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Expression of CD44 Variant Exons 8–10 in Colorectal Cancer and Its Relationship to Metastasis

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Splice variants of CD44 are overexpressed in human lung, breast, and colon carcinoma cell lines. This study was conducted to clarify the association between the expression of CD44 variant exons 8–10 and metastatic potential in human colorectal cancer. We found that the expression of a CD44 splice variant containing exons v8–10 was increased in all of 60 colorectal cancer specimens examined compared with matched normal colorectal mucosa, as determined by Northern blotting. Expression of CD44 variant exons 8–10 did not significantly correlate with histological type, depth of tumor invasion, lymphatic invasion, venous invasion, or lymph node metastasis. However, the level of CD44 variant exon 8–10 expression was significantly higher in carcinomas associated with liver metastasis than in those without liver metastasis. In addition, expression of CD44 variant exons 8–10 in the liver metastases was more intense than that in the primary colorectal cancers. These findings indicated that this domain of the CD44 glycoprotein encoded by exons v8–10 may play an important role in tumor hematogenous metastasis of human colorectal cancer.

Key words: Liver metastasis — Colorectal cancer — CD44 variant exons 8–10 — Adhesion molecule

The metastasis of carcinomas is one of the most intractable problems in clinical medicine. The process of metastasis has been elucidated at the molecular level,[1] and has been shown to occur in a 7-step process: (1) detachment of tumor cells from the primary tumor, (2) migration into the extracellular matrix, (3) invasion of adjacent structures, including lymphatic and blood vessels, (4) transportation via these vessels, (5) adhesion to the capillaries of distant organs, (6) infiltration from the capillaries, and (7) proliferation in the organs. Recent studies have elucidated the nature of intercellular adhesion and adhesion molecules; adhesion molecules belonging to the cadherin family, the immunoglobulin superfamily, the integrin and selectin families and others appear to play important roles in the control of such cell activities as adhesion, mobility, migration, growth, and differentiation. Certain highly metastatic cells have been shown to adhere strongly, via these molecules, to endothelial cells and basement membrane components such as laminin, fibronectin, and type IV collagen.[2,3] These adhesion molecules may be involved in cancer metastasis via adhesive cell-cell interactions and via interactions with the extracellular matrix.

In addition to the above groups of adhesion molecules, more recent studies have revealed a unique group of CD44 surface molecules. Human CD44 glycoprotein has been proposed to function as a homing lymphocyte receptor[4–8]; the molecule is believed to play a role in binding to endothelial cells in the post capillary venules of lymphoid organs. The CD44 molecule also binds the extracellular matrix components hyaluronic acid, fibronectin, and collagen.[9–11] Several groups have now isolated and sequenced a number of different CD44 isoforms produced by alternative splicing.[12–16] CD44 isoforms carry up to 10 exons (exon v1–v10) encoding a total of 338 amino acids in the membrane proximal extracellular region of the standard CD44. Several variants of CD44 have been detected in various human tumor cell lines and human tumors. Although the function of such CD44 isoforms is largely unknown, it is thought that splice variants of CD44 may play an important role in tumor growth and metastasis. One CD44 isoform expressed in metastasizing rat tumor cell lines has been shown to confer metastatic potential on non-metastatic variants of a rat pancreatic carcinoma line.[13] Further, highly invasive human bladder carcinoma cells have been shown to express high levels of CD44, whereas a noninvasive bladder carcinoma cell line expressed low levels of the molecule.[17]

In this study, using reverse transcriptase-polymerase chain reaction (RT-PCR) and Northern blot analysis, we investigated the SW480 cell line, as well as surgical specimens of human colorectal cancers and matched adjacent normal mucosae to determine their expression of variant CD44. Our findings suggest that CD44 vari-

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ants may play a role in the hematogenous metastasis of human colorectal cancer.

MATERIALS AND METHODS

Patients and tissue samples Tumor samples and the adjacent normal mucosa were obtained from 60 colorectal patients; 36 patients with colonic cancer and 24 with rectal cancer including 7 with liver involvement, receiving treatment in the First Department of Surgery, Fukui Medical School. A representative tumor specimen and non-neoplastic mucosa from the surgical margin were immediately frozen in liquid nitrogen within 10 min of removal at operation and stored at −80°C until use. All patients with colorectal cancer underwent resection of the colon or rectum with extensive lymph node dissection. Twenty-seven (45.0%) patients with colorectal cancers had lymph node involvement, and 13 (21.7%) had liver metastases.

