Molecular Genetic Characterization of Alternatively Spliced CD44 Transcripts in Human Stomach Carcinoma

CD44 is a member of cell surface glycoproteins which are involved in cell-matrix adhesion and tumor metastasis. Certain types of tumors express complex CD44 isoforms generated by alternative splicing of 2v-10v exons, and their expression appears to promote metastasis of tumor cells. Using a nested RT-PCR, we analyzed expression of CD44 variants in 26 stomach carcinoma, 21 matched normal tissues, and 2 carcinoma cell lines. We observed frequent and complex patterns of CD44 variant expression in tumor tissues. While exons 6v and 7v expression was detected in most normal and tumor tissues, exon 9v was most rarely detected. Exon 5v showed a significantly frequent expression in carcinoma, suggesting that its expression might contribute to the malignant progression. While exon 9v was frequently observed in diffuse-type tumors, the other 8 variant exons including 6v showed more frequent expression in intestinal-type tumors. Exons 9v and 10v were predominantly expressed in advanced tumor tissues and exon 8v was expressed more frequently in tumors of lymph node metastasis. We believe that series with a longer follow-up now need to be tested to clarify the association between CD44 splice variant expression and distant metastasis or long-term prognosis. *(JKMS 1997; 12: 505~13)*

Key Words : *Stomach neoplasms; Antigens, CD44; Neoplasm metastasis*

Yong-Sik Kim*, Sung-Gil Chi, Youn-Wha Kim, Yong-Koo Park, Choong Yoon*

Departments of General Surgery* and Pathology, College of Medicine, Kyung Hee University, Seoul, Korea

Received : July 16, 1997
Accepted : August 26, 1997

Address for correspondence
Youn-Wha Kim, M.D.
Department of Pathology, College of Medicine, Kyung Hee University, #1 Hoeki-dong, Dongdaemoon-gu, Seoul 130-702, Korea
Tel : (02) 961-0543, Fax : (02) 957-0489

INTRODUCTION

CD44 is a heterogeneous family of molecules with putative functions in cell-cell and cell-matrix adhesion and a major receptor for hyaluronate (1-3). In the rat system, highly metastatic pancreatic and mammary carcinoma cell lines were found to express splice variants of the CD44 glycoprotein (4). These variants differ from the standard CD44 molecule in that they contain additional peptide domains inserted into the extracellular portion of the transmembrane protein. Some of these variant CD44 proteins have been shown to be causally involved in tumor metastasis formation (1).

CD44 protein isoforms range in size from Mr 80,000 to 250,000 (3). This heterogeneity results from post-translational modifications (5) and from a phenomenon known as alternative mRNA splicing (6). The gene contains a total of 20 exons. The standard CD44 mRNA isoform lacks exons 6 through 15 which are known as variant exons, designated as 1v through 10v. Different CD44 isoforms are generated by alternative usage of amino acid sequences encoded by 10 variant exons. Larger variant isoforms contain a segment encoded by one or more of exons 1v through 10v spliced together in different combinations (alternative splicing). The variation occurs in the extracellular portion of the molecule, leading to a considerable number of theoretically possible primary structures. Several of these variants have indeed been detected (7).

Variant CD44 protein isoforms are normally expressed on a variety of epithelial cells in a regulated tissue-specific pattern (8). However, a number of cancers demonstrate deregulated alternative CD44 mRNA splicing (9, 10), resulting in the production of novel isoforms. Most interestingly, cells of certain tumors express large CD44 splice variants, and their expression appears to promote metastasis formation during tumor progression (11). For example, two CD44 isoforms containing sequences encoded by variant exon 6v confer full metastatic potential to non-metastasizing pancreatic tumor cells of the rat (4). Aberrant expression of splice variants containing exon 6v has also been found on carcinoma cell lines from the lung, breast, and colon (12-14). Subsequently, several tumors including human colorectal carcinomas, non-Hodgkin's lymphomas, and melanomas have been screened for the expression of CD44 isoforms. These studies revealed that certain splice variants of CD44 may play a crucial role in the progression of vari-

ous tumors, particularly in the process of tumor progression.

Stomach cancer is one of the most frequent cancers in Korea and accounts for one sixth of all cancer deaths in Western countries including the United States (15). The mortality rates in different countries and ethnic groups differ considerably. The populations in Korea, Japan, and Costa Rica currently suffer from the highest stomach cancer motality rates. In this study a detailed analysis of RNA expression of CD44 isoforms was performed in twenty-six human stomach cancer tissues, twenty-one matched normal tissues and two established human stomach carcinoma cell lines to determine its prognostic relevance.

