AURKA and 12 cases showed low expression. The median expression score for p-AURKA was 5. P-AURKA staining was not associated with histologic type such as endometrioid, serous, or mucinous CCs, and homologous or heterologous SC types. Expression of AURKA was not different between CCs and SCs.

Fig. 2 demonstrates high expression of AURKB and p-AURKB in CCs and SCs. More cases with high expression of AURKB were observed for CCs compared to that of SCs (P=0.008). The number of cases with high and low expression of AURKB was 11 and 13, respectively. The median expression score for AURKB was 4. AURKB expression was positively correlated

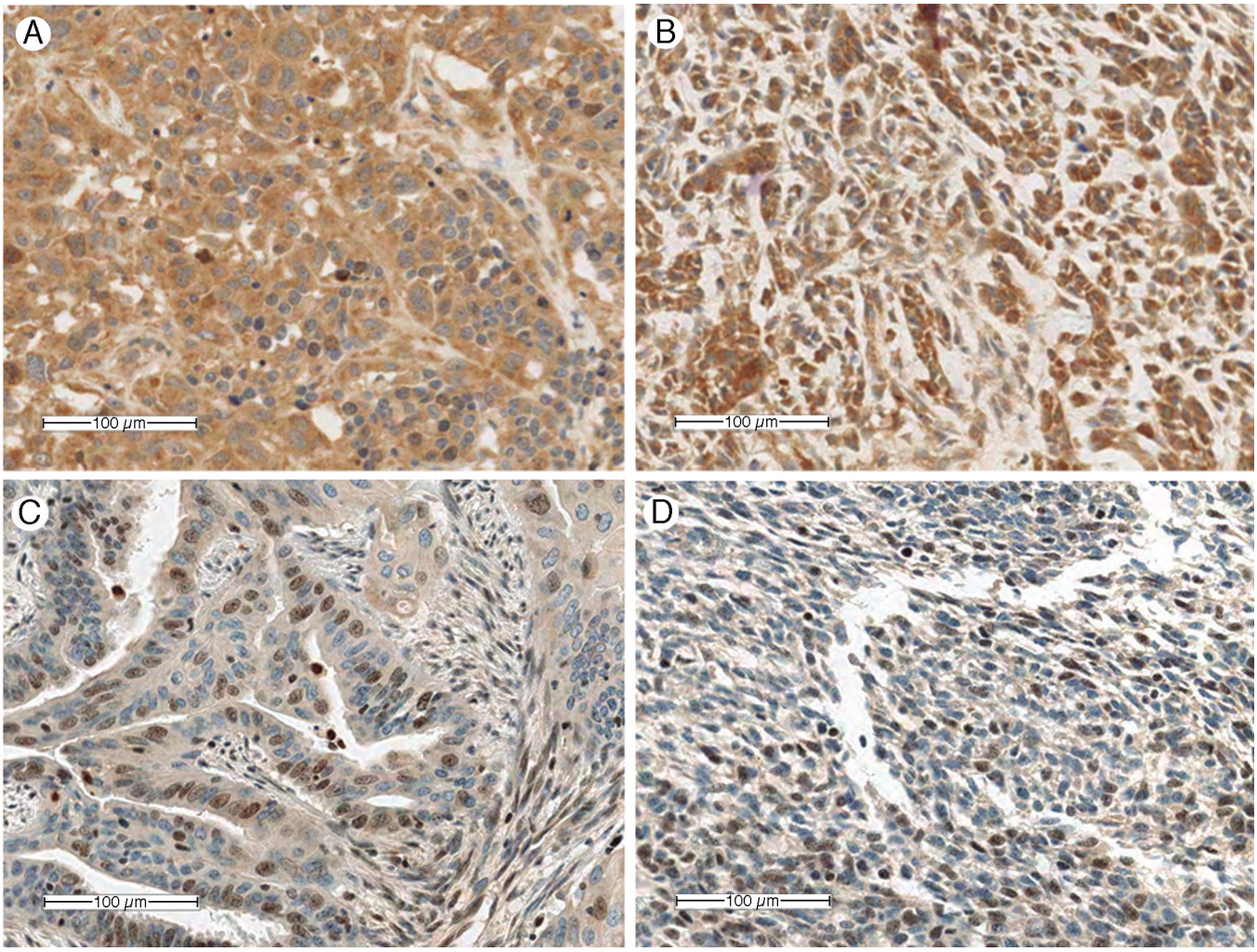

Fig. 1. Immunohistochemistry analysis of expression of aurora kinase A (AURKA) and phospho-aurora kinase A (p-AURKA) in carcinomatous components (CCs) and sarcomatous components (SCs). This figure illustrates overexpression of AURKA in CCs **(A)**, AURKA in SCs **(B)**, p-AURKA in CCs **(C),** and p-AURKA in SCs **(D)**. Whereas AURKA showed predominantly cytoplasmic staining, p-AURKA was expressed in the nucleus. Overall, p-AURKA expression was significantly different in the CCs and SCs.

with homologous histology in SCs (P=0.033). Expression of p-AURKB was not different between CCs and SCs.

Overexpression of aurora kinases positively correlated with lymphatic and hematogenous metastasis

Table 2 shows the clinical significance of p-AURKA and AURKB expression in CCs. P-AURKA expression was associated with in advanced stage, positive pelvic lymph node metastasis, and omental metastasis (P=0.027, 0.012, and 0.037, respectively). P-AURKA overexpression was related to a high mitotic count in CCs (P=0.026). High expression of AURKB was associated with positive vascular invasion (P= 0.011). However, other aurora kinases, AURKA and p-AURKB, were not significantly correlated with clinical parameters of UCS in this study.

Survival of patients with carcinosarcoma according to aurora kinases expression

Among 24 patients, 10 experienced treatment failure during the follow-up period. The median follow-up period was 42.5±29.8 months. Of 5 patients with stage IV disease, 3 had immediate postoperative recurrence before adjuvant treatment. Disease progression during adjuvant therapy occurred in 3 cases. Only 4 patients had disease recurrence after surgery and adjuvant therapy. The modality used to identify treatment failure included chest, abdomen, and pelvis CT. The areas of recurrence included the peritoneum (6/10, 60%), lung (2/10, 20%),

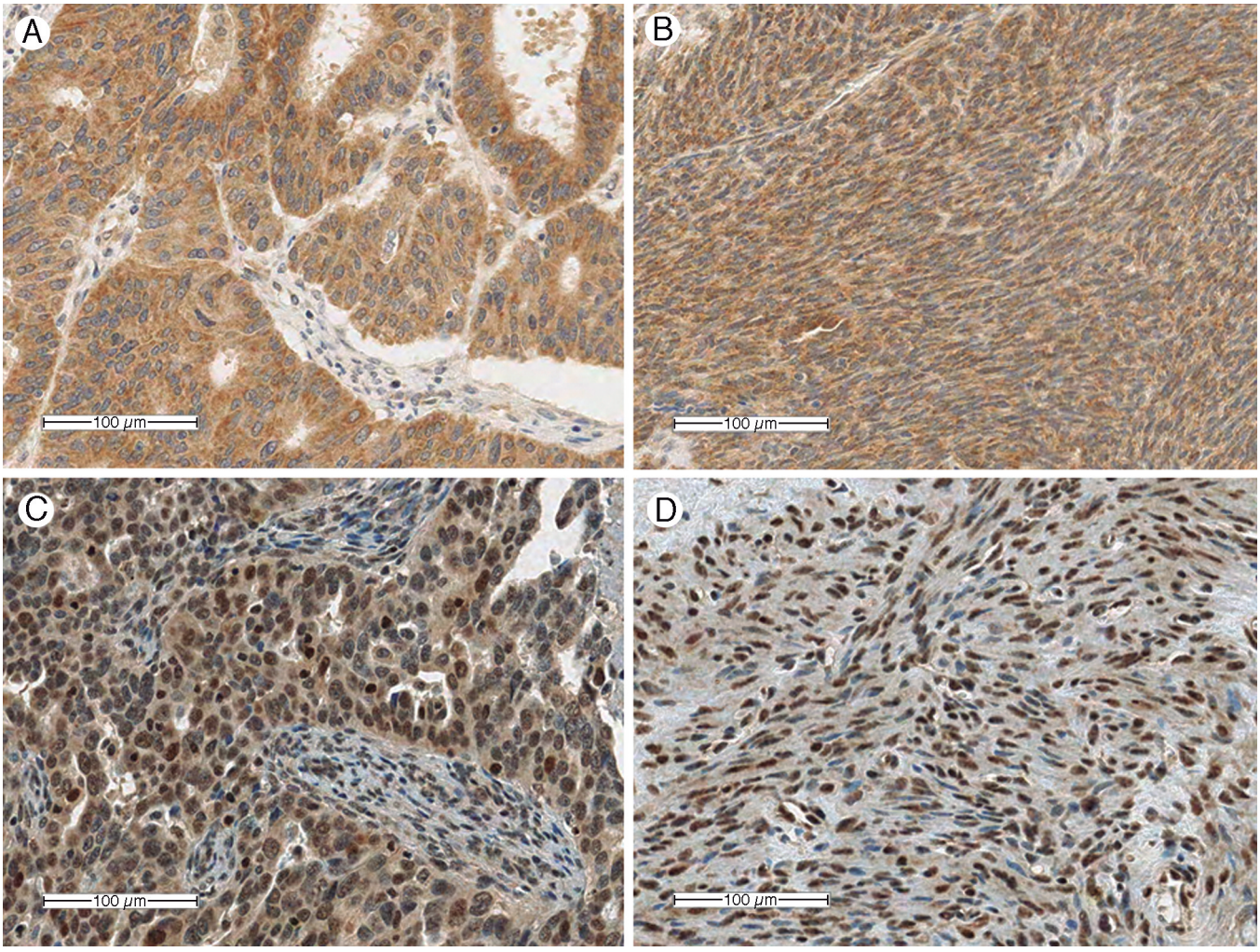

Fig. 2. Immunohistochemistry analysis of expression of aurora kinase B (AURKB) and phospho-aurora kinase B (p-AURKB) in carcinomatous components (CCs) and sarcomatous components (SCs). This figure illustrates overexpression of AURKB in CCs **(A)**, AURKB in SCs **(B)**, p-AURKB in CCs **(C)**, and p-AURKB in SCs **(D)**. Whereas AURKB showed predominant staining in the cytoplasm, p-AURKB was expressed in the nucleus. Overall, AURKB expression was significantly different in the CCs and SCs.

lymph node (1/10, 10%), and vaginal vault (1/10, 10%). Fig. 3 shows the survival function based on Kaplan-Meier analysis. In the high expression group, for both AURKB and p-AURKA, the median progression-free survival was 7.77±5.08 months, and the overall survival was 17.3±5.17 months (P= 0.049 and 0.232).

Discussion

