

## Expression of CD44 Splice Variant mRNA in Bladder Cancers and its Diagnostic Application

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—국문초록—

### 방광암에 있어서 CD44유전자 변이형의 발현과 조기진단에의 응용

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대부분의 암은 조기에 발견할 수만 있다면 외과적 절제술 등의 방법에 의해 치유가 가능하다. 암의 조기 진단법에는 여러가지가 있는데 주로 암세포에서 발현되는 tumor marker가 이용되고 있다. 최근에 lymphocyte homing receptor로 알려진 CD44의 변이형(CD44v)이 주로 암세포에서 발현됨으로 새로운 tumor marker로의 이용 가능성이 보고되고 있다. 본 연구는 방광암 환자의 뇨로부터 암세포를 분리한 후 역전사중합효소연쇄반응(RT-PCR) 및 Southern blot analysis를 실시하여 CD44v의 발현을 조사하여 방광암진단 및 조기 진단의 가능성을 알아보았다. 정상뇨(15명)의 경우 15명 전부에서 CD44표준형(CD44s)의 발현은 관찰되었으나 CD44v의 발현은 검출 전부터 볼 수 없었다(특이도=100%). 방광암 환자의 뇨(15명)의 경우에서는 CD44s의 발현이 관찰된 12명 중 10명에서 CD44v의 발현이 관찰되었다(민감도=83%). 이상의 결과를 미루어 볼때 CD44v는 방광암세포에서만 특이적으로 발현됨으로 tumor marker로서의 이용 가능성을 알 수 있었으며, RT-PCR방법 또한 신속하고 정확한 방법으로 알려져 있어 이 방법을 이용한 뇨에서 CD44v의 검출은 방광암의 조기검출 및 진단에 이용 가능성이 있음을 알 수 있었다.

**Key Words:** CD44v, Bladder cancer

### INTRODUCTION

The CD44 glycoprotein is known to be a re-

ceptor for lymphocyte homing and is expressed not only on lymphoid tissues but also on epithelial tissues, hematopoietic tissues, and various cancer cell lines and/or cancer tissue specimens<sup>1~5</sup>). The CD44 gene which has been mapped to the short arm of chromosome 11 is composed of at least 19 exons containing at least 12

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alternatively-spliced exons<sup>9)</sup>.

Cancer patients mainly die of cancer metastasis. Early diagnosis and surgical removal of cancer enhance the chance of survival, thus early detection of cancer is desirable. Of the approach to the cancer control that could decrease the mortality, screening program is the most important one. Recently Matsumura et al<sup>10)</sup> reported the detection of CD44v transcripts in urine samples suggested that it might be helpful for screening of bladder cancers. Certain specific spliced variants of CD44 transcripts (CD44v) containing exon 11 (V6) have been suggested to play a potential role in tumor metastasis and the variants containing a newly identified exon 6 was reported to be effective in distinguishing various neoplastic and non-neoplastic tissues<sup>5-8, 10-15)</sup>. Thus, these splice variants were proposed as a new tumor marker or a marker of metastatic potential of various cancers<sup>8, 10-15)</sup>.

In present study, we have sought to investigate the occurrence of CD44 transcripts in urine samples of bladder cancer patients using reverse transcription-polymerase chain reaction (RT-PCR) and Southern blot analysis in order to assess the possibility of screening the bladder cancer cells in the urine.

## METHODS

### Urine samples and Total RNA extraction

About 50 ml of naturally voided urine samples from 15 patients with known bladder cancer and 15 with no evidence of the disease were transported to our laboratory within one hour after collection. The samples were centrifuged to pellet the urinary epithelial cells and the pellet was suspended for extraction of total RNA. In case of gross hematuria, red blood cells were lysed by Tris-ammonium chloride buffer (0.144 M NH<sub>4</sub>Cl and 0.0017 M Tris, pH 7.2)<sup>9)</sup>. Total cel-

lular RNAs were isolated by using RNeasy (Biotext lab Inc, Texas, USA).