MATERIALS AND METHODS

Stomach cancer cell lines

Two human stomach cell lines derived from metastatic cancers, KATO III and AGS (ATCC), were maintained in Dulbecco's modified Eagle's or RPMI-1640 supplemented with 5-10% fetal bovine serum. Cells were grown at 37℃ in a humidified 5% CO_2 atmosphere (16).

Tumor specimens

Twenty-six primary stomach cancers diagnosed at the Department of Pathology, Kyung Hee University were obtained fresh from surgical resections, and either RNA was immediately extracted or the specimens were snap-frozen in liquid N_2 and stored at -70℃ until use. From 21 of these 26 patients, normal stomach tissues which were found to not contain cancerous cells upon review of the slide taken adjacent to the piece analyzed were obtained and also subjected to RT-PCR analysis for comparison (17).

RNA extractions from fresh or frozen tissues and cell lines

Total cellular RNA was extracted from cell lines and tumor tissues by a slightly modified single-step method (18, 19). The concentration of extracted RNA was determined by a spectrophotometric measurement of an absorbance at 260 and 280 nm and by setting the ratio of optical density 1.0 (260/280) equal to 40 μg/ml of RNA.

cDNA synthesis

One μg of total cellular RNA was reverse transcribed into cDNA using MoMuLV reverse transcriptase and random hexamer primers. cDNA was diluted 1:4 with distilled, sterile H_2O prior to PCR amplification (20, 21). Two separate cDNAs were prepared from each RNA to check reproducibility.

Table 1. Oligonucleotide primers for RT-PCR

Primer	Sequence	Exonic Location	Direction
1	5'-GACGAAGACAGTCCCTGGAT-3'	5s	S
2	5'-CAGCCATTTGTGTTGTTGTG-3'	2v	AS
3	5'-TGGTGCTGGAGATAAAATCT-3'	3v	AS
4	5'-CAGTCATCCTTGTGGTTGTC-3'	4v	AS
5	5'-TTGTGCTTGTAGAATGTGGG-3'	5v	AS
6	5'-CAGCTGTCCCTGTTGTCGAA-3'	6v	AS
7	5'-TCCAGGCAACTCCTAGTAGT-3'	6v	S
8	5'-CAGCCTCAGCTCATACCAGC-3'	7v	S
9	5'-ATATGGACTCCAGTCATAGT-3'	8v	S
10	5'-AGCAGAGTAATTCTCAGAGC-3'	9v	S
11	5'-ATAGGAATGATGTCACAGGT-3'	10v	S
12	5'-GGGTGGAATGTGTCTTGGTC-3'	15s	AS
13	5'-AAGACATCTACCCCAGCAAC-3'	5s	S
14	5'-TTTGCTCCACCTTCTTGACT-3'	16s	AS
15	5'-CCATCCTTCTTCCTGCTTGA-3'	7v	AS
16	5'-GCGTTGTCATTGAAAGAGGT-3'	8v	AS
17	5'-TGCTTGATGTCTGAGTAGAA-3'	9v	AS
18	5'-CTGATAAGGAACGATTGACA-3'	10v	AS

S: sense, A: antisense

Strategy of reverse transcription-polymerase chain reaction (RT-PCR)

The RT-PCR approach used in this study has been previously reported (21, 22). The oligonucleotides used as amplification primers and probes are listed in Table 1. Oligonucleotide primers were designed according to the published human CD44 cDNA sequences (23). Cycling parameters for reproducible quantitation were determined depending on the exonic regions to be amplified and the primer sets used. PCR was usually performed with 30 μl reactions with 2 μl of 1:4 diluted cDNA as template and 1 unit of Taq polymerase and PCR buffer using a thermal cycler (Perkin Elmer Cetus, CA, USA). All the cDNA sample concentrations were equilibrated semi-quantitatively using the human GAPDH sequences as a housekeeping standard marker transcript to yield a PCR product of 580 bp.

Specific amplification of variant CD44 isoforms using nest-PCR

The CD44-specific PCR was performed using primers

homologous to the standard region directly flanking the variant sequences (primer #13 and #14, Table 1). The PCR regime used was denaturation at 95℃ for 1 min, annealing at 60℃ for 45 sec, and extension at 72℃ for 1 min 30 sec for 36 cycles, followed by 10 min at 72℃. For detection of variant exon-specific transcript expression, primers were chosen at the 3' or 5' side of each variant exon and nested PCR reactions were carried out using 1 μl of original PCR products as templates and each of the variant exon-specific primers shown in Table 1. The nested PCR was done in 36 cycles: denaturation at 95℃ for 1 min, annealing at 60℃ for 45 sec, and extension at 72℃ for 1 min 30 sec for 36 cycles, followed by 10 min at 72℃. Nested PCR was repeated at least three times for each exonic region.