Aurora kinase is a serine-threonine kinase that is involved in mitosis. Aurora kinases are associated with the mitotically active phase, and are overexpressed in proliferative organs such as the testes, thymus, and fetal liver (Katayama et al., 2003). AURKA initiates cellular mitosis by forming bipolar spindles at the centrosome during prophase (Lens et al., 2010). AURKA is associated with the separation of centrosomes and the arrangement of chromosomes during metaphase (Marumoto et al., 2005; Hoar et al., 2007). Overexpression of AURKA is associated with genomic instability, multi-nucleation, polyploidy, and cellular senescence (Meraldi et al., 2002; Vader and Lens, 2008). Previous studies have demonstrated overexpression of aurora kinases in uterine cancer, in which AURKA was highly expressed in non-endometrioid endometrial carcinoma (Moreno-Bueno et al., 2003). A recent study reported that AURKA was a candidate for targeted therapy in endometrial cancer (Umene et al., 2015). In addition, AURKA and p-AURKA were shown to be overexpressed in leiomyosarcoma (Brewer Savannah et al., 2012; Shan et al., 2012).

AURKB produces the cohesive form of sister chromatids during prophase, helps microtubules bind to the kinetochore during the prometaphase-metaphase transition, and participates in cytokinesis through interactions with other molecules (Lens et al., 2010; Alushin et al., 2012). Overexpression of AURKB results in polyploidy (Fu et al., 2007), and has been reported to be a prognostic factor for poor outcome in endometrial cancer (Kurai et al., 2005). However, aurora kinase expression has not been studied, even though mitosis-related factors are important in UCS. Therefore, the aim of the current study was to investigate the expression pattern of aurora kinases using immunohistochemistry, and to identify their clinical significance to UCS.

The pathogenesis of UCS can be explained by the conversion theory, which states that SCs originate from CCs via sarcomatous metaplasia (McCluggage, 2002). CCs control metastatic potential and determine tumor aggressiveness (Sreenan et al., 1995; de Jong et al., 2011). In this study, aurora kinases were mainly expressed in CCs compared to SCs. Because aurora kinases play an important role in mitosis, the expression of aurora kinases is expected to contribute to cellular proliferation and disease progression. Our study indirectly suggests sarcomatous metaplasia from the epithelial portion to the mesenchymal area. Because there has been no previous study for describing the expression of aurora kinases in uterine carcinosarcoma, we could not apply standard criteria for immunohistochemical scoring. Thus, we assessed the expression pattern of aurora kinases with suitable criteria according to each aurora kinase. Similar to previous research that reported cytoplasmic expression of