### Reverse transcription-polymerase chain reaction

Oligonucleotides were synthesized on a PCR-MATE<sup>TM</sup> DNA synthesizer (Applied Biosystems Inc, Foster, CA, USA). Primer sequences to amplify standard form of CD44 were: S1, 5'-GACACATATTGCTTCAATGCTTCAGC-3' and S2, 5'-GATGCCAAGATGATCAGCCATTC TGAAT-3'. Primers for amplification for variant form of CD44 were: V1, TTGATGAGC ACTAGTGCTACAGCA-3' and V2, 5'-TCCT GCTTGATGACCTCGTCCCAT-3' which will amplify the regions of exon 6 to 11. Reverse transcriptions were carried out in a total volume of 20  $\mu$ l containing 0.75 mM antisense primers (S2 or V2), 1 mM deoxyribonucleotides, 20 unit RNase inhibitor, and 25 unit reverse transcriptase in the reaction buffer (20 mM Tris-HCl, pH 8.3; 50 mM KCl; 5 mM MgCl<sub>2</sub>). First strand cDNAs were obtained after 60 min at 42°C, then denatured for 5 min at 99°C and 5 min at 5°C in DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA). The total first strand cDNA products were then used as template for PCR in a reaction mixture that comprised of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl<sub>2</sub>, 250 mM deoxyribonucleotides, 25 unit/ml AmpliTag DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA), and 0.75 mM sense primers (S1 or V1). The PCR was run 35 cycles according to the following cycle parameters; denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and elongation for 1 min at 72°C. 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 products were

electrophoresed through 1.2% agarose gel at 50V for 1 h in  $1\times$  TAE buffer (0.04 M Tris-acetate and 0.001 M EDTA). After staining with ethidium bromide (5  $\mu$ g/ml), the gels were photographed under ultraviolet (UV) light. For Southern blot analysis, the DNA in a agarose gel was denatured at room temperature for 30 min with a solution containing 0.2 N NaOH and 1.5 M NaCl and was then neutralized with 1 M Tris-HCl (pH 7.0) and 1.5 M NaCl. DNA was transferred onto a nitrocellulose filter (Schleicher and Schuell, Dassel, Germany) and immobilized by UV irradiation using CL-1000 UV crosslinker (UVP, San Gabriel, CA, USA). After pre-hybridization at 42°C for 12 h 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), 5 mM EDTA, 75 mM NaCl, and 250  $\mu$ g/ml denatured sonicated salmon sperm DNA, the filters were hybridized with the 5'-end labeled probes with [ $\gamma$ - $^{32}$ P]ATP in same solution at 42°C for 18 h<sup>16-18)</sup>. Probes for standard and variant form were P1 (5'-CCTG-AAGAAGATTGTACATCAGTCACAGAC-3') and P2 (5'-TGAGATTGGGTTGAAGAAATC-3'), respectively. The filters were subsequently washed in  $2\times$  standard saline citrate (SSC, 0.3 M NaCl and 0.03 M sodium citrate), 0.1% SDS for 20 min at room temperature with two changes of buffer, then in  $1\times$  SSC, 0.1% SDS for 15 min at 65°C, and in  $0.1\times$  SSC, 0.1% SDS for 15 min at 65°C. Autoradiographs were obtained after the exposure to Kodak X-Omat film (Eastman Kodak Co., Rochester, NY, USA) at -70°C for 2 days in the presence of an intensifying screen.