Agarose gel electrophoresis

The RT-PCR products were resolved on 2% Nusieve (FMC, Rockland, ME), 1% agarose gels at 110 volts for 1.5 hours. The gels were stained in 500 ml of ethidium bromide solution (0.5 μg/ml of 1 X Tris-borate EDTA, TBE) for 30 minutes and destained in 500 ml of 1X TBE for 30 minutes (24). The PCR products were visualized using ultraviolet light, and photographs of the gels were taken at a setting of f=10 for 120 seconds on Polaroid, type 55 film using a land camera. The photographic negatives were soaked in distilled water until clean and air dried.

Laser densitometry

Measurement of signal intensity was performed with a densitometer (Bio-Rad, CA, USA) using the gelscan program on an IBM compatible computer (17, 21). The RT-PCR products and the negative control areas on the photographic negative film were scanned sequentially, and the negative control background was then subtracted. After subtraction of the background, the ratio of the area under each curve to that of the housekeeping gene (GAPDH) was quantitatively related to expression of the gene.

RESULTS

Standardization of stomach tissue cDNA for PCR

To verify the quantity of cDNA which was synthesized from stomach tissue mRNA, expression levels of GAPDH, a housekeeping endogenous control gene, were analysed. All 47 tissue cDNAs showed variable but detectable levels of GAPDH expression. Relative expression levels of GAPDH reflected by intensities of PCR products were measured by densitometric scanning of each band on gels. Based on the GAPDH expression levels, cDNA quantities were adjusted by redilution with ddH_2O. Using 2 μl of these rediluted cDNAs, PCR and agarose gel electrophoresis analyses were repeated and similar levels of GAPDH expressions were observed. Fig. 1 shows a representative of GAPDH PCR characterized with rediluted cDNA specimens. Thus, 2 μl of these cDNAs was used for the detection of variant CD44 mRNA isoforms from these tissue specimens.

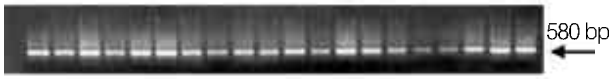

Fig. 1. RT-PCR analysis of mRNA expression of an endogenous housekeeping gene, GAPDH in human stomach cancer tissues. 10 μl of GAPDH PCR products (580 bp) were resolved on a 2% agarose gel, stained with ethidium bromide, and photographed using a Polaroid film.

Expression of CD44 variants in stomach cancer cell lines

Expression of CD44 mRNA variants in 2 stomach cell lines, AGS and KATO III was analyzed using nested RT-PCR. As described above, the first-round PCR was performed with primers 13 and 14 whose sequences are complementary to standard (S) exons 5 and 16, respectively. One μl of these PCR products were used as amplification templates for 10 separate nested PCR reactions in which specific sets of primers are included for sequence-specific amplification of 10 variant exons. As shown in Fig. 2, nest-PCR using each variant exon-specific primer demonstrated that stomach cell line AGS expressed several CD44 variant transcripts in which all variant exons (2v-10v) were differentially incorporated. Intensities and sizes of nested PCR products indicate that at least two or three types of variant transcripts are differentially expressed in these cell lines. Expression of the standard form of CD44 transcripts was also detected from this cell line (Fig. 2, lane 10; 74 bp).

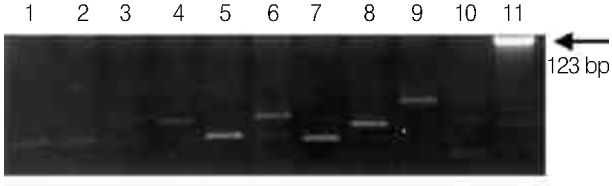

Fig. 2. Expression of CD44 variant exons in the stomach cell line, AGS. 10 μl of nest-PCR products were electrophoresed through a 2% agarose gel and stained with ethidium bromide. Lanes 1 through 9 represent variant exons 2v to 10v. Lane 10; 1st-round PCR products, lane 11; 123 bp DNA size marker.