RT-PCR Total RNA was extracted from each sample using guanidinium thiocyanate.[18] cDNA prepared from 3 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (BRL) with an oligo(dT)^{14} primer was used as a template for PCR. The 5′ and 3′ primers encompassed positions 646–662 (containing a *Bam*HI site) and 731–747 (containing an *Eco*RI site) of the published human standard CD44 sequence, respectively. These primers and an oligo(dT)^{14} primer were prepared using a Model 394 DNA synthesizer (Applied Biosystems).

To amplify the CD44 gene, 35 cycles of denaturation (93°C, 1 min), annealing (50°C, 1.5 min), and extension (72°C, 1 min) were performed in a thermal cycler (Program Temp Control System PC-700, Astec Inc., Fukuoka). PCR products were analyzed by PAGE in 12% gels. The PCR products were cloned into pBluescript II SK (Stratagene). DNA was sequenced using a Sequenase version 2.0 kit (USB) with [α-^{32}P]dCTP.

Northern blotting Twenty micrograms of isolated RNA was electrophoretically separated on a 1% agarose gel containing formaldehyde and transferred onto nylon membranes (Gene-Screen Plus, Du Pont). Hybridization and stringency of washing were as recommended by the manufacturer. cDNA probes were labeled with [α-^{32}P]-dCTP using a multiprime labeling kit (Amersham). For hybridization studies, the *Bam*HI-*Eco*RI fragment of PBSK-CD44 variant exons 8–10 was used as the probe. Blots were exposed to an imaging plate (IP) and analyzed using a Bio Imaging Analyzer BAS 2000 (Fujix). β-Actin probe was used as an internal control. The comparative intensity of RNA signals between cancer and normal mucosa was determined by one-dimensional densitometric tracing after normalizing with respect to the internal control.

RESULTS

The 5′ primer encompassed positions 646–662 (containing a *Bam*HI site) and the 3′ primer encompassed positions 731–747 (containing an *Eco*RI site) of the published human CD44 sequence. The PCR product of the standard CD44 sequence has a length of 119 base pairs. Fig. 1 shows variant CD44 mRNA detection by PCR amplification from samples of the SW480 cell line, normal colon mucosa, and colorectal cancers; there were fragments of 515 base pairs, 311 base pairs, and 119 base pairs. The largest PCR product, obtained from SW480 cells and colorectal cancers, was sequenced. This variant contained an additional sequence of 396 base pairs inserted between positions 667 and 668 of the standard CD44 molecule. The sequence can be divided into three exons (exons v8, 9, and 10). The 311 base pair band contained the CD44 variant exons 8 and 9, but the 119 base pair band contained no CD44 variant exons. The PCR products of standard CD44 were detected in tumors and normal tissues as a band of 119 base pairs.

The expression of CD44 variant exons 8–10 in the 60 colorectal tissue samples was analyzed in relation to its expression in 60 matched samples of morphologically normal mucosa obtained at colectomy or rectal resection. Northern blot hybridization detected 5.0, 2.7, and 2.1 kbp mRNA species, which corresponded to the transcripts of variant CD44 in both colorectal cancer and normal mucosal specimens (Fig. 2). Of the mRNA bands, the 5.0 and 2.7 kbp bands contained the CD44 variant exons 8–10 and 8 and 9 respectively. Expression of the

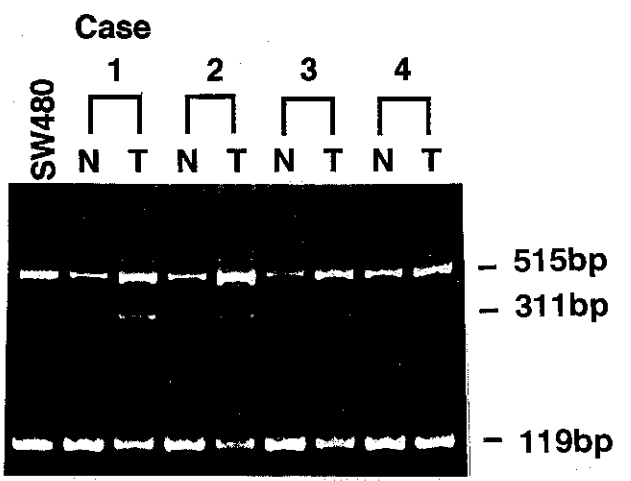

Fig. 1. Variant CD44 mRNA detection by RT-PCR amplification from samples of the SW480 cell line, normal colonic mucosa (N), and colorectal cancer (T). Fragments of 515, 311, and 119 bp were amplified in all samples, but to different levels.