## RESULTS

In the preliminary investigation of urinary cancer cells, immunocytological detection of

CD44v positive cancer cell was attempted with variable results, therefore we turned to the analysis of CD44v mRNA in the shedded cancer cells. To analyze the expression of CD44s and CD44v mRNA in bladder cancer, we used RT-PCR. To confirm that our RT-PCR system was able to amplify CD44s mRNA transcripts, S1 and S2 primers were used to amplify segments of CD44s; hematopoietic (expected copy size, 482bp) and epithelial isoform (expected copy size, 878bp), from urine samples of normal and bladder cancers. The 482bp and 878bp band of CD44s isoform transcripts were observed in most cases of normal and cancer samples (Figure 1 and 2). And we have also investigated specific expressions of known tumor related exons (exon 6 to 11) in various cancers which included colon, 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. Also, RT-PCR products of CD44s and CD44v were cloned into the pGEM-T vector (Promega, Madison, WI). The inserts were sequenced according to the dideoxy chain termination method. Sequences of isoforms of CD44s and CD44v of RT-PCR products were exactly identical to the hematopoietic isoform (GeneBank Access number, X56794), the epithelial isoform (GeneBank Access number, X55105), and CD44v containing exon 6 to 11 (sequence data from Screaton et al<sup>9)</sup>, Matsumura et al<sup>10)</sup>, and GeneBank Access number, X62739). Thus, we considered primers designed to amplify CD44s and CD44v transcripts, to be specific for the detection of neoplastic form of CD44 as stated by Matsumura et al<sup>10)</sup>. The expression of CD44s was investigated using primers, S1 and S2. Figure 1 shows details of the 15 normal samples. All of normal sample expressed CD44s (482bp), and 12 samples of bladder cancer patients ex-

**Fig. 1.** (A) Electrophoretic analysis of reverse transcription PCR amplification products from normal urine samples. N, M, and P represent negative control, 100 base pair(bp) molecular size standard, and positive control(HT-29 cell lines), 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 482 bp band present in normal samples corresponds to the expected region of CD44s amplification product. (B) Southern blot analysis of gel shown in panel A with internal probe P1. (C) Autoradiograph of filter amplified with primers, V1 and V2, and probed with internal probe P2.

pressed CD44s. Neither CD44s(Fig. 2, lane 5, 14, 15) nor CD44v(Fig. 3, lane 5, 14, 15) were amplified in three urine samples from cancer patients. We believe that the absence of the bands may have been due to the RNA degradation. The 735bp band, the expected tumor related

CD44v amplification product, was not found in any case of normal samples(Figure 1) and was only detected in 10 of the 12 cancer specimens which were confirmed the presence of satisfactory mRNA by CD44s amplification products in urine. Southern blot analysis using internal

**Fig. 2.** (A) Electrophoretic analysis of reverse transcription PCR amplification products from urines samples of bladder cancer patients. N, M, and P represent negative control, 100 base pair(bp) molecular size standard, and positive control(HT-29 cell lines), 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 482 bp band present in cancer samples corresponds to the expected region of CD44s amplification product. (B) southern blot analysis of gel shown in panel A with internal probe P1.

probes, P1 and P2, also verified the same results of RT-PCR.

These results supports the conclusion that transcripts from the exon 6 to 11 of CD44 gene is uniquely expressed by the tumors.

## DISCUSSION

The CD44 gene is known as one of the most extensively alternatively spliced genes, thus the alternative splicing of this gene transcripts has led to an extensive size heterogeneity of this cell surface maker. Originally, CD44 was known

as lymphocyte homing receptor. But recently, some specific splice variants were expressed in tumor-specific and tumor metastasis-associated manner, thus these variants were proposed as a diagnostic marker of various cancers. In present study, we have investigated the expression of the splice variants(CD44v) in bladder cancers by using RT-PCR method and assessed the possibility of this method as a screening method of bladder cancer. Using RT-PCR technique, we demonstrated the cancer associated expression of CD44v containing exon 6 to 11. Most of our tumor samples(83%) expressed CD44v contain-

**Fig. 3.** (A) Electrophoretic analysis of reverse transcription PCR amplification products from urine samples of bladder cancer patients. N, M and P represent negative control, 100 base pair(bp) molecular size standard, