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Original Paper

CD44 Exon v6 Correlates with Cellular Differentiation but not with Progression and Metastasis of Cervical Cancer

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The purpose of this study was to investigate whether CD44v6 expression correlates with progression or metastasis of cervical cancer. The presence of mRNA for CD44v6 was examined, the association with clinicopathological features was assessed in 80 patients with cervical cancer by reverse transcriptase–polymerase chain reaction (RT–PCR) and subsequent Southern blot hybridisation with an oligonucleotide probe specific for v6. The standard form of CD44 was expressed in all specimens and 53 of 80 cervical cancers expressed an isoform containing exon v6 in combination with other variant exons. In addition, longer size transcripts of more than 1350 bp (long form) were identified in 22 of the 53 CD44v6 positive patients. The expression of CD44v6 and CD44v6 long form in squamous cell carcinomas was significantly higher than that in non-squamous cell carcinomas ($P < 0.001$). The expression of CD44v6 long form in histological grade 1 and 2 was significantly higher than that in grade 3 ($P < 0.05$). 47 patients in stage Ib–IIb cervical cancers were treated by radical hysterectomy and pelvic lymphadenectomy. We did not find any association between the expression of the long form or the short form of CD44v6 and any pathological features, except for histological cell type. These findings suggest that the regulation of CD44v6 seems to be different between different histological cell types and different tumour grades, and the expression of CD44v6 might not be implicated in the progression and metastasis of cervical cancer.

Key words: CD44v6, cervical cancer, metastasis
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INTRODUCTION

CD44 is a cell surface transmembrane glycoprotein and is involved in cell adhesion, serving as a receptor for extracellular matrix components such as hyaluronic acid, fibronectin, collagen and osteopontin [1–4]. Although CD44 plays a significant role in lymphocyte homing, lymphocyte activation, and extracellular matrix adhesion [5], the precise functions of CD44 in the metastatic process are largely unknown. It was recently found that 20 exons encode CD44 and 10 exons, exon 6 (v1) to exon 15 (v10), are variably expressed by alternative splicing of nuclear RNA to produce variable isoforms carrying different membrane proximal inserts [6]. The standard form does not contain any of these exons. In particular, CD44 variants sharing exon v6 appear to be of major importance for tumour progression and metastasis in rat pancreas carcinoma [7, 8] and also in several human carcinomas [9, 10].

Several reports have demonstrated that the expression of CD44v6 is significantly correlated with the presence of pelvic lymph node metastasis or adverse clinical outcome of cervical cancer. Some reports, however, did not identify a positive correlation between CD44v6 expression and progression or metastasis of this malignancy [11–17]. There have been few studies investigating the expression of CD44 mRNA isoforms variably expressed by alternative splicing of hn RNA in cervical cancer. We have examined the expression of CD44v6 in a panel of 80 cervical cancers by reverse transcriptase–polymerase chain reaction (RT–PCR) and subsequent Southern blot hybridisation with a v6 specific probe. The association between CD44v6 expression and clinicopathological features was analysed. We also investigated whether the expression of CD44v6 correlates with progression or metastasis of cervical cancer.

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MATERIALS AND METHODS

Tissue samples

Our patient population consisted of 80 patients with a diagnosis of invasive cervical cancer at the Department of Obstetrics and Gynaecology of Okayama University Medical School, Okayama, Japan who underwent treatment during the years 1995–1996. Samples of cervical tissue were obtained under colposcopic control from areas with apparent invasive disease. These specimens were bisected, and one portion was submitted for standard histopathological diagnosis to verify the presence of pure tumour, whilst the other portion was snap frozen and stored at −80°C for RNA extraction. The histology of tumours was assigned according to the WHO classification: 49 were classified as squamous cell carcinoma, 20 as adenocarcinoma and 11 as adenosquamous carcinoma. Staging was thoroughly reviewed based on the International Federation of Gynecology and Obstetrics (FIGO) staging system: 20 were stage 1, 40 were stage II, 14 were stage III, six were stage IV.