Table 2. Expression of variant CD44 exons in human stomach tissues

Patient number	Tissue type	Tumor Types	Variant exons								
			2v	3v	4v	5v	6v	7v	8v	9v	10v
1	T	Intestinal				+	+	+			+
2	T	Diffuse	+		+			+	+		+
3	T	Diffuse				+	+	+			
4	T	Diffuse					+	+			
5	T	Intestinal					+	+	+		
6	N						+	+			
	T	Intestinal	+			+	+	+	+	+	+
7	N					+	+	+	+		
	T	Intestinal					+	+	+		
8	N					+	+	+	+		
	T	Diffuse				+	+	+	+		
9	N					+	+	+	+		
	T	Diffuse	+			+	+	+			+
10	N					+	+	+	+		
	T	Intestinal				+	+	+	+		+
11	N						+	+	+		
	T	Intestinal	+	+	+	+		+	+		+
12	N		+	+		+	+	+			
	T	Intestinal			+	+	+	+			+
13	N				+	+	+	+	+		+
	T	Intestinal			+	+	+	+			+
14	N					+	+	+			
	T	Intestinal				+	+	+			+
15	N			+		+	+				+
	T	Intestinal				+	+	+			
16	N		+			+	+	+			
	T	Diffuse		+		+	+	+			
17	N		+	+	+		+	+	+	+	+
	T	Diffuse	+	+	+	+	+	+	+	+	+
18	N			+	+		+	+		+	+
	T	Intestinal		+	+		+	+	+		+
19	N		+				+	+	+		+
	T	Intestinal	+	+	+	+	+	+	+		+
20	N			+				+	+		+
	T	Diffuse	+				+	+	+	+	+
21	N		+				+	+	+		+
	T	Diffuse	+	+	+	+	+	+	+	+	+
22	N			+	+		+	+			+
	T	Intestinal	+	+	+	+	+	+	+		+
23	N		+					+	+	+	+
	T	Intestinal	+	+	+	+	+	+	+	+	+
24	N		+		+		+	+	+		
	T	Intestinal	+	+	+	+	+	+	+	+	+
25	N		+		+				+		+
	T	Diffuse		+	+	+	+	+	+	+	+
26	N				+		+	+	+		+
	T	Diffuse		+	+	+	+	+	+	+	+

N; normal, T; tumor

Expression of variant exon(s)-containing transcripts in stomach tissue

For sensitive detection of variant exon(s)-containing CD44 transcripts, PCR was first performed with primers 13 and 14 which covered the entire variant exonic region (2v-10v). One μl of these PCR products were subjected to nest-PCR with variant exon(s)-specific primers. Primer combinations used for these nested PCR are as followed; 1/2 for exon 2v (181 bp), 1/3 for exon 3v (307 bp), 1/4 for exon 4v (421 bp), 1/5 for exon 5v (538 bp), 7/6 for exon 6v (129 bp), 8/15 for exon 7v (132 bp), 9/16 for exon 8v (102 bp), 10/12 for exon 9v (316 bp), 11/12 for exon 10v (226 bp), and 1/12 for standard form (74 bp) of CD44 transcripts. Expression of variant exons in tumor and normal tissues was summarized in Table 2. Representative agarose gel electrophoresis analysis of the nest-PCR products (exon 7v and 10v) were shown in Fig. 3.

As shown in Table 3, all CD44 variant exons were detected in both cancerous and noncancerous tissue. Among 9 variant exons, 7v expression was most frequently observed in both cancerous (96.2%) and noncancerous (95.2%). In contrast, exon 9v was most infrequently expressed in both cancerous (30.8%) and noncancerous (14.3%) tissue. In general, expressions of variant exons were more frequently detected in cancerous tissue than in noncancerous tissue. Among 21 matched sets of tissue, 12 tumor tissue specimes expressed more than 6 different variant exons and only 6 of these 12 matched noncancerous tissue specimes expressed more than 6 different exons. While no association with cancer or noncancer regions was observed in exons 2v, 6v, 7v, and 8v expression, exon 5v showed significantly frequent expression in cancerous regions compared to matched noncancerous tissue regions (76. 9% vs 42.9%). Expressions of exons 3v, 4v, and 9v were more frequently detected in cancerous tissue than noncancerous tissue (Table 3).

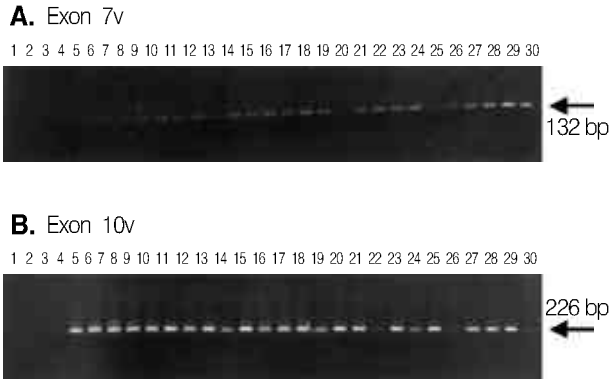

Fig. 3. Nest-PCR analysis of CD44 variant exon 7v (132 bp) (A) and 10v (226 bp) (B) expression in human stomach tissues. 15 μl of PCR products were resolved a 2% agarose gels, stained with ethidium bromide, and photographed using a Polaroid film. (A) lanes 1 to 30; 15N to 27T, AGS, and KATO III, N; normal, T; tumor). (B) lanes 1 to 29; 16T to 27T, AGS, and KATO III, lane 30; 123 bp size marker. N; normal, T; tumor).

