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TITLE

Usefulness of separately evaluating lymphatic and venous vessel invasion in cervical adenocarcinoma

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Running title

Yusuke T et al: EVALUATING LYMPHATIC AND VENOUS VESSEL IN CERVICAL ADENOCARCINOMA.

ABSTRACT

Uterine cervical adenocarcinoma is less sensitive to radiation and chemotherapy than squamous cell carcinoma, and thus, its management includes surgical treatment. We evaluated lymphatic and venous vessel invasion separately in surgical specimens to predict prognosis and recurrence. We retrospectively analysed data from the medical records of 108 patients who were diagnosed with cervical adenocarcinoma or adenosquamous carcinoma and underwent abdominal radical hysterectomy in our institution from January 1993 to April 2017. Lymphatic and venous vessel invasion was separately evaluated in all patient samples using immunohistochemical staining with D2-40 and haematoxylin and eosin (H&E)–Victoria blue double staining, respectively. Clinicopathological and prognostic findings were classified by ly and v status, 66 (61.1%) were both ly (-) and v (-), invasion in ly and/or v were observed in 42 patients (38.9%); specifically, 7 ly (−) / v (+) (6.5%), 24 ly (+) / v (−) (22.2%), and 11 ly (+) / v (+) patients (10.2%), suggesting that deep stromal invasion and pelvic lymph node metastasis was significantly higher in the ly(+) and v (+) groups. Multiple logistic regression analysis of risk factor for local and distant recurrences identified FIGO stage IB2 or higher (odds ratio (OR); 6.437, p = 0.0143) and v (OR; 9.290, p = 0.0036) as independent risk factors for distant recurrence. In conclusion, we propose that by separately evaluating lymphatic and blood vessel invasions, useful information on prognosis about distant recurrence in cervical adenocarcinoma can be obtained.

Key words： uterine cervical adenocarcinoma, lymph vessel invasion, venous vessel invasion

INTRODUCTION

Uterine cervical adenocarcinoma is less sensitive to radiation and chemotherapy than squamous cell carcinoma, and thus, its management includes surgical treatment. Pathological evaluation of lymphatic and venous vessel invasion separately in surgical specimens has been used to predict prognosis and recurrence in several types of cancers, such as gastric cancer [1], colorectal cancer [2,3], and breast cancer [4,5]. However, in gynecological oncology, both lymphatic and blood vessel invasions are judged as positive or negative based on lymphovascular space involvement. Nonetheless, some studies have reported on the usefulness of separately evaluating lymphovascular invasion for predicting recurrence and prognosis in endometrial cancer [6,7]. Even though the importance of evaluating vascular invasion has been reported in cervical cancer [8], no studies have addressed the use of separately evaluating lymphatic and blood vessel invasions for predicting recurrence and prognosis in cervical adenocarcinoma.

Therefore, this study aimed evaluate the prognostic effects of separately evaluating lymphatic and venous invasion in cervical adenocarcinoma.

PATIENTS and METHODS

We retrospectively analysed data from the medical records of 108 patients who were diagnosed with cervical adenocarcinoma or adenosquamous carcinoma and underwent abdominal radical

hysterectomy at the University of the Ryukyus Hospital from January 1993 to April 2017. Before surgery, all patients underwent chest-abdominal computed tomography and pelvic magnetic resonance imaging, and diagnosis was confirmed histopathologically. Patients were considered eligible for radical hysterectomy if they conformed to stage IB-II criteria established by the International Federation of Gynaecology and Obstetrics (FIGO) 2008. Patients treated before 2007 were appropriately reclassified. Data from patients with double cancer or incomplete resection was excluded from this study as were patients with neuroendocrine tumors because of its poor prognosis. Abdominal radical hysterectomy was performed by gynecologic oncologists and ovaries were preserved in young patients with shallow cervical stromal invasion at stage IB1 and normal ovarian function (ideally, age < 35 years). Postoperative adjuvant treatment was provided to patients with less than 2/3 cervical stromal invasion but with lymphovascular space invasion, greater than 2/3 cervical stromal invasion, or pelvic lymph node metastasis or more than stage IB2. Adjuvant treatment included radiotherapy before 2005 and systemic chemotherapy (paclitaxel＋carboplatin) after 2006.

