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The Significance of CD44 Variants Expression in Colorectal Cancer and Its Regional Lymph Nodes

CD44 is a cell adhesion molecule with numerous isoforms created by mRNA alternative splicing. Expression of CD44 variants has been suggested to play a potential role in tumor progression and metastasis. We designed primers CD44V, CD44V6/7, CD44R1 and CD44V6-10 to analyze and compare the roles of each CD44 variants. Expressions of CD44 variants were investigated in normal colonic mucosa, the lymph nodes which was histopathologically free of cancer cell, and cancer tissues of 44 human colorectal cancer patients by RT-PCR method. The expression of CD44V was observed in 28 out of 39 (71.8%) tumors and 7 out of 11 (63.6%) N1 normal regional lymph nodes, and CD44V6/7 was observed in 28 out of 39 (71.8%) tumors and 9 out of 11 (81.8%) N1 normal regional lymph nodes. The expressions of CD44V and CD44V6/7 were most frequently observed compared with any other CD44 variants. In normal colonic mucosa, the expression of CD44 variants are low but in cancer tissue and its regional lymph node, the expression of CD44V and CD44V6/7 were significantly higher and more frequent than any other CD44 variants ($p<0.05$). These results suggest that CD44V and CD44V6/7 can be a molecular marker for colorectal cancer and its micrometastasis to the regional normal lymph node.

Key Words: *Antigens, CD44; Colorectal Neoplasms; Neoplasm Metastasis; CD44 Variants*

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INTRODUCTION

Colorectal carcinoma is one of the most common fatal neoplasms in Koreans. Surgical treatment is very effective if the tumor is removed before spreading to other abdominal organs (1). Despite the recent advances in the diagnostic methods of colonic cancer including ultrasonography, colonoscopy, CT and MRI, early detection rate is low.

The metastasizing tumor cell will detatch from colorectal cancer tissue and migrate into lymphatic vessels and harbor in regional lymph nodes. Multistep hypothesis of gene abnormalities has been accepted as the basis of colorectal carcinogenesis but it is unknown which gene abnormalities is related in order to metastasize. Loss or modification of cell-cell adhesion molecules is needed for detachment of tumor cell from the cancer tissue. Of adhesion molecules, CD44, membrane associated protein, contributes tumor cell adhesion. The CD44 splice variants are distinguished by additional sequences in the extracellular portion of the protein. These isoforms share function of metastatic cells and lymphocytes during lymphocyte homing processing and are expressed on both primary tumor and metastatic cells. Metastatic lymph nodes have been comfirmed by histopathological method, but it is difficult to confirm micrometastasis by this method.

Histochemical study has improved but still has its limit in making accurately determining the stage of colonic cancer because of the lack of exact information on metastases to the regional lymph nodes. Since the colorectal cancers spread to other organs through lymph nodes and blood stream, it would be desirable to have metastatic markers that can confirm effective assessment of lymph node metastases.

CD44 is a multifunctional polymorphic transmembrane glycoprotein that plays an important role in matrix adhesion, lymphocyte activation and lymph node homing (2). The *CD44* gene has 20 exons, and the region encoding the insertion is composed of 10 exons (exon 6 to 15, or V1 to V10) that are alternatively spliced to produce variable isoforms carrying different membrane proximal inserts (3). These isoforms are detectable in tumor cells with metastatic potential in many cancers including

colorectal cancer (4). Of the variant isoforms, exon 6 has been known to have metastatic potential but some scientists reported that none of CD44 variants correlated with tumor progression and metastasis (5-8). To examine the controversial role of CD44 variants, we studied the relationship between each variant isoforms and colorectal cancer.

MATERIALS AND METHODS

Patients and tissue samples

Tumor, normal colonic mucosa and N1 regional lymph nodes which were located within 5 cm from the cancer were obtained from 44 colorectal cancer patients who were treated at the Department of General Surgery, Keimyung University Medical Center. All specimens were immediately frozen in liquid nitrogen within 20 min after the removal of specimens.

RNA preparation and RT-PCR

Total RNA was extracted from each samples by the method of Chomczynski and Sacchi (9). cDNA prepared from 2 μg of total RNA using RT-PCR with primers was used as a template for polymerase chain reaction (PCR). The reverse transcription reaction was performed at 42℃ for 60 min. The cDNA was denatured at 99℃ for 5 min and stored at 5℃ for 5 min. PCR was done with 34 cycles of denaturation (94℃ for 1 min), annealing (58℃ for 1 min), elongation (72℃ for 1 min) and extension after completion of all cycles. We designed primers of CD44V, CD44V6/7, CD44R1 and CD44V6-10 to elucidate the roles of CD44 variants in colorectal cancer and its metastasis to the regional nodes (Table 1, Fig. 1).

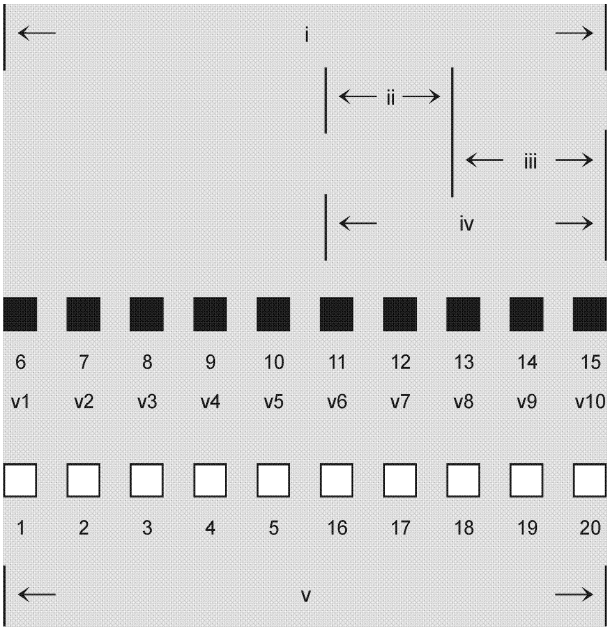

Fig. 1. Map of CD44V(i), CD44V6/7(ii), CD44R1(iii), CD44V6-10(iv) and CD44S(v). Open boxes represent constitutive 5' and 3' exons and solid boxes represent extracellular alternatively spliced exons.

Gel electrophoresis

10 μL of each PCR product were electrophoresed through 1.2% agarose gel at 50V for 1 hr in 1X TAE buffer. After staining with ethidium bromide (5 μg/mL), the gels were photographed under ultraviolet light.

Statistical analysis

The statistical differences between groups were analyzed by χ^2 test. A value of $p<0.05$ was considered sig-

Table 1. Sequence of oligonucleotide primer, exon and molecular size

Primer		Sequence (5'-3')	Exon	Molecular size
CD44S	S	GACACATATTGCTTCAATGCTTCAGC	3-17	482
	AS	GATGCCAAGATGATCAGCCATTCTGGAAT		
CD44V	S	TTGATGAGCACTAGTGCTACAGCA	6-15	735
	AS	TCCTGCTTGATGACCTCGTCCCAT		
CD44V6/7	S	CAGCCTCAGCTCATACCAGCCAT	11-12	230
	AS	TGTCATTGAAAGAGGTCCTGT		
CD44R1	S	TCCCAGACGAAGACAGTCCCTGGAT	13-15	482
	AS	CACTGGGGTGGAATGTGTCTTGGTC		
CD44V6-10	S	CAGCCTCAGCTCATACCAGCCAT	11-15	555
	AS	CACTGGGGTGGAATGTGTCTTGGTC		

*S and AS represent sense and antisense primers, respectively

nificant. All statistical analysis were made using a commercial statistical software package (SAS 6.0).

RESULTS

The expression rates of each *CD44* variants in cancer tissues were 28/39 (71.8%) for CD44V, 28/39 (71.8%) for CD44V6/7 and 28/39 (71.8%) for CD44R1. The expression rates of each CD44 variants in N1 regional normal lymph nodes were 9/11 (81.8%) for CD44V6/7, 7/11 (63.6%) for CD44V and 4/11 (36.4%) for CD44R1.

The expression rates of each *CD44* variants in normal colorectal mucosa were low. The expression rates of CD44V, CD44V6/7 and CD44R1 were high in colorectal cancer tissues, but in N1 regional lymph nodes, the expression rate of CD44R1 was lower than CD44V and CD44V6/7. Among *CD44* variants, CD44V and CD44V6/7 were expressed significantly at colorectal cancer tissues and N1 regional lymph nodes ($p<0.05$, Table 2, Fig. 2).

DISCUSSION

The *CD44* gene is about 60 kb in size and contains 20 exons. The variations on the most proximal extracellular region of the CD44 molecule may alter cell-to-cell and cell-to-matrix adhesion properties, increasing the affinity between CD44 variants and hyaluronate and thus modifying the metastatic potential of tumor cells (5). Droll (10) demonstrated that the chondroitin sulphate moiety attached to exon 20 can be recognized and bound by other CD44 molecules. This interaction was shown to promote homotypic and/or heterotypic cell-cell adhesion in vitro, and by extension may potentiate the adhesive interactions between circulating tumor cells and the vascular endothelium in vivo. These findings support the hypothesis that CD44V, V6/7, R1 and V6-10 play an important role in the metastatic process after migration from the primary tumor.

Published reports regarding the relationship between the expression of CD44 variants and the progression of colorectal cancer have been controversial. A report by Imazeki et al. (8) failed to detect any differences in the expressions of CD44V and CD44V6 in adenomatous polyps compared to primary and metastatic colon cancers. Gotley et al. (3) reported findings which did not support the role of CD44R1 as a consistent prognostic indicator for colonic tumor progression. In contrast, Tanabe et al. (11) suggested that CD44R1 may possibly be a metastatic marker for colorectal cancer.