Tumor types and expression of variant exons

To characterize any correlation of expression patterns of specific variant exons with tumor types in stomach cancer, we analyzed the expression frequencies of each variant exon in two types of stomach cancer, intestinal and diffuse. The most significant difference of expression in tumor types was recognized in exon 9v (Table 3). Exon 9v expression was observed in 62.5% of diffuse-type tumors while only 37.5% of intestinal-type tumors express this exon. The 9v was the only exon that showed more frequent expression in diffuse-type compared to intestinal-type. The other 8 variant exons were more frequently detected in intestinal-type than in diffuse-type tumors. Among these, exons 5v, 6v, 7v, 8v, and 10v were found to be expressed in intestinal-type tumors with approximately 17-22% higher frequency compared to those in diffuse-type tumors. The 6v was recognized as the exon that is expressed most frequently in intestinal-type tumors (60.9%) and that showed the most significantly different expression compared to that in diffuse-type tumors (39.1%).

Expression of CD44 variants and patients' sex and ages

No significant difference in expressions of variant CD44 exons between male and female was observed (Table 3). To determine any correlation of variant exon expression with patient's age, we arbitrarily divided patients into 3 age groups; 30-49, 50-59, and over 60. While no age relationship was recognized with expression of 2v, 4v, 5v, 6v, 7v 8v, and 10v, significant negative correlations were found with expression of 3v and 9v. As shown in Table 3, expression frequencies of 3v and 9v exons were decreased with an increase of age.

Progression of tumor and expression of variant CD44 exons.

The relationship of variant CD44 exons with cancer progression was analysed based on the histopathologic characteristics of tumor invasion and existence of lymph node metastasis. No significant differences between early and advanced cancers were found in expression of 2v-8v exons. In contrast, exons 9v and 10v were predominantly

Table. 3. Histopathologic correlation of expression of variant CD44 isoforms

	Variant exons								
	2v	3v	4v	5v	6v	7v	8v	9v	10v
Expression									
Normal	8/21(38.1)	6/21(28.6)	7/21(33.3)	9/21(42.9)	19/21(90.5)	20/21(95.2)	14/21(66.7)	3/21(14.3)	13/21(61.9)
Tumor	11/26(42.3)	11/26(42.3)	13/26(50.0)	20/26(76.9)	23/26(88.5)	25/26(96.2)	17/26(65.4)	8/26(30.8)	17/26(65.4)
*T. Types									
Intestinal									
¶Exp/Int	6/15(40.0)	6/15(40.0)	8/15(53.3)	12/15(80.0)	14/15(93.3)	15/15(100)	10/15(66.7)	3/15(20.0)	10/15(66.6)
†Int/Exp	6/11(54.5)	6/11(54.5)	7/13(53.8)	12/20(60.0)	14/23(60.9)	15/25(60.0)	10/17(58.8)	3/8(37.5)	10/17(58.8)
Diffuse									
¶Exp/Dif	5/11(45.5)	5/11(45.5)	5/11(45.5)	8/11(72.7)	9/11(81.8)	10/11(90.9)	7/11(63.6)	5/11(45.5)	7/11(63.6)
†Dif/Exp	5/11(45.5)	5/11(45.5)	6/13(46.2)	8/20(40.0)	9/23(39.1)	10/25(40.0)	7/17(41.2)	5/8(62.5)	7/17(41.2)
Sex									
Male	4/8(50.0)	3/8(37.5)	4/8(50.0)	7/8(87.5)	7/8(87.5)	8/8(100)	4/8(50.0)	2/8(25.0)	5/8(62.5)
Female	7/18(38.9)	8/18(44.4)	9/18(50.0)	13/18(72.2)	16/18(88.9)	17/18(94.4)	13/18(72.2)	6/18(33.3)	12/18(66.7)
*Tumor Depth									
Early	2/4(50.0)	2/4(50.0)	2/4(50.0)	3/4(75.0)	4/4(100)	4/4(100.0)	3/4(75.0)	0/4(0.0)	2/4(50.0)
Advanced	9/22(40.9)	9/22(40.9)	11/22(50.0)	17/22(77.3)	19/22(86.4)	21/22(95.5)	14/22(63.6)	8/22(36.4)	17/22(77.3)
Metastasis									
*LN +	3/7(42.9)	3/7(42.9)	4/7(57.1)	6/7(85.7)	6/7(85.7)	6/7(85.7)	6/7(85.7)	2/7(28.6)	4/7(57.1)
−	8/19(42.1)	8/19(42.1)	9/19(47.4)	14/19(73.7)	17/19(89.5)	19/19(100)	11/19(57.9)	6/19(31.6)	13/19(68.4)
Age (year)									
30 - 49	3/7(42.9)	4/7(57.1)	3/7(42.9)	6/7(85.7)	6/7(85.7)	6/7(85.7)	4/7(57.1)	4/7(57.1)	4/7(57.1)
50 - 59	5/11(45.5)	5/11(45.5)	7/11(63.6)	7/11(63.6	9/11(81.8)	11/11(100)	9/11(81.8)	3/11(27.3)	7/11(63.6)
60 - 79	3/8(37.5)	2/8(25.0)	3/8(37.5)	7/8(87.5)	8/8(100)	8/8(100)	4/8(50.0)	1/8(12.5)	6/8(75.0)