Lymphatic and venous vessel invasion was separately evaluated in all patient samples using immunohistochemical staining with D2-40 and haematoxylin and eosin (H&E)–Victoria blue double staining, respectively. Immunohistochemical staining and evaluation were performed by pathologists after surgery, and the results were confirmed by gynecological oncologists. The former was used for identifying lymphatic invasion (ly) while the latter was for the venous invasion (v). Samples were classified as double-positive, i.e., D2-40 (ly) positive and Victoria blue–H&E (v) positive, only when

tumor clusters at the deepest point of stromal invasion were detectable within both the D2-40-positive lymphatic vessel and the Victoria blue positive venous vessel. For Victoria blue–H&E staining, deparaffinized sections were washed in a graded alcohol series for 1 min each, stained with Victoria blue solution (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) overnight, rinsed in 70% ethanol, and washed with water. Subsequently, the slides were stained with H&E dye (Mayer's hematoxylin solution and pure eosin; Muto Pure Chemicals Co., Ltd.). D2-40 staining was performed on the Bench Mark XT autostainer (Ventana Medical Systems, Inc., Tucson, AZ, USA). Briefly, slides were blocked using an endogenous Biotin Blocking kit (cat. no. 760-050; Ventana Medical Systems, Inc.) for 20 min and incubated with anti-D2-40 mAb (mouse monoclonal, clone D2-40, dilution 1:100; DAKO, Tokyo, Japan) for 32 min after antigen retrieval with CC1 buffer at 95 °C for 60 min. Slides were subsequently incubated with a pre-diluted Discovery Universal Secondary Antibody cocktail (cat. no. 760-4205; Ventana Medical Systems, Inc.) for 32 min and treated with biotinylated horse anti-mouse immunoglobulin (Ig) G, followed by an avidin–biotin–peroxidase complex. Slides were finally stained with diaminobenzidine and 0.15% hydrogen peroxidase, and counterstained with Mayer's hematoxylin and pure eosin (Figure 2).

All statistical analyses were performed using the JMP software package, version 13.0 (SAS Institute, Cary, NC, USA). Fisher's exact test was used for rate comparisons. The 5-year disease-free survival (DFS) and overall survival (OS) curves were estimated using the Kaplan–Meier method, and differences were examined using the log-rank test. The Cox proportional hazards model and multiple

logistic regression analysis were used to perform multivariate analysis. A p-value of < 0.05 was considered statistically significant.

This retrospective study was conducted according to the principles stated in the Declaration of Helsinki, 1964, and its subsequent revisions, and was approved by the Institutional Review Board of our university on December 17, 2018 (#1378).

RESULTS

Patient characteristics and DFS/OS based on each variable are shown in Table 1. The median age was 46 years (range, 24–68) and the median follow up period was 59 months (range, 4–288). FIGO stage distribution was as follows: 82 patients (75.9%) in stage IB1, 17 (15.7%) in stage IB2, 1 (0.9%) in stage IIA1, and 8 (7.4%) in stage IIB. Based on histopathology, 92 patients (85.2%) had adenocarcinoma and 16 patients (14.8%) had adenosquamous carcinoma. All patients underwent abdominal radical hysterectomy with 2 patients (1.9%) doing so after a caesarean section. Ovarian preservation was possible in 5 patients (4.6%). The median number of dissected pelvic lymph nodes was 25 (range, 16-41) and pelvic lymph node metastasis was observed in 16 patients (14.8%). Ovarian metastasis was observed in only 1 patient (0.9%). Less than 2/3 cervical stromal invasion was observed in 69 patients (63.9%) while 39 patients (36.1%) had deeper than 2/3 stromal invasion. Lastly, 50 patients (46.2%) received adjuvant therapy with 38 patients (76.0%) provided systemic chemotherapy, and 12 patients (11.1%) provided radiotherapy (8 patients, radiotherapy alone; 1 patient, concurrent

chemoradiotherapy; 3 patients, sequential chemoradiotherapy).

Recurrence was observed in 22 patients (20.4%). The median time to relapse was 20.5 months (range, 2–132). With respect to site of recurrence, 9 patients (40.9%) showed pelvic recurrence only, 8 patients (36.4%) had distant organ recurrence only, and 5 patients (22.7%) showed both pelvic and distant organ recurrence. The prognosis in 92 patients (85.2%) was no evidence of disease, while 7 patients (6.5%) were alive with disease, and 9 patients (8.3%) had died of disease.

Clinicopathological and prognostic findings were classified by ly and v status, and are shown in Table 2, and Figure 1 and 2. While 66 (61.1%) were both ly (-) and v (-), invasion in ly and/or v were observed in 42 patients (38.9%); specifically, 7 ly (−) / v (+) (6.5%), 24 ly (+) / v (−) (22.2%), and 11 ly (+) / v (+) patients (10.2%), suggesting that deep stromal invasion and pelvic lymph node metastasis was significantly higher in the ly(+) and v (+) groups. The 5-year DFS rate was 34.3% in ly (-) / v (+), 66.9% in ly (+) / v (-), and 53.0% in ly (+) / v (+), respectively, which was also significantly poorer than that seen in the ly (-) / v (-) (92.2%) group ($p = 0.0001$). Additionally, 5-year OS rates were 83.3% in ly (-) / v (+), 75.0% in ly (+) / v (-), and 72.7% in ly (+) / v(+), respectively, which was significantly lower than that in ly (-) / v (-) group (98.5%) ($p = 0.0015$).

