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Identification of CD44 Splice Variant in Korean Colorectal Cancers and Cell Lines

Seong-Il Suh, M.D.,[1),4)] Won-Ki Baek, M.D.,[1),4)]
Jong-Wook Park, M.D.,[2),4)] Ok-Suk Bae, M.D.,[3),4)]
Min-Ho Suh, M.D.,[1),4)] Byung-Kil Choe, M.D.[2),4)]

Departments of Microbiology,[1)] Immunology,[2)] General Surgery,[3)] and Institute for Medical Science,[4)] School of Medicine, Keimyung University, Taegu, Korea

CD44 is a glycoprotein expressed in a wide variety of cell types. Recently expression of some alternatively-spliced variants of CD44 transcripts(CD44v) has been suggested to play a potential role in tumor metastasis and the detection of CD44v containing exon 6 to 11 may be helpful for the diagnosis of cancers. Expressions of CD44v containing exon 6 to 11 were investigated in 20 human colorectal cancer samples, peripheral blood leukocytes isolated from colorectal cancer patients, and 4 colorectal cancer cell lines using reverse transcription-polymerase chain reaction and Southern blot analysis. The standard form of CD44 transcripts was expressed in all samples tested. CD44v containing exon 6 to 11 was expressed in 18 cases of colorectal cancers(sensitivity=90%), 3 out of 4 cell lines, and one normal tissue(specificity=95%). These results suggest that the expression of CD44v containing exon 6 to 11 can be regarded as tumor specific and that this marker may be helpful for the early diagnosis of colon cancers, if specimens from the early stage are available.

Key Words : *CD44 splice variant, Colorectal cancer*

INTRODUCTION

Colorectal cancers remain one of the most serious health burdens not only in Korea but throughout the world.

The CD44 glycoprotein has been known as a lymph node homing receptor on circulating lymphocytes and is expressed on a wide variety of tissues (Picker et al., 1989). cDNAs encoding the standard form of CD44 transcripts(CD44s) and the alternatively-spliced variants of CD44(CD44v) have been cloned from human tissues including lymphoid tissues, epithelial tissues, hematopoietic tissues, and various cancer cell lines and/or cancer tissue specimens(Brown et al., 1991 : Dougherty et al., 1991 : Hofmann et al., 1991 : Jackson et al., 1992 : Stamenkovic et al., 1991 : Wielenga et al., 1993). The family of CD44 glycoproteins is encoded by a gene that is composed of at least 19 exons containing at least 12 alternatively-spliced exons(Screaton et al., 1992). The CD44 gene is known as one of the most extensively alternatively spliced genes, leading to an extensive size heterogeneity of this cell surface marker. Certain

Address for correspondence : *Byung-Kil Choe, M.D., Ph.D., Professor and Director, Institute for Medical Science, School of Medicine, Keimyung University, 194, Dongsan-dong, Jung-gu, Taegu, 700-310, Korea.*
Tel : (053) 250-7846, Fax : (053) 255-1398

specific splice variants have been found to be overexpressed in human non-metastatic and metastatic cancers(Heider et al., 1993 : Hofmann et al., 1991 : Matsumura and Tarin, 1992 : Tanabe et al., 1993 : Wielenga et al., 1993). Variants containing exon v6(exon 11) have been suggested to play a potential role in tumor metastasis and a newly identified exon 6 was reported to be effective in distinguishing neoplastic and non-neoplastic tissues(Heider et al., 1993 : Herrlich et al., 1993 : Koopman et al., 1993 : Matsumura et al., 1994 : Ponta et al., 1994). Thus, these splice variants were proposed as a diagnostic marker for various cancers or a marker for metastatic potential of cancers(Guo et al., 1994 : Heider et al., 1993 : Herrlich et al., 1993 : Koopman et al., 1993 : Matsumura and Tarin, 1992 : Matsumura et al., 1994 : Ponta et al., 1994 : Tanabe et al., 1993 : Wielenga et al., 1993).

In this study, we examined 20 human colorectal adenocarcinomas and 4 colorectal carcinoma cell lines from Korean patients to assess the expression of CD44v containing exon 6 to 11 as a preliminary study for the early detection of colorectal cancer cells in stool.