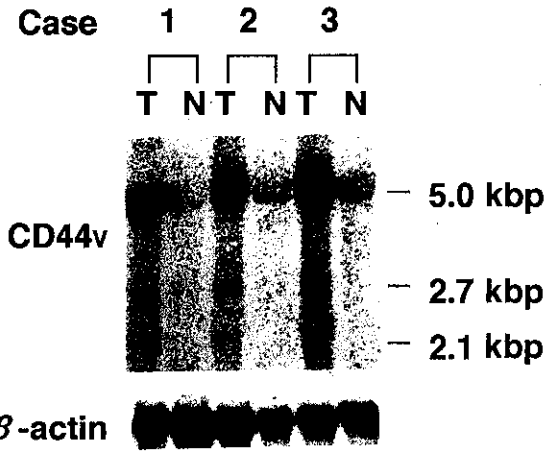

Fig. 2. Northern blot analysis of variant CD44 containing exons v8–10, utilizing RNA from colorectal cancers (T) and adjacent normal colonic mucosae (N). The hybridization probe used is specific for the cDNA portion of CD44 variant exons 8, 9 and 10. Hybridization detected bands of 5.0, 2.7, and 2.1 kbp. Expression of variant CD44 RNA in colorectal cancers exceeded that in the adjacent normal mucosae.

Table I. Correlation between Pathological Variables and Expression of Variant CD44

Variable	No. of cases	Variant CD44 expression (carcinoma/normal)	
Histological type			
well	15	1.97±0.64	
mod	21	2.17±0.95	NS
por,muc	6	1.65±0.50	
Depth of tumor invasion			
pm≧	4	1.66±0.70	
ss, a1	44	1.99±0.66	NS
s, a2	9	2.54±1.34	
si, ai	3	1.51±0.58	
Lymphatic invasion			
negative	14	2.06±0.91	NS
positive	46	2.02±0.79	
Venous invasion			
negative	32	2.04±0.72	NS
positive	28	2.02±0.71	
Lymph node metastasis			
negative	33	1.94±0.72	NS
positive	27	2.14±0.92	

CD44 variant exons 8–10 was determined by analyzing radioactivity of the 5.0 kbp band. The expression of CD44 variant exons 8–10 in each of the colorectal carcinoma samples exceeded that in the matched normal colorectal mucosae in all 60 patients for whom we analyzed paired tissue samples, even after normalization with respect to β-actin as the internal control.

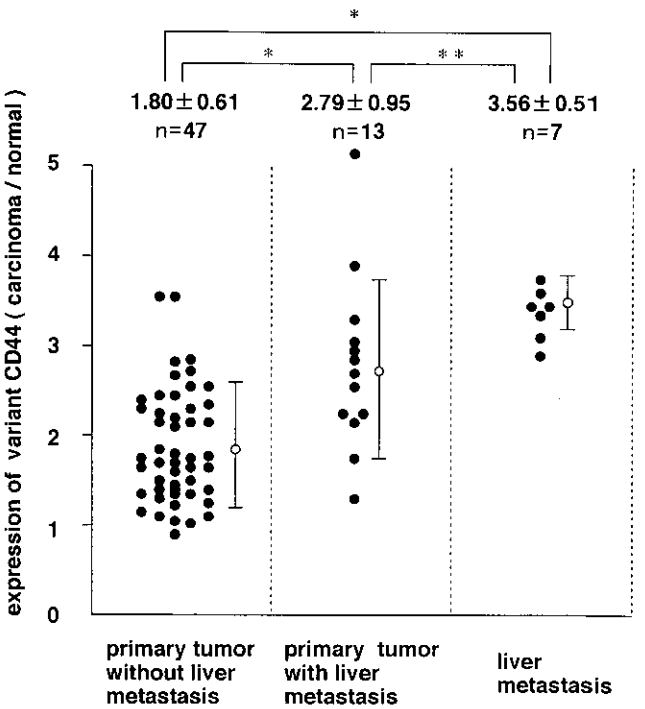

Fig. 3. Relationship between expression of variant CD44 and liver metastasis. Variant CD44 RNA levels were significantly higher in tumors with liver metastasis than in those without. The expression of variant CD44 RNA in the liver metastasis was more intense than that in primary tumors. *, $P<0.01$; ** $P<0.05$.