RNA extraction and RT–PCR

Total RNA was extracted from tumour tissues using the RNeasy total RNA kit (Qiagen, California, U.S.A.) according to the manufacturer's protocol. Tissues with RNA displaying high quality 18S and 28S bands on ethidium bromide stained gels were selected. RT–PCR for CD44 using 1 μg total RNA as the template was carried out using an RNA PCR kit (Takara, Kyoto, Japan). The sense primer P1 5′-GACACATATTGCTTCAATGCTTCAGC-3′ and the antisense primer P2 5′-GATGCCAAGATGATCAGCCATTCTGGAAT-3′ [18] were used for amplification of the cDNA. The PCR primers P1 and P2 were designed from exons encoding the standard form sequence adjacent to the variant insert, where variable exons can be included in the mRNA transcript to produce alternate forms of CD44. mRNA encoding the standard CD44 isoform (CD44s) yields a 482 bp fragment and longer fragments are supposed to be amplified from variant forms. The reaction mixture for PCR has been described elsewhere [19, 20]. The PCR profile consisted of a 3 min initial denaturation at 94°C followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C, 2 min extension at 72°C, and finally a 15 min extension at 72°C. All oligodeoxynucleotides were synthesised on a Model 394 DNA synthesizer (PE Applied Biosystems, California, U.S.A.).

Southern hybridisation

The final PCR products were Southern transferred and hybridised with the CD44 specific oligonucleotide probe. The probe for the standard part of the CD44 gene was S1; 5′-CCTGAAGAAGATTGTACATCAGTCACAGAC-3′ [18]. The oligonucleotide probe specific for exon v6 was K8; 5′-GAATGGGAGTCTTCTCTGGGTGTT-3′ [21]. The details of the Southern blot hybridisation have been described elsewhere [19]. The membranes were first hybridised with the K8 probe and then hybridised with the S1 probe. Successful stripping of the membranes was confirmed by exposure to film before new hybridisation.

Statistical analysis

The chi-square test was used to analyse the correlation between CD44v6 expression and clinicopathological factors. Probability values less than 0.05 were considered statistically significant.

RESULTS

The corresponding fragment of CD44s was amplified in all surgical specimens (Figure 1a) and the expected size of the 482 bp fragment was abundantly displayed by the S1 probe. Figure 1(b) shows RT–PCR–Southern blot analysis of cervical cancer specimens hybridised with the K8 probe. Overall, 53 of 80 cervical cancers expressed isoforms containing exon v6 with variations of the size of the fragments (CD44v6). In addition, longer size transcripts of more than 1350 bp (long form) were identified in 22 of the 53 CD44v6 positive patients. The expression of CD44v6 and CD44v6 long form in squamous cell carcinomas was significantly higher than that in non-squamous cell carcinomas ($P < 0.001$) (Table 1). The expression of CD44v6 long form in histological grade 1