*LN, lymph node
Numbers in parentheses are percentages
¶Exp/Int ; expression/intestinal type tumors, †Int/Exp ; intestinal type tumors/expression total
¶Exp/Dif ; expression/diffues type tumors, †Dif/Exp ; diffuse type tumors/expression total

expressed in advanced tumor tissues. While none of 4 early stage of cancers expressed 9v exons, 8 of 22 (36.4 %) advanced stage of cancers showed expression of this exon. However, no significant difference of 9v expression was recognized between lymph node positive (28.6%) and negative (31.6%) patients. Overall, differences in expression frequency were not observed between lymph node positive and negative tumors. However, exon 8v was found to be expressed in 6 of 7 (85.7%) tumor tissue specimens of lymph node positive patients but in only 11 of 19 (57.9%) tumors of lymph node negative patients.

DISCUSSION

Variant CD44 protein isoforms are normally expressed on a variety of epithelial cells in a regulated tissue-specific pattern (8). However, a number of cancers demonstrate deregulated alternative CD44 mRNA splicing (9), resulting in the production of novel isoforms. Overexpression of CD44v is sufficient to confer metastatic potential to nonmetastasizing tumor cells (4, 25). Since several tumor lines, irrespective of their histology, acquired metastatic potential with CD44v expression, it appears that CD44v implements a common limiting function for tumor progression. So far, the smallest version of CD44v conferring metastatic properties carries only 85 amino acids of extra sequence in the variant part of the molecule. Expression of some variant isoforms associated with metastasizing tumor cells is transiently induced in lymphocytes upon antigenic stimulation (10). Most interestingly, cells of certain tumors express large CD44 splice variants, and their expression appears to promote metastasis formation during tumor progression (11). For example, two CD44 isoforms containing sequences encoded by variant exon 6v confer full metastatic potential to nonmetastasizing pancreatic tumor cells of the rat (4). Aberrant expression of splice variants containing exon 6v has also been found on carcinoma cell lines from the lung, breast, and colon (12-14). Subsequently, several tumors including human colorectal carci-

noma, non-Hodgkin's lymphoma, and melanoma have been screened for expression of CD44 isoforms. These studies revealed that certain splice variants of CD44 may play a crucial role in the progression of various tumors, particularly in the process of tumor progression.

In this study we performed a detailed analysis of RNA expression of CD44 isoforms in 26 human stomach cancer tissue and 21 matched normal tissue specimens to determine its prognostic relevance, and in 2 established human stomach carcinoma cell lines. The methods of reverse transcription-PCR (RT-PCR) amplification with variant exon-specific primers permit semi-quantitative and qualitative conclusions.

PCR analysis of RNA derived from different primary tumors and normal mucosa revealed that a more complex pattern of different splice variants is expressed in tumors compared to matched non-cancerous counterparts from the same patients. All CD44 variant exons were detected in both cancerous and non-cancerous tissues but the pattern of variant exon expression differs from one tumor to another. Among 9 variant exons, 7v expression was most frequently observed in both cancerous (96.2%) and noncancerous (95.2%) and exon 9v was most rarely detected in both cancerous (30.8%) and noncancerous (14.3%) tissue. In general, numbers of variant exons detected were higher in cancerous tissue than in noncancerous tissue. For example, of 21 matched sets of tissue, 12 tumor tissue specimes expressed more than 6 different variant exons and only 6 of these 12 matched non-cancerous tissue specimens expressed more than 6 different exons. Interestingly, exon 5v showed the most significant difference in expression frequency between cancerous and noncancerous tissue (76.9% vs 42.9%). This result is in contrast to the observation of Karl-Heinz et al. (26) who detected exons 5v and/or 6v expression in most normal and tumor tissue specimes of stomach cancer. However, our findings of frequent expression of exon 6v in both normal (90.5%) and tumor tissue (88.5%) is consistent with their observation. Our results using nest-PCR indicate that alternatively spliced CD44 variants can be readily detected in most normal stomach tissue.