Table I shows the relationship between clinicopathological findings and the level of CD44 variant exon 8–10 RNA expression, determined from the ratio of CD44 variant exon 8–10 transcripts in cancer tissue to that in the adjacent normal mucosa. The CD44 variant exon 8–10 expression ratio (carcinoma/normal mucosa) did not significantly correlate with histological type, depth of invasion, or lymphatic or vascular invasion. The mean variant CD44 expression ratio was 1.94±0.72 for lesions without lymph node metastasis and 2.14±0.92 for those with lymph node metastasis; this difference was not significant (Table I). There was also no correlation between the grade of lymph node metastasis and the variant CD44 ratio.

However, the mean variant CD44 ratio for lesions associated with liver metastasis (2.79±0.95) was significantly higher than that for lesions without such metastasis (1.80±0.61) (Fig. 3). Further, the expression of variant CD44 in lesions associated with liver metastases was 3.56-fold greater than that in the matched normal colon mucosae, and this figure was significantly higher than that in the primary carcinoma without liver metas-

Table II. Correlation between Level of Variant CD44 RNA and Rate of Liver Metastasis

Variant CD44	No. of cases	No. of cases with liver metastasis (%)
High[a]	28	11 (42.9) *
Low[b]	32	2 (6.3) *

a) High: variant CD44 RNA level (carcinoma/normal) $\geqq 2$.
b) Low: variant CD44 RNA level (carcinoma/normal) < 2.
$*P < 0.01$.

Table III. Correlation between Dukes' Stage and Expression of Variant CD44

Stage	No. of cases	Variant CD44 expression (carcinoma/normal)
A	3	** 1.32±0.27
B	28	** 1.95±0.69
C	18	1.79±0.54 * *
D	11	2.82±1.07 *

$*P < 0.01$. $**$ $P < 0.05$.

tasis. Tumors with a variant CD44 transcript ratio of 2.0 or more were designated high CD44 tumors, and those with a ratio of less than 2.0 as low CD44 tumors. We then examined the relationship between variant CD44 expression and hematogenous metastasis in terms of high and low CD44 tumors. Liver metastasis was positive in 2 (6.3%) of the 28 patients with low CD44 tumors and in 12 (42.9%) of the 20 patients with high CD44 tumors; the difference between the two groups was significant ($P < 0.01$) (Table II).

Table III shows the variant CD44 expression ratio in relation to Dukes' stage. The ratio increased with advancing stage, values being 1.32±0.27 in stage A, 1.95± 0.69 in stage B, 1.79±0.54 in stage C, and 2.82±1.07 in stage D. The ratio in Dukes' stage D was significantly higher than that in stages A, B, and C ($P < 0.01$).

DISCUSSION

A number of functions have been assigned to standard CD44, e.g., binding to endothelial cells in the postcapillary venules of lymphoid organs, involvement in lymphocyte recirculation, binding to collagen, fibronectin, and hyaluronate to confer cell matrix contact, and signal transfer in lymphocytes and macrophages.[4-11] Several variants of the CD44 molecule generated by alternative splicing have now been shown to be causally involved in metastasis. Homologues of rat CD44 splice variants have been shown to be expressed at the RNA level in human lung, breast, and colonic carcinoma cell lines.[12] Tumor cells transfected with overexpressing CD44 alternative splice variants gain access to lymph nodes and hematogenous metastatic sites in animal models; direct evidence of this phenomenon was provided by Günthert *et al.*,[13] who showed that overexpression of one CD44 variant isoform, encoded by the cDNA clone pMeta-1, sufficed to confer metastatic behavior on a non-metastasizing rat pancreatic adenocarcinoma cell line. As stated above, it has also been shown that highly invasive human bladder carcinoma cells expressed high levels of CD44, whereas a noninvasive bladder carcinoma cell line expressed low levels.[17]