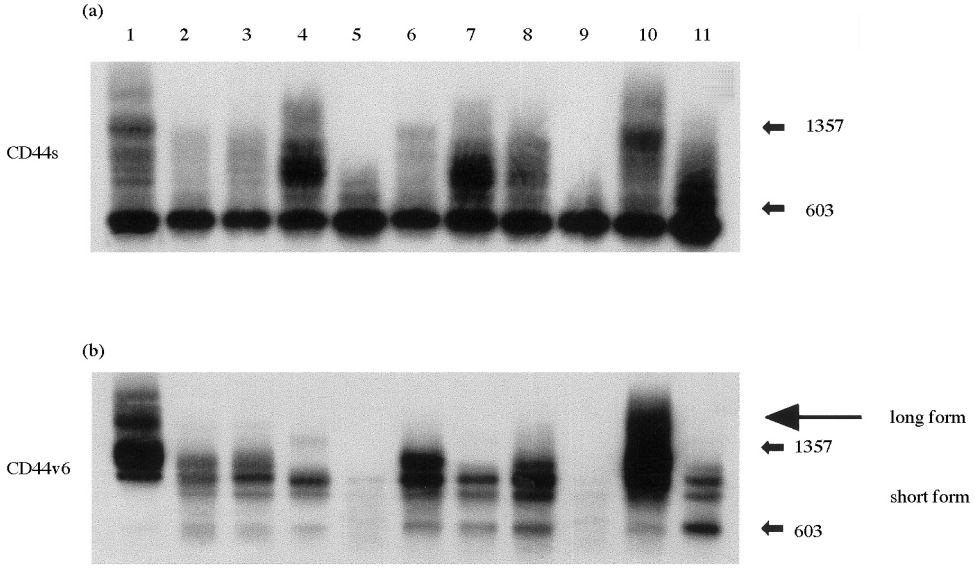

Figure 1. Detection of mRNA for CD44 in cervical cancer specimens (lanes 1–11). Total RNAs from tissues were extracted, transcribed to cDNAs and subjected to polymerase chain reaction (PCR) for any forms of CD44 and subsequent Southern blot hybridisation with oligonucleotide probes S1 (a) and K8 (b).

and 2 was significantly higher than that in grade 3 ($P < 0.05$) (Table 1). 47 patients in stage Ib–IIb cervical cancers underwent radical hysterectomy and pelvic lymphadenectomy. We did not find any association between the expression of the long form or the short form of CD44v6 and any pathological features, except for histological cell type (Table 2).

Paired tumour specimens were available from the primary tumours and metastatic lymph nodes in 3 cases. 2 of these patients did not express CD44v6 in either the primary or the metastatic site. Although CD44v6 was expressed in one primary tumour, it was not detected in the metastatic site (Figure 2, case no. 1). The observation period was so short that survival of the patients could not be analysed in the current study.

Table 1. *Association between* CD44v6 *expression and clinicopathological factors in cervical cancer*

Variables	No.	CD44v6 expression	CD44v6 long form expression
Stage			
I	20	12 (60.0%)	3 (15.0%)
II	40	26 (65.0%)	12 (30.0%)
III	14	10 (71.4%)	5 (35.7%)
IV	6	5 (83.3%)	2 (33.3%)
Histology			
SCC	49	40 (81.6%)*	20 (40.8%)*
LK	6	6 (100%)	6 (100%)
LNK	41	34 (82.9%)	14 (34.1%)
SNK	2	0	0
Non-SCC	31	13 (41.9%)	2 (6.5%)
AD	20	7 (35.0%)	1 (5.0%)
ADSQ	11	6 (54.5%)	1 (9.1%)
Grade			
G1	17	12 (70.6%)	6 (35.3%)†
G2	53	37 (69.8%)	16 (30.2%)†
G3	10	4 (40.0%)	0

SCC, squamous cell carcinoma; LK, large cell keratinising, LNK, large cell non-keratinising; SNK, small cell non-keratinising; AD, adenocarcinoma; ADSQ, adenosquamous carcinoma. *$P < 0.001$; †$P < 0.05$.

Table 2. *Association between* CD44v6 *expression and clinicopathological factors in cervical cancer treated by radical hysterectomy and pelvic node dissection*

Variables	No.	CD44v6 expression	CD44v6 long form expression
Cervical infiltration depth			
$> 2/3$	26	16 (61.5%)	7 (26.9%)
$\leq 2/3$	21	11 (52.4%)	3 (14.3%)
Parametrial involvement			
Positive	17	11 (64.7%)	5 (29.4%)
Negative	30	16 (53.3%)	5 (16.7%)
Vaginal involvement			
Positive	10	6 (60.0%)	2 (20.0%)
Negative	37	21 (56.8%)	7 (18.9%)
Lymph node metastasis			
Positive	13	8 (61.5%)	2 (15.4%)
Negative	34	19 (55.9%)	8 (23.5%)
Lymph-vascular space involvement			
Positive	23	15 (65.2%)	6 (26.1%)
Negative	24	12 (50.0%)	4 (16.7%)
Histology			
SCC	25	19 (76.0%)*	8 (32.0%)
Non-SCC	22	8 (36.4%)	2 (9.1%)
Grade			
G1	12	7 (58.3%)	4 (33.3%)
G2	27	17 (63.0%)	6 (22.2%)
G3	8	3 (37.5%)	0 (0%)

*$P < 0.01$. SCC, squamous cell carcinoma.