Serum markers such as CEA, CA19-9 and AFP have been used frequently for follow up of patients after surgical treatment (12, 13), but they are not sensitive enough to reliably detect the recurrence of the cancer. It would be helpful to confirm the exact metastatic status to the regional and distant lymph nodes, but so far

Table 2. Expression rates of CD44V, CD44V6/7, CD44R1 and CD44V6-10 in 44 colorectal cancer patients

Genes	Expressed patient no. / total patient no. (%)		
	Normal colorectal mucosa	Colorectal cancer tissue	M
CD44V	9/36 (25.00)	28/39 (71.79)*	7/11 (63.63)*
CD44V6/7	6/36 (16.66)	28/39 (71.79)*	9/11 (81.81)*
CD44R1	12/36 (33.33)	28/39 (71.79)*	4/11 (36.36)
CD44V6-10	10/36 (27.77)	21/39 (53.84)	7/11 (63.63)

M: N1 regional normal lymph node (epicolic and paracolic nodes within 5 cm from the colon cancer)
*Show significant differences between normal colorectal mucosa and compared tissues ($p<0.05$)

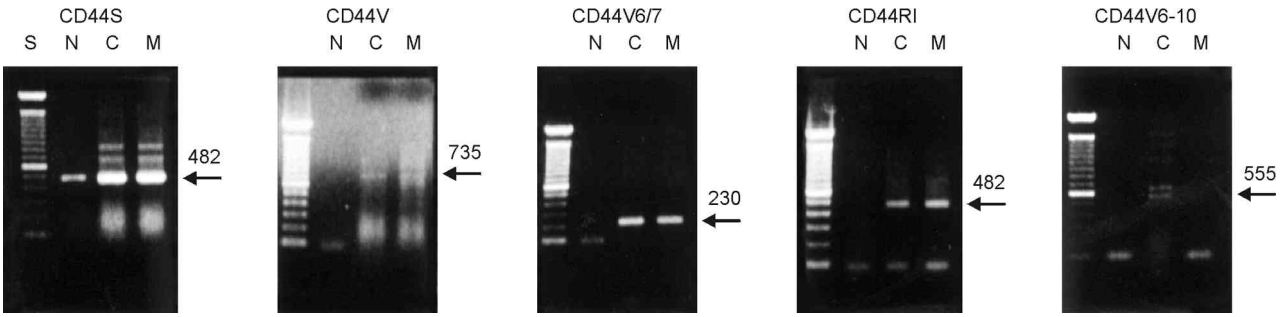

Fig. 2. Electrophoretic analysis of RT-PCR amplification products from normal colorectal mucosa (N), colorectal cancer (C) and regional normal lymph node (M). The PCR products were obtained with primers specific for each CD44 variants (S: 1,2,3 size marker).

histochemical studies have shown limitations in confirming the micrometastasis to the lymph nodes. Recently immunohistochemical method has been used most commonly for the detection of colorectal cancer micrometastasis (15), but it is not considered adequate for the exact diagnosis of micrometastasis, since not all tissues are examined and the interpretations are subjective.

RT-PCR is much more sensitive for detecting metastatic cancer cells in lymph nodes and, therefore, it can be used to detect micrometastasis in lymph nodes (14, 15). The method of RT-PCR targetted at CD44V allows the sensitive detection of few cancer cells in blood or tissues, and helps the diagnosis of colorectal cancer and micrometastasis to the regional lymph node (16). The possibility of using CD44V6/7 as a metastatic marker for the colorectal cancer was reported by many researchers (17-19). Although it has yet to be demonstrated, micrometastasis of regional lymph node has definite relationship with poor prognosis in colorectal cancer patients. The confirmation of micrometastasis in regional lymph node is considered important to know the exact cancer stage and for better care of the patients. Reported role of the CD44V gene in colorectal cancer and its metastasis has been controversial. Several authors reported the association of the expression of CD44 isoforms with the increased progression of human colon cancer and metastatic potential (5, 20-22), and Yamao et al. (23) reported that variant isoforms are expressed in a tissue-specific manner. On the other hand, other studies suggested that none of the CD44 variants correlated with tumor progression or with colorectal tumor metastasis (3, 7, 8).

In our study, we found that the rates of the CD44V and CD44V6/7 expression were significantly high in colorectal cancer and in histologically uninvolved regional lymph nodes of colorectal cancer patients. These histologically normal lymph nodes may well harbor micrometastases, although this is not confirmed in this study. Although CD44R1 was not significantly expressed in normal regional lymph nodes, it seems that CD44R1 is related to tumor progression of colorectal cancer. These findings suggest that CD44V and CD44V6/7 might be useful for the diagnosis of colorectal cancer and the detection of micrometastasis in normal regional lymph nodes of colorectal cancer patients. Further studies are needed to verify whether these histologically "normal" regional nodes are involved in micrometastases and to evaluate the significance of micrometastasis of regional lymph nodes in relation to the prognosis of these patients.

In conclusion, among the various CD44 isoforms, expressions of CD44V and CD44V6/7 were significantly increased than other isoforms in colorectal cancer and in histologically "normal" nodes possibly harboring micrometastases. The analysis of CD44V and CD44V6/7 might be useful for the diagnosis of colorectal cancer and for the detection of micrometastasis in regional lymph nodes of colorectal cancer patients.

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