MATERIALS AND METHODS

Tumor samples and cell lines

Fresh tumor and normal tissues taken from the vicinity of each cancer as the pairing controls were obtained from patients undergoing surgery for colorectal adenocarcinoma at the Department of General Sugery, Keimyung University Dong San Medical Center, Taegu, Korea. Age, gender, location of tumors, clinical stage, and distant metastases for each patient are shown in Table 1. The samples had been snap-frozen in liquid nitrogen immediately after surgical resection and were transferred to the laboratory, then total RNA was extracted within 30 min of arrival. Four SNU colorectal cancer cell lines established from primary(SNU-C2A, C4, and C5) and metastatic tumor(SNU-C1), and colon adenocarcinoma cell line(HT-29) were obtained from the Korean Cell Line Bank(Seoul, Korea). These cell lines were grown in RPMI 1640 medium supplemented with heat inactivated 10% fetal bovine serum. Cultures were maintained in a humidified incubator at 37 ℃ in an atmosphere of 5% CO_2 and 95% air.

Table 1. Clinical features and results of RT-PCR and Southern blot analysis against CD44v of 20 colorectal cancer tissues

Patient No	Gender	Age	Location	Stage[a)]	Distant metastasis	Expression of CD44v[c)]
1	F	34	rectum	C	−	−
2	F	53	rectum	B	−	+
3	F	65	rectum	A	−	+
4	F	67	sigmoid	C	−	+
5	F	60	rectum	C	−	+
6	M	59	transverse colon	B	−	+
7	F	53	rectum	D	+[b)]	−
8	M	47	rectum	C	−	+
9	M	57	sigmoid	C	−	+
10	M	46	cecum	B	−	+
11	F	52	transverse colon	C	−	+
12	M	57	ascending colon	A	−	+
13	M	70	rectum	C	−	+
14	F	57	rectum	C	−	+
15	M	60	cecum	D	+[b)]	+
16	M	65	cecum	C	−	+
17	M	45	cecum	A	−	+
18	M	61	sigmoid	B	−	+
19	M	62	descending colon	C	−	+
20	F	72	rectum	D	−	+

a) Stages are presented according to the Dukes' classification.

b) Liver metastasis.

c) Results of RT-PCR and Southern blot analysis against CD44v containing exon 6 to 11.

Total RNA extraction

Total cellular RNAs were isolated by homogenizing the tissue in guanidine-HCl buffer(4M guanidium-HCl, 25mM sodium citrate, 10mM ethylendiaminetetraacetic acid, 0.33% antifoam A(Sigma, Saint Louis, MO, USA), 0.1M 2-mercaptoethanol, and 0.5% sarcosyl, pH 7.0) according to the method of Chomczynski and Sacchi(1987). To isolate cellular RNAs from cell lines in suspension cultures, cell pellets were lysed by the addition of 1 ml of RNAzolB(Biotext lab Inc, Texas, USA) per 10^7 cells and cells grown in monolayer were lysed by the addition of 2 ml of RNAzolB per 5 cm^2 flask. To isolate RNAs from peripheral blood leukocytes(PBL) of colorectal adenocarcinoma patients, PBL were purified from 10 ml of whole blood by lysing the red blood cells with Tris-ammonium chloride(0.144 M NH_4Cl and 0.0017 M Tris, pH 7.2), and then PBL were lysed by the addition of 1 ml of RNAzolB per 10^7 cells.

Reverse transcription-polymerase chain reaction (RT-PCR)

Oligonucleotides were synthesized on a PCR-MATE™ DNA synthesizer(Applied Biosystems Inc, Foster, CA, USA). Sequence information for the oligonucleotides used as primers for RT-PCR and probes for Southern blot analysis are given in Table II. Reverse transcriptions were carried out using 4*u*g of total cellular RNA in a total volume of 20 *u*ℓ containing 0.75mM antisense primers(S2 or V2), 1mM deoxyribonucleotides, 20 unit RNase inhibitor, and 25 unit reverse transcriptase in the reaction buffer(20mM Tris-HCl, pH 8.3 ; 50mM KCl ; 5mM $MgCl_2$). First strand cDNAs were obtained after 60 min at 42℃, then denatured for 5 min at 99℃ and 5 min at 5℃ in DNA thermal cycler(Perkin-Elmer Cetus, Norwalk, CT, USA). The total first strand cDNA products were then used as a template for PCR in a reaction mixture that was comprised of 10mM Tris-HCl(pH 8.3), 50mM KCl, 2mM $MgCl_2$, 250mM deoxyribonucleotides, 25 unit/ml AmpliTaq DNA polymerase(Perkin-Elmer Cetus, Norwalk, CT, USA), and 0.75*u*M sense primers(S1 or V1). The PCR was run for 35 cycles according to the following cycle parameters ; denaturation for 1 min at 94℃, annealling for 1 min at 55℃, and elongation for 1 min at 72℃. Positive(HT-29 cell line) control was included in each set of samples tested.