DISCUSSION

Although there have been many recent reports examining the implication of CD44 variants in primary tumours and metastases [22], the functions of each CD44 variant isoform are largely unknown. The expression of the variant region, v6, has been particularly confirmed in highly metastatic cell lines [7, 23, 24]. Indeed, introduction of a variant form of CD44 conferred metastatic potential to a non-metastatic rat pancreas carcinoma cell line [7]. Furthermore, this effect could be inhibited by a monoclonal antibody specific for the variant region [8]. These experimental results were supported by findings of elevated levels of CD44 mRNA in malignancies of diverse types of organs [10, 18, 25].

Several reports have demonstrated that the expression of CD44v6 protein is significantly correlated with pelvic lymph node metastasis in cervical cancer [11, 13]. Additionally, a positive correlation between CD44v6 protein expression and adverse clinical outcome of cervical cancer patients has been confirmed by recent immunohistochemical studies [12, 14]. In contrast, Woerner and colleagues indicated no correlation between the expression of distinct CD44 variant isoforms and progression and metastatic spread of cervical cancers [17]. Various antibodies, different cut-off levels and intratumoral

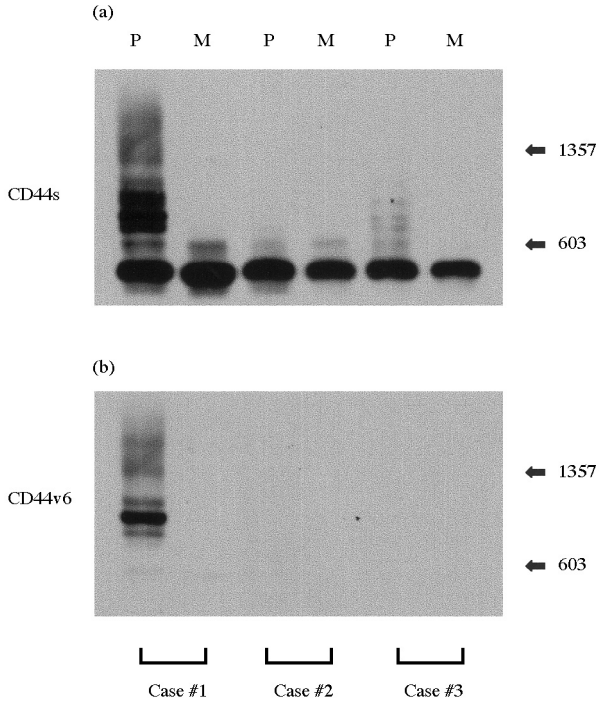

Figure 2. Detection of mRNA for CD44 in paired specimens from the primary tumour (P) and metastatic lymph node (M) in 3 patients. The probes for these hybridisations were S1 (a) and K8 (b).

heterogeneity of expression makes the immunohistochemical assessment of CD44 complicated. In fact, Friedrichs and associates reported that a wide spectrum of expression levels of CD44v isoforms and CD44s is obvious, reflecting the tumour heterogeneity of individual patients rather than an association with parameters of aggressive tumour growth in breast cancer [26].

To our knowledge, there have been few studies investigating CD44v6 mRNA expression in a relatively large group of human cervical cancers. We studied the presence of mRNA for standard form and for variants containing exon v6 by RT–PCR and subsequent Southern blot analysis in diverse histological types of cervical cancers. It was demonstrated that CD44s was expressed in all specimens, while CD44v6 and CD44v6 long form were expressed in 53 (66%) and 22 (28%) of these 80 specimens, respectively. Dall and colleagues reported that large CD44 mRNA isoforms containing v3 to v10 were produced in approximately half the uterine cervical cancers and only patients with lymph node metastases were in this group [15]. In contrast, we could find no association between the expression of CD44v6 long form and lymph node metastasis. Furthermore, a CD44v6 negative metastatic lymph node was found in 1 patient, although this patient's primary tumour did express CD44v6. It was presumed, therefore, that the expression of v6 exon was not implicated in the progression and metastatic process in this patient. Shimabukuro and associates also reported a lymph node metastasis case without the larger molecular size transcripts. It might be concluded from these facts that CD44v6 long form is not essential for lymph node metastasis in cervical cancer. Contamination of cancer cell RNAs with RNAs from normal cervical epithelium may influence the result because normal cervical epithelium can express CD44v6 [16, 17, 27]. We believe that such contamination was eliminated in the present study because all specimens were obtained from easily detectable tumours and the majority of specimens were confirmed to be cancer cells themselves according to the histological review of tissue sections.