Several groups have analyzed CD44 splice variants in tumor tissues obtained from patients with colonic and gastric cancers.[19,20] Tanabe *et al.*[21] analyzed CD44 splice variants in specimens from normal colonic mucosa, primary colorectal cancers, normal liver, and metastases, and suggested that overexpression of CD44R1 variant may have increased the metastatic potential of those cancers. Very recently, Guo *et al.*[22] reported that levels of soluble CD44 in serum were elevated in patients with advanced gastric or colon cancer, and that levels of serum CD44 were correlated with tumor metastasis and tumor burden. An immunohistochemical study in colorectal tumors using monoclonal antibodies against a bacterially expressed fusion protein encoded by exons v3–v10 was performed by Wielenga *et al.*,[23] who found that expression of variants containing exon v6 sequences was largely restricted to the advanced stages of tumor development and, in addition, was more prevalent and intense in metastatic than in nonmetastatic cancers. In gastric cancers, isoforms of variant CD44 containing exon v9 were found to be associated with distant metastases, and were significantly and positively correlated with tumor recurrence and mortality.[24]

In a previous study using rat cDNA sequences that encode metastasis-specific extracellular exons v8, 9, and 10 of the CD44 surface glycoprotein, we isolated human cDNA sequences by PCR amplification. The PCR clones obtained from human colonic cancer and from the SW480 cell lines identified an entire range of different RNA species. The PCR products confirmed that the species in normal mucosae were amplified to a lesser extent than variant CD44 in matched colorectal cancer specimens. We also found that expression of the CD44 variant exons 8–10 was increased in colorectal cancer compared to the normal mucosae. In this study, we demonstrated by Northern blot analysis that CD44 variant exon 8–10 expression was increased in human colorectal cancers in comparison with the expression in matched normal colon mucosae, and that the expression increased in cancers as the pathological stage progressed. We also found that the expression level was greater in cancers with liver metastasis than in those without.

Variant CD44 glycoproteins have been reported to play a role in the lymphatic spread of carcinomas in the rat, a finding that prompted Hofmann *et al.*[12] to study the expression of variant CD44 in spontaneous human tumors. In our study, however, CD44 variant exon 8–10 expression was not related to lymph node metastasis.

Metastasis is a multistep process in which cells migrate from the primary tumor, invade blood/lymphatic vessels, travel through the circulation, are arrested in the capillaries of distant organs, penetrate the endothelial basement membrane, and proliferate to form secondary deposits. A number of studies have indicated that variant CD44 plays a role in the steps of the metastatic process. Seiter *et al.*[25] showed that an anti-CD44v monoclonal antibody interfered strongly with the outgrowth of metastases, and suggested that variant CD44 catalyzed the embedding/outgrowth of tumor cells in draining lymph nodes. Arch *et al.*[26] found that T cells express CD44v and that activation can be inhibited efficiently by anti-CD44v. These findings support the hypothesis that variant CD44 plays an important role in the metastatic process after migration from the primary tumor. In our univariate analysis, the expression of CD44 variant exons 8–10 did not correlate with lymphatic or vascular invasion. This finding suggests that variant CD44 is not involved in the early steps of metastasis, i.e. in the detachment of tumor cells from the primary tumor, migration into the extracellular matrix, on invasion of adjacent structures including lymphatic and blood vessels. In contrast, Heider *et al.*[20] suggested that variant CD44 was already expressed at a relatively early stage of colorectal carcinogenesis and tumor progression. Our findings, however, showed that variant CD44 expression increased with the progression of the pathological stage of cancer. We also found that CD44 variant exon 8–10 expression was significantly more intense in samples from patients with liver metastasis than in samples from those without liver metastasis. The incidence of liver metastasis significantly correlated with CD44 variant exon 8–10 expression. Furthermore, in all 7 patients who showed metastasis in the liver, variant CD44 expression in the liver was more intense than that in the matched primary tumor. Our findings suggest that the expression of the CD44 splice variant containing exons v8–v10 is related to the progression of cancer and that cancer cells are in a state of constant change, finally acquiring metastatic potential. We do not know whether patients with high expression without synchronous liver metastasis will heterochronously metastasize in the future, and such patients should be observed closely.

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