Gel electrophoresis and Southern blot analysis

Ten microliters of each PCR product were electrophoresed through 1.2% agarose gel at 50V for 1h in 1x TAE buffer(0.04M Tris-acetate and 0.001M EDTA). After staining with ethidium bromide(5 μg/ml), the gels were photographed under ultraviolet(UV) light. For Southern blot analysis(Southern, 1975), the DNA in agarose gel was denatured at room temperature for 30 min with a solution containing 0.2N NaOH and 1.5M NaCl and was then neutralized with 1M Tris-HCl(pH 7.0) and 1.5M NaCl. DNA was transferred onto a nitrocellulose filter(Schleicher and Schuell, Dassel, Germany) and immobilized by UV irradiation using a CL-1000 UV crosslinker(UVP, San Gabriel, CA, USA). After prehybridization at 42℃ for 12h in a solution containing 50%(V/V) formamide, 0.1% sodium dodecyl sulfate(SDS), 5% Denhardt's solution(0.1% each of polyvinylpyrrolidone, bovine serum albumin, and Ficoll), 5mM EDTA, 75mM NaCl, and 250*u*g/ml denatured sonicated salmon sperm DNA, the filters were hybridized with the 5'-end

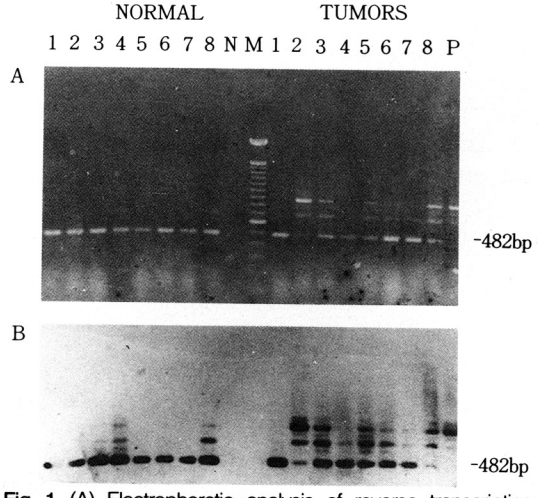

Fig. 1. (A) Electrophoretic analysis of reverse transcription PCR amplification products from normal colorectal mucosa and colorectal adenocarcinomas. N, M, and P represent negative control, 100 base pair(bp) molecular size standard, and positive control(HT-29 cell line), respectively. The PCR products obtained with primers for CD44s, S1 and S2, were resolved on 1.2% agarose gel, stained with ethidium bromide, and visualized under UV light. The 482bp band present in both normal and tumor samples corresponds to the expected region of CD44s amplification product. (B) Southern blot analysis of the gel shown in panel A with the internal probe P1.

labeled probes with [γ-^{32}P]ATP in the same solution at 42℃ for 18h(Arrand, 1985 : Sambrook et al., 1989). The filters were subsequently washed in 2x standard saline citrate(SSC, 0.3M NaCl and 0.03M sodium citrate), 0.1% SDS for 20 min at room temperature with two changes of buffer, then in 1x SSC, 0.1% SDS for 15 min at 65℃, and in 0.1x SSC, 0.1% SDS for 15 min at 65℃. Autoradiographs were obtained after exposure of Kodak X-Omat film(Eastman Kodak Co., Rochester, NY, USA) at -70℃ for 2 days in the presence of an intensifying screen.

RESULTS

To confirm that our RT-PCR system was able to amplify CD44 mRNA transcripts of various sizes, two primers, S1 and S2, were used to amplify segments of CD44s ; hematopoietic(expected copy size 482bp) and epithelial form(expected copy size 878bp), from normal colorectal mucosa and colorectal adenocarcinomas. The 482bp band was present both in normal and tumor samples(Fig. 1A). The 878bp band was observed in 2 cases of normal and 19 cases(95%) of tumor samples(Fig. 1B). However, it is noteworthy to mention that the 878bp fragment and several other larger fragments were overexpressed in 19 cases of tumor samples, suggesting the presence of alternatively spliced transcripts. Southern blot analysis(Fig. 1B), using internal probe(P1) to CD44s, has confirmed that these extra bands were authentic copies derived from S1 and S2 primers.

In our preliminary experiments using V1 and V2 primers, we have also investigated specific expressions of tumor related exons(exon 6 to 11, see Table 2) in various cancers which included colon, bladder, stomach, and thyroid cancers. Significant overexpression of CD44v containing exon 6 to 11 were observed in most of the tumor samples but not in any of the adjacent normal tissues(unpublished data). We considered therefore, V1 and V2 primers designed to amplify exon 6 to 11 transcripts, to be specific for the detection of the neoplastic form of CD44 as stated by Matsumura et al(1994).

The expression of CD44v containing exon 6 to 11 was investigated using primers, V1 and V2. Table 1 and Fig. 2 show details of the 20 patients with colorectal adenocarcinoma. The 735bp band which is the expected tumor related CD44v amplification product, was found in 18 cases of adenocarcinomas and 1 case of normal colorectal mucosa. Southern blot analysis using internal probe, P2, also verified the same results as Fig. 2A.

To test the possibility that non-epithelial cells such as leukocytes infiltrating into the carcinomatous tissues might have expressed CD44v mRNA, the expression of CD44v containing exon 6 to 11 in buffy coat cells of colorectal adenocarcinoma patients was investigated. As shown in Fig. 3, the standard hematopoietic isoform was observed but CD44v containing exon 6 to 11 was undetectable in these samples. These

Table 2. Oligonucleotide primers, internal probes, and exon dentification in the CD44 gene

Oligonucleotides designation in this study	Sequences (5′-3′)	Sequence locations in exon	Reference
Primers for CD44s			
S1	GACACATATTGCTTCAATGCTTCAGC	3-4	Matsumura and Tarin(1992) Mastsumura et al.(1994) Screaton et al.(1992)
S2	GATGCCAAGATGATCAGCCATTCTGGAAT	16-17	Matsumura and Tarin(1992) Matsumura et al.(1994) Screaton et al.(1992)
Primers for CD44v[a)]			
V1	TTGATGAGCACTAGTGCTACAGCA	6	Matsumura et al.(1994)
V2	TCCTGCTTGATGACCTCGTCCCAT	11	Matsumura et al.(1994)
Probe for CD44s			
P1	CCTGAAGAAGATTGTACATCAGTCACAGAC	4	Matsumura et al.(1994) Screaton et al.(1992)
Probe for CD44v[a)]			
P2	TGAGATTGGGTTGAAGAAATC	11	Matsumura et al.(1994)

a) Primers and probe for CD44v containing exon 6 to 11.