The expression of CD44v6 in squamous cell carcinomas was significantly higher than that in non-squamous cell carcinomas and the expression of CD44v6 long form in histological grade 1 and 2 was significantly higher than that in grade 3. Furthermore, it is noteworthy that all large cell keratinising squamous cell carcinomas expressed CD44v6 long form and none of the small cell non-keratinising squamous cell carcinomas expressed CD44v6. These facts suggest that the regulation of CD44v6 might be different between different histological cell types and different tumour grades. This result is compatible with the report that CD44v6 is apparently a marker for cellular differentiation but not for tumour progression in human mammary carcinoma [26].

It has been reported that downregulation of CD44v6 expression was observed in squamocellular tumours of the head and neck, whereas the squamous epithelium seems to be the only nonmalignant tissue in which expression of CD44v6 was readily detectable [28, 29]. In fact, Shimabukuro and associates reported that the intensity of CD44v6 immunostaining is strongest in normal cervical epithelium followed by cervical intraepithelial neoplasia (CIN), invasive squamous cell carcinoma, and adenocarcinoma. Moreover, poorly differentiated and undifferentiated carcinomas from patients having poor prognosis did not stain at all [16]. Further investigations will be necessary to determine whether the downregulation of CD44v6 expression is related to progression and metastasis in cervical cancer.

In conclusion, the CD44 variant sharing exon v6 was found in approximately two-thirds of cervical cancers by RT–PCR–Southern blot analysis. The regulation of CD44v6 seems to be different between different histological cell types and different tumour grades, and the expression of CD44v6 might not be implicated in the progression and metastasis of cervical cancer.

1. Wayner EA, Carter WG, Piotrowicz RS, Kunicki TJ. The function of multiple extracellular matrix receptors in mediating cell adhesion to extracellular matrix: preparation of monoclonal antibodies to the fibronectin receptor that specifically inhibits cell adhesion to fibronectin and reacts with platelet glycoproteins Ic-IIa. *J Cell Biol* 1988, **107**, 1881–1891.
2. Stamenkovic I, Aruffo A, Amiot M, Seed B. The hematopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronate-bearing cells. *EMBO J* 1991, **10**, 343–348.
3. Jalkanen S, Jalkanen M. Lymphocyte CD44 binds the COOH-terminal heparin-binding domain of fibronectin. *J Cell Biol* 1992, **116**, 817–825.
4. Weber GF, Ashkar S, Glimcher MJ, Cantor H. Receptor-ligand interaction between CD44 and osteopontin (Eta-1). *Science* 1996, **271**, 509–512.
5. Lesley J, Hyman R, Kincadr PW. CD44 and its interaction with extracellular matrix. *Adv Immunol* 1993, **54**, 271–335.
6. Screaton GR, Bell MV, Bell JI, Jackson DG. The identification of a new alternative exon with highly restricted tissue expression in transcripts encoding the mouse Pgp-1 (CD44) homing receptor. Comparison of all 10 variable exons between mouse, human, and rat. *J Biol Chem* 1993, **268**, 12235–12238.
7. Gunthert U, Hofmann M, Rudy W, *et al.* A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. *Cell* 1991, **65**, 13–24.
8. Seiter S, Arch R, Reber S, *et al.* Prevention of tumor metastasis formation by anti-variant CD44. *J Exp Med* 1993, **177**, 443–455.
9. Mulder JWR, Kruyt PM, Sewnath M, *et al.* Colorectal cancer prognosis and expression of exon-v6-containing CD44 proteins. *Lancet* 1994, **344**, 1470–1472.
10. Kaufmann M, Heider KH, Sinn HP, Minckwitz G von, Ponta H, Herrlich P. CD44 variant exon epitopes in primary breast cancer and length of survival. *Lancet* 1995, **345**, 615–619.
11. Kainz C, Kohlberger P, Sliutz G, *et al.* Splice variants of CD44 in human cervical cancer stage IB to IIB. *Gynecol Oncol* 1995, **57**, 383–387.
12. Kainz C, Kohlberger P, Tempfer C, *et al.* Prognostic value of CD44 splice variants in human stage III cervical cancer. *Eur J Cancer* 1995, **31**, 1706–1709.
13. Biesold C, Køhler U, Horn LC, Bilek K, Kade R, Emmert C. CD44 exon v6 as a predictor of lymphatic metastases in cervical carcinoma—an immunocytochemical study of 94 cases. *Arch Gynecol Obstet* 1995, **256**, 147–153.
14. Speiser P, Wanner C, Tempfer C, *et al.* CD44 is an independent prognostic factor in early-stage cervical cancer. *Int J Cancer* 1997, **74**, 185–188.
15. Dall P, Heider KH, Hekele A, *et al.* Surface protein expression and messenger RNA-splicing analysis of CD 44 in uterine cervical cancer and normal cervical epithelium. *Cancer Res* 1994, **54**, 3337–3341.
16. Shimabukuro K, Toyamo-Sorimachi N, Ozaki Y, *et al.* The expression patterns of standard and variant CD44 molecules in normal uterine cervix and cervical cancer. *Gynecol Oncol* 1997, **64**, 26–34.
17. Woerner SM, Givehchian M, Dürst M, *et al.* Expression of CD44 splice variants in normal, dysplastic, and neoplastic cervical epithelium. *Clin Cancer Res* 1995, **1**, 1125–1132.
18. Matsumura Y, Tarin D. Significance of CD44 gene products for cancer diagnosis and disease evaluation. *Lancet* 1992, **340**, 1053–1058.
19. Seki A, Kodama J, Miyagi Y, Kamimura S, Yoshinouchi M, Kudo T. Amplification of the mdm-2 gene and p53 abnormalities in uterine sarcomas. *Int J Cancer* 1997, **73**, 33–37.