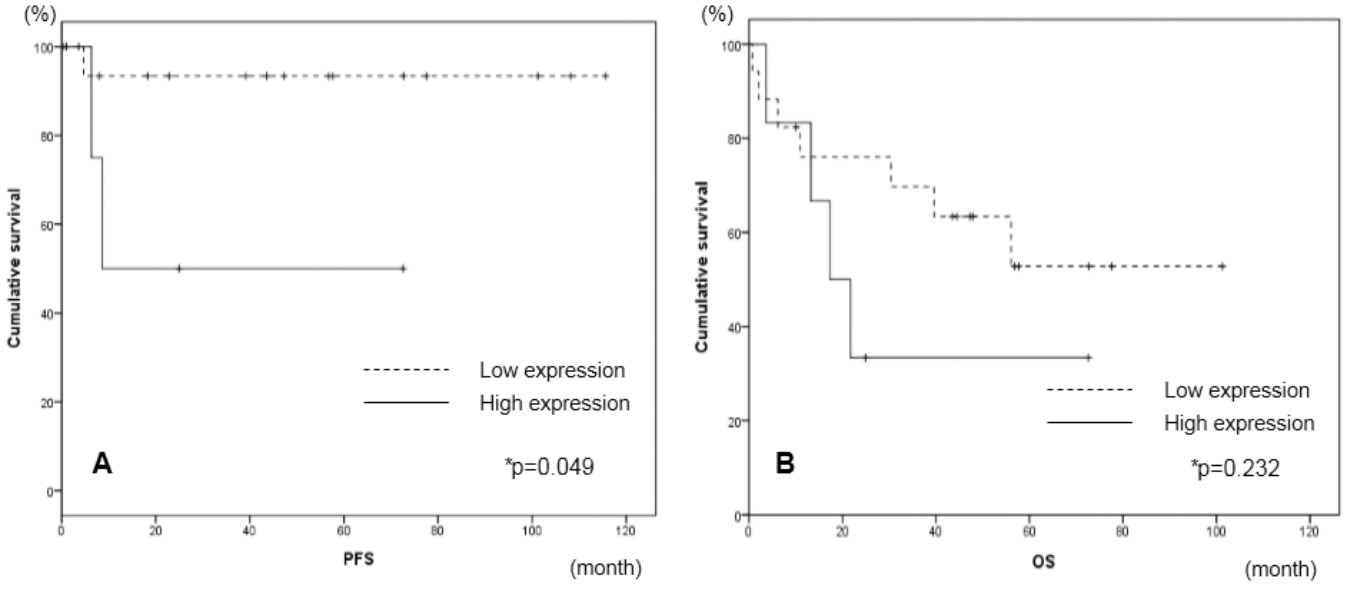

Fig. 3. Survival function based on Kaplan-Meier analysis. Progression-free survival **(A)** and overall survival **(B)** according to the expression pattern of both phospho-aurora kinase A (p-AURKA) and aurora kinase B (AURKB). The high-expression group had poor prognosis compared to that of the low-expression group in terms of progression-free survival.

AURKA and AURKB in the neoplastic endometrium, AURKA and AURKB showed positive expression in the cytoplasm of UCS (Kurai et al., 2005). Phosphorylated aurora kinases such as p-AURKA and p-AURKB were expressed in the nuclei. Although the exact mechanism of aurora kinase phosphorylation is not known, phosphorylated forms are thought to be active in the nucleus.

The expression patterns of aurora kinases were correlated with clinical features of UCS. Specifically, overexpression of p-AURKA in CCs was associated with lymphatic tumor metastasis with pelvic lymph node and omental involvement. In squamous cell carcinoma of the head and neck, p-AURKA was associated with metastasis to the lymph nodes (Reiter et al., 2006). The autophosphorylation site of p-AURKA is a target for aurora kinase inhibitors that are used for cancer therapy (Ohashi et al., 2006; Lin et al., 2008). Epithelial expression of p-AURKA is expected to be an indicator of lymph node metastasis in UCS. In our study, overexpression of AURKB in CCs was associated with vascular invasion. In UCS, the correlation between vascular invasion and AURKB was explained via the action of vascular endothelial growth factor (VEGF). An AURKB inhibitor binds receptor of the VEGF family and blocks vascular permeability to attenuate angiogenesis (Glaser et al., 2012). An abundance of localized VEGF and angiopoietin has been observed in UCS compared to expression of these factors in endometrial carcinoma (Emoto et al., 2004).

High expression of p-AURKA and AURKB is a prognostic factor for progression-free survival in UCS. Although the sample size was too small to apply multivariate analysis in this study, our data revealed correlations between aurora kinase expression and prognosis in UCS. Because expression of p-AURKA and AURKB was associated with bidirectional dissemination via the lymphatic and hematogenous pathways, it could be an overall good predictor of tumor prognosis. In bladder cancer, p-AURKA was associated with an advanced stage and poor histologic differentiation (Bufo et al., 2010). In addition, AURKB overexpression was associated with cell proliferation and poor prognosis in melanoma, breast, ovarian, and colon cancer (Hegyi et al., 2012; Hetland et al., 2012).

This study was the first to explore the expression patterns of aurora kinases, and to investigate their predictive role in UCS. Existing treatments have shown poor efficacy in overcoming the poor prognosis of UCS, a disease with a high rate of recurrence and a low response rate to many different types of treatment. Novel mechanisms to inhibit tumor growth are crucial for the development of new therapeutic methods. Treatment of UCS could target aurora kinases in CCs to prevent disease progression and to increase patient survival rates.

Author declaration. We confirm that the manuscript has been read and approved by all authors, and that there are no other persons who satisfied the criteria for authorship.

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