In this study, the most significant difference of variant exon expression between two tumor types was recognized in exon 9v. Exon 9v expression was observed in 62.5% of diffuse-type tumors while only 37.5% of intestinal-type tumors express this exon. Also, the 9v was the only exon that showed more frequent expression in diffuse-type compared to intestinal-type. The other 8 variant exons were more frequently detected in intestinal-type than in diffuse-type tumors. The 6v was recognized as the exon that is expressed most frequently in intestinal-type tumors (60.9%) and that showed the most significantly different expression compared to that in diffuse-type tumors (39.1%). Overall, this result is consistent with the previous report of Karl-Heinz et al. (26), which described the frequent expression of exon 6v in intestinal-type tumors. However, In contrast to our detection of 6v, they could not observe expression of 6v in diffuse-type tumors. It is likely that this discrepancy could be due to the difference of sensitivity of detection methods used. They performed RT-PCR with primers which were homologous to standard exonic regions and these PCR products were subjected to Southern hybridization using variant exon-specific oligonucleotides as probes. We believe that our RT-PCR followed by nest-PCR that uses the first-round PCR products as templates and variant exon-specific primers might significantly improve the detection sensitivity, especially for low levels of expression. Additionally however, false-positive data can be generated by contaminating resting and activated lymphocytes (27). It is also difficult to draw quantitative conclusions from PCR data.

No significant difference in expressions of variant CD44 exons between male and female was observed. While no age relationship was recognized with expression of 2v, 4v, 5v, 6v, 7v 8v, and 10v, significant negative correlations were found with expression of 3v and 9v. Expression frequencies of 3v and 9v exons were decreased with an increase of patients' age.

Since several tumor lines acquired metastatic potential with CD44v expression, it appears that CD44v implements a common limiting function for tumor progression. It was found that cells of certain tumors express large CD44 splice variants, and their expression appears to promote metastasis formation during tumor progression (10). Thus, we analyzed the relationship of variant CD44 exons with cancer progression based on the histopathologic characteristics of tumor invasion and existence of lymph node metastasis. While no significant differences between early and advanced cancers were found in expression of 2v - 8v exons, exons 9v and 10v were predominantly expressed in advanced tumor tissue. None of 4 early stage of cancers expressed 9v exons, 8 of 22 (36.4%) advanced stage of cancers showed expression of this exon. However, no significant difference of 9v expression was recognized between lymph node positive (28.6%) and negative (31.6%) patients. Although differences in expression frequency were generally not observed between lymph node positive and negative tumors, exon 8v was found to be expressed in 6 of 7 (85.7%) tumor tissue specimens of lymph node positive patients but only in 11 of 19 (57.9%) tumors of lymph node negative patients.

In conclusion, we observed a much more frequent and complex pattern of CD44 splice variant expression at the

RNA levels in tumor tissue compared to normal tissue in human stomach cancers. Exons 6v and 7v expression was observed in most normal and tumor tissue, and exon 9v was most rarely detected in both types of tissue. Exon 5v showed a significantly frequent expression in cancerous tissue, suggesting that 5v plays a role in the progression of stomach cancer. While exon 9v expression was frequently observed in diffuse-type tumors, the other 8 variant exons, including 6v which is expressed most frequently in intestinal-type tumors, showed more frequent expression in diffuse-type tumors. While no significant differences between early and advanced cancers were found in expression of 2v-8v exons, exons 9v and 10v were predominantly expressed in advenced tumor tissue and exon 8v was expressed more frequently in tumor tissue of lymph node positive patients. Series with a longer follow-up now need to be tested to clarify the association between CD44 splice variant expression, distant metastasis, and long-term prognosis.

REFERENCES

1. Hughes EN, Mengod G, August JT. *Murine cell surface glycoproteins. Characterization of a major component of 80,000 daltons as a polymorphic differentiation antigen of mesenchymal cells. J Biol Chem 1981 ; 256 : 7023-9.*
2. Brown TA, Bouchard T, St John T, Wayner E, Carter WG. *Human keratinocytes express a new CD44 core protein (CD44E) as heparan-sulfate intrinsic membrane proteoglycan with additional exons. J Biol Chem 1991 ; 113 : 207-21.*
3. Aruffo A, Stamenkovic I, Mennick M, Underhill CB, Seed B. *CD44 is the principal cell surface receptor for hyaluronate. Cell 1990 ; 61 : 1303-13.*
4. Gunthert U, Hofmann M, Rudy W, Reber S, Zoller M, Haussmann L, Matzku S, Wenzel A, Ponta H, Herrlich P. *A new variant of glycoproteins CD44 confers metastatic potential to rat carcinoma. Cell 1991 ; 65 : 13-24.*
5. Lesely J, Hyman R, Kincade 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. J Biol Chem 1993 ; 268 : 12235-38.*
7. Gunthert U. *CD44 : a multitude of isoforms with diverse functions. Curr Top Microbiol Immunol 1993 ; 184 : 47-63.*
8. Fox SB, Fawcett J, Jackson DG, Collins I, Gatter KC, Harris AL, Gearing A, Simmons DL. *Normal human tissues, in addition to some tumors, express multiple different CD44 isoforms. Cancer Res 1994 ; 54 : 4539-46.*
9. Matsumura Y, Tarin D. *Significance of CD44 gene products for cancer diagnosis and disease evaluation. Lancet 1992 ; 340 : 1053-8.*
10. Arch R, Wirth K, Hoffmann M, Ponta H, Matzku S, Herrlich P, Zoller M. *Participation in normal immune responses of a splice variant of CD44 that encodes a metastasis-inducing domain. Science 1992 ; 257 : 682-5.*
11. Sherman L, Sleeman J, Herrlich P, Ponta H. *Hyaluronate receptors : key players in growth, differentiation, migration and tumor progression. Curr Opin Cell Biol 1994 ; 6 : 726-33.*
12. Friedrichs K, Franke F, Lisboa BW, Kugler G, Gille I, Terpe HJ, Holzel F, Maass H, Gunthert U. *CD44 isoforms correlate with cellular differentiation but not with progression in human breast cancer. Cancer Res 1995 ; 55 : 5424-33.*
13. Tanabe K, Ellis L, Saya H. *Expression of CD44R1 adhesion molecule in colon carcinomas and metastases. Lancet 1993 ; 341 : 725-6.*
14. Aβmann V, Kern HF, Elsasser HP. *Differential expression of the hyaluronan receptors CD44 and RHAMM in human pancreatic cancer cells. Clin Cancer Res 1996 ; 2 : 1607-18.*
15. Correa P. *Human gastric carcinogenesis; a multistep and multifactorial process : First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. Cancer Res 1992 ; 52 : 6735-40.*
16. Maeng YH, Chi SG, Park JH, Yang MH. *Molecular genetic characterization of the pathway of p53 tumor suppressor gene in human stomach cancer. Kyung Hee Univ Med J 1996 ; 21 : 59-69.*
17. Chi SG, devere White RW, Meyers FJ, Siders D, Lee F, Gumerlock PH. *p53 in prostate : frequent expressed transition mutations. J Natl Cancer Inst 1994 ; 86 : 926-33.*
18. Chomczynski P, Sacchi N. *Single-step method of RNA isolation by acid-guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987 ; 162 : 156-9.*
19. Madewell BR, Gumerlock PH, Saunders KA, Meyers FJ, Kraegel SA, Kawasaki ES. *Canine and bovine ras family expression detected and discriminated by the use of polymerase chain reaction. Anticancer Res 1989 ; 9 : 1743-9.*
20. Gumerlock PH, Poonamallee UR, Meyers FJ, deVere White RW. *Activated ras alleles in human carcinoma of the prostate are rare. Cancer Res 1991 ; 51 : 1632-7.*
21. Fishman JR, Gumerlock PH, Meyers FJ, deVere White RW. *Quantitation of NM23 expression in human prostate tissue. J Urol 1994 ; 152 : 202-7.*
22. Meyers FJ, Gumerlock PH, Kawasaki ES, Wang AM, deVere White RW, Erlich HA. *Bladder cancer : Human leukocyte antigen II, interleukin-6, and interleukin-6 receptor expression determined by the polymerase chain reaction. Cancer 1991 ; 67 : 2087-95.*
23. Screaton GR, Bell MV, Jackson DG, Cornelis FB, Gerth U, Bell JI. *Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. Proc Natl Acad Sci USA 1992 ; 89 : 12160-4.*
24. Gumerlock PH, Tang YJ, Meyers FJ, Silva J Jr . *Use of the polymerase chain reaction for the specific and direct detection of Clostridium difficile in human feces. Rev Inf Dis 1991 ; 13 : 1053-60.*
25. Reber S, Matzku S, Gunthert U, Ponta H, Herrlich P, Zoller

M. *Retardation of metastatic tumor growth after immunization with metastasis-specific monoclonal antibodies. Int J Cancer 1990 ; 46 : 919-27.*

26. Heider KH, Dammrich J, Skroch-Angel P, Muller-Hermelink HK, Vollmers P, Herrlich P, Ponta H. *Differential expression of CD44 splice variants in intestinal- and diffuse-type human gastric carcinomas and normal gastric mucosa. Cancer Res 1993 ; 53 : 4197-203.*
27. Koopman G, Heider HH, Horst E, Adolf GR, van den Berg F, Ponta H, Herrlich P, Pals ST. *Activated human lymphocytes and aggressive non-Hodgkin lymphomas express a homologue of the rat metastasis-associated variant of CD44. J Exp Med 1993 ; 177 : 897-904.*