20. Seki A, Nakamura K, Kodama J, *et al.* A close correlation between the c-erbB-2 gene amplification and local progression in endometrial adenocarcinoma. *Eur J Gynecol Oncol* 1998, **19**, 90–92.
21. Kan M, Aki M, Akiyama K, Naruo S, Kanayama H, Kagawa S. High-level expression of the CD44 variant sharing exon v10 in renal cancer. *Jpn J Cancer Res* 1995, **86**, 847–853.
22. Gunthert U, Stauder R, Mayer B, Terpe H, Finke L, Friedrichs K. Are CD44 variant isoforms involved in human tumor progression? *Cancer Surv* 1995, **24**, 19–24.
23. Herrlich P, Zoller M, Pals ST, Ponta H. CD44 splice variants: metastases meet lymphocytes. *Immunol Today* 1993, **14**, 395–399.
24. Rudy W, Hofmann M, Schwartz AR, *et al.* The two major CD44 proteins expressed on a metastatic rat tumor cell line are derived from different splice variants: each one individually suffices to confer metastatic behavior. *Cancer Res* 1993, **53**, 1262–1268.
25. Stauder R, Eistere W, Thaler J, Gunthert U. CD44 variant isoforms in non-Hodgkin's lymphoma: a new independent prognostic factor. *Blood* 1995, **8**, 2885–2899.
26. Friedrichs K, Franke F, Lisboa BW, *et al.* CD44 isoforms correlate with cellular differentiation but not with prognosis in human breast cancer. *Cancer Res* 1995, **55**, 5424–5433.
27. Higashikawa K, Yokozaki H, Ue T, *et al.* Evaluation of CD44 transcription variants in human digestive tract carcinomas and normal tissues. *Int J Cancer* 1996, **66**, 11–17.
28. Salmi M, Grøn-Vita K, Sointu P, Grenman R, Kalimo H, Jalkanen S. Regulated expression of exon v6 containing isoforms of CD44 in man: downregulation during malignant transformation of tumors of squamocellular origin. *J Cell Biol* 1993, **122**, 431–442.
29. Soukka T, Salmi M, Joensuu H, *et al.* Regulation of CD44v6-containing isoforms during proliferation of normal and malignant epithelial cells. *Cancer Res* 1997, **57**, 2281–2189.

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