Multivariate analyses using the Cox proportional hazards model identified FIGO stage IB2 or higher (DFS: hazard ratio (HR); 3.192, $p = 0.0199$; OS: HR 5.144, $p = 0.0370$) and pelvic lymph node metastasis (DFS: HR, 4.798, $p = 0.0022$; OS: HR; 7.865, $p = 0.014$) as independent prognostic factors for DFS and OS. However, ly and v were not significant predictors (Table 3). Multiple logistic regression

analysis of risk factor for local and distant recurrences identified FIGO stage IB2 or higher (odds ratio (OR); 6.437, p = 0.0143) and v (OR; 9.290, p = 0.0036) as independent risk factors for distant recurrence (Table 4), while lymph node metastasis was an independent risk factor (OR; 16.98, p = 0.0002) for pelvic recurrence only.

DISCUSSION

Our analysis showed that deep cervical stromal invasion, pelvic lymph node metastasis, v (+), and ly (+) are significant prognostic factors for DFS and OS in the log-rank test, but multivariate analysis did not identify v (+) and ly (+) as independent prognostic factors. However, v (+), but not ly (+), was a significant risk factor for distant recurrence. Lymphatic invasion alone was not a predictor of recurrence. It is necessary to accumulate the cases of only v (+) which were few in this study, these results suggest that, in cervical adenocarcinoma, it may be useful to separately evaluate lymphatic and venous invasion.

As it is sometimes difficult to distinguish between lymphatic and blood vessels with HE staining alone, the Japanese Gastric Cancer Association and the Japanese Society for Cancer of the Colon and Rectum recommend separate evaluation by immunostaining for lymphatic vessel and blood vessel invasions [9,10]. Compared with H&E staining, immunohistochemical staining is useful for visualizing both lymphatic vessels and veins as it increases the sensitivity of vascular invasion assessment [11,12]. Kahn et al. [11] have reported that immunohistochemical staining using an antibody against D2-40 (podoplanin) is the most sensitive method for identifying lymphatic vessels. Victoria blue

H&E staining was devised as a simpler alternative to the Elastica–Masson staining method (combined Verhoeff and Masson trichrome stain) and the Elastica-Verhoeff-van Gieson staining, which is widely used in clinical practice for staining elastic fibers of thin veins and not those of arteries or lymphatic vessels like CD31 [13]. Thus, in this study, routine H&E, Victoria blue–H&E, and D2-40 immunohistochemical staining methods were used to separately evaluate lymphatic and venous invasion.

In gynecologic cancers other than cervical cancer, detailed assessment of vascular invasion is currently being attempted. Wakayama et al. have examined 189 cases of stages I-IV endometrial cancer and have found that lymphatic vessel invasion was both a strong prognostic factor and an independent risk factor for distant recurrence [6]. Sato et al. analyzed 376 cases of stage I-III endometrial cancer and showed that venous invasion is an independent risk factor for recurrence [7]. Yamanishi et al. used Victoria blue and D2-40 staining to map the route from the primary lesion to metastatic ovarian cancer [14]. The results from these studies imply that separate evaluation of vessel invasion is both important and useful in making decision in the clinical settings.

In reports on cervical cancer, Sakuragi et al. [15] reported that venous invasion, which was assessed by only H&E staining, was an independent prognostic factor in 239 cases of stage IB-IIB cervical cancer treated with abdominal radical hysterectomy. Zhang et al. used CD34 and D2-40 immunohistochemical staining to separately evaluate venous and lymphatic vascular invasion and state that both invasions are independent prognostic factors in an early stage squamous cell carcinoma of the cervix [16]. These studies mainly focused on squamous cell carcinoma while our study is the first report

that is focused on cervical adenocarcinoma alone.

Cervical adenocarcinoma has a poorer prognosis than squamous cell carcinoma and tends to spread into the lymphatic and venous drainage system even at an early stage [17-20]. A significantly greater extent of venous invasion was observed in adenocarcinoma compared to squamous cell carcinoma, which may cause hematogenous distant metastasis [15]. These are consistent with our results focused on cervical adenocarcinoma that v (+) but not ly (+) was a significant risk factor for distant recurrence.

The strengths of our study include the use of D2-40, which is the most reliable marker for lymphatic endothelium, and use of a specific marker for lymphatic (D2-40) and blood vessel (Victoria blue) endothelium. There has been no report in the past on the assessment of vascular invasion using similar immunohistochemical staining in cervical cancer, especially cervical adenocarcinoma. However, the relatively small number of patients is a limitation of this study、evaluation of infiltration into venules without elastic fibers may be overlooked.

In conclusion, we propose that by separately evaluating lymphatic and blood vessel invasions, useful information on prognosis about distant recurrence in cervical adenocarcinoma can be obtained.

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