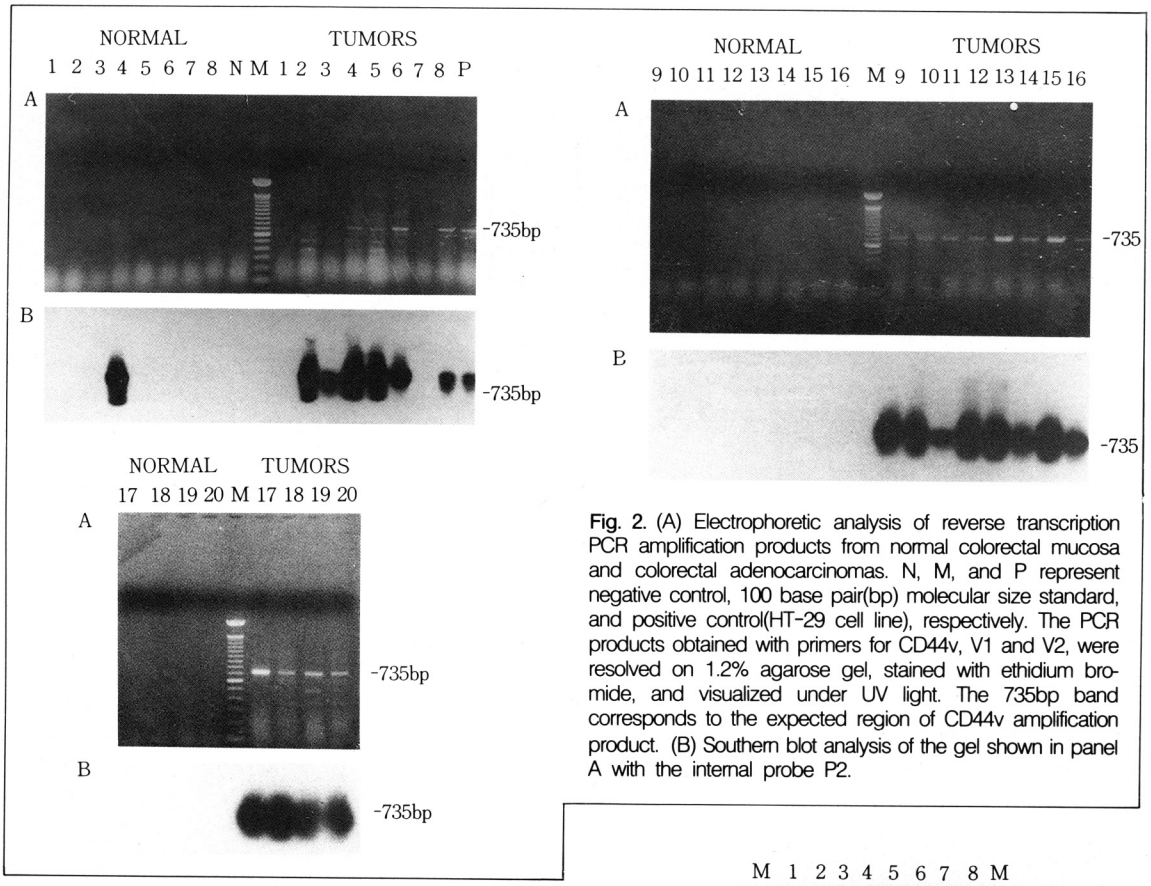

Fig. 2. (A) Electrophoretic analysis of reverse transcription PCR amplification products from normal colorectal mucosa and colorectal adenocarcinomas. N, M, and P represent negative control, 100 base pair(bp) molecular size standard, and positive control(HT-29 cell line), respectively. The PCR products obtained with primers for CD44v, V1 and V2, were resolved on 1.2% agarose gel, stained with ethidium bromide, and visualized under UV light. The 735bp band corresponds to the expected region of CD44v amplification product. (B) Southern blot analysis of the gel shown in panel A with the internal probe P2.

results support the conclusion that transcripts from the exon 6 to 11 of the CD44 gene are uniquely expressed by the tumors. Exceptions to this conclusion were observed ; one tissue from normal colorectal mucosa expressed CD44v containing exon 6 to 11, whereas two cancer tissues each from Dukes' stage C and D patients did not.

To extend our conclusions to the colorectal carcinoma cell lines established from Korean patients, expression of CD44 transcripts was investigated in 4 SNU cell lines. Three SNU cell lines expressed several larger CD44s isoforms as well as CD44v containing exon 6 to 11(Fig. 4A and 4C). The SNU-C1, C2A and C5 cell lines expressed CD44v containing exon 6 to 11 ; particularly the C1 cell line originated from metastatic tumor overexpressed CD44v containing exon 6 to 11(Fig. 4A and 4C). The SNU-C4 cell line expressed only CD44s(Fig. 4B and 4D). Thus the frequency of CD44v(containing exon 6 to 11) expression among Korean colorectal cancer

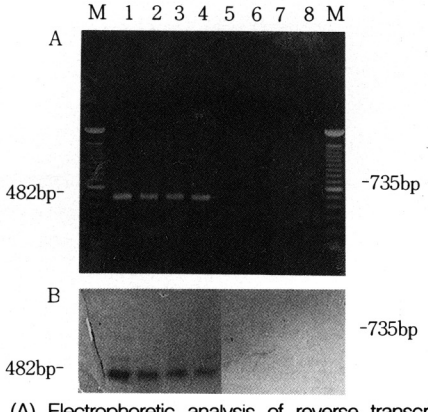

Fig. 3. (A) Electrophoretic analysis of reverse transcription PCR amplification products from PBL of colorectal adenocarcinoma patients. M represents 100 base pair(bp) molecular size standard. The PCR products obtained with primers for CD44s, S1 and S2(lane 1 - 4) and for CD44v, V1 and V2(lane 5 - 8), were resolved on 1.2% agarose gel, stained with ethidium bromide, and visualized under UV light. The 482 bp and 735bp bands correspond to the expected region of CD44s and CD44v amplification products, respectively. (B) Southern blot analysis of the gel shown in panel A with the internal probe P1 and P2, respectively.

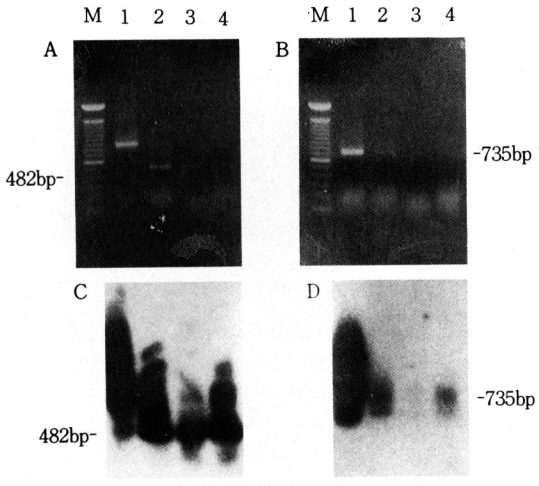

Fig. 4. (A and B) Electrophoretic analysis of reverse transcription PCR amplification products from SNU colorectal cancer cell lines. M represents 100 base pair(bp) molecular size standard. Lanes : 1, SNU-C1 ; 2, SNU-C2A ; 3, SNU-C4 ; and 4, SNU-C5. The PCR products obtained with primers for CD44s, S1 and S2(A) and for CD44v, V1 and V2(B), were resolved on 1.2% agarose gel, stained with ethidium bromide, and visualized under UV light. The 482bp and 735bp bands correspond to the expected region of CD44s and CD44v amplification products, respectively. (C and D) Southern blot analysis of the gel shown in panel A and B with the internal probe P1 and P2, respectively.

cell lines(75%) seems to reflect the frequency of the CD44v(containing exon 6 to 11) of clinical cancers in Korea.

DISCUSSION

Most of our tumor samples(90%) overexpressed a number of larger CD44s isoforms and CD44v containing exon 6 to 11. While one case of normal control expressed CD44v containing exon 6 to 11, rest of the normal controls expressed only CD44s. Thus, the CD44v containing exon 6 to 11 seems to be uniquely related to colorectal adenocarcinoma. However, our data could not establish the correlation between expressions of CD44v(containing exon 6 to 11) and metastatic behavior of the cancer. In the one instance where a normal control expressed CD44v containing exon 6 to 11, we do not know whether this is a false-positive case, or an indication of imminent malignant transformation. Furthermore two cases of tumor samples and SNU-C4 did not express CD44v containing exon 6 to 11. Non-detection is not likely to be due to technical failure because the CD44s were observed in these cases.

Some years ago, Butcher and his collaborators indicated that lymphocytes may migrate through activated high-endothelial venules presumably using CD44 as one of the homing devices(Picker et al., 1989). When Stamenkovic et al.(1989) cloned CD44 cDNA sequences, they found that CD44 was not only expressed by lymphoid cells but also by a number of cancer cell lines and by a variety of solid tumor cells. Since then, a number of groups indicated that alternatively spliced CD44 transcripts play a role in the metastasis of certain human tumor cell lines and rat pancreatic adenocarcinoma lines(Gunthert et al., 1991 : Heider et al., 1993 : Herrlich et al., 1993 : Koopman et al., 1993 : Ponta et al., 1993). Herrlich et al.(1993), therefore, proposed a hypothesis that both activated lymphocytes and metastasizing tumor cells bind a specific ligand in the lymph nodes through CD44 v6 variants. The establishment of a correlation between the overexpression of CD44v and the metastatic potential of the tumor may require further exhaustive clinical investigation.

The strong association between altered CD44 gene expression and neoplasia reported by us as well as by others(Guo et al., 1994 : Hofmann et al., 1991 : Koopman et al., 1993 : Matsumura and Tarin, 1992 : Matsumura et al., 1994 : Tanabe et al., 1993 : Wielenga et al., 1993) establishes that CD44v will be a useful marker for the malignancy. We found that the detection of CD44v(containing exon 6 to 11) was quite specific in tumor samples and SNU cell lines, therefore we did not titrate the detection limit of tumor cells in this investigation. However, in order to extend the applicability of this method to colorectal cancer screening, we are now trying to find out if this method can be extended to the detection of shedded cancer cells in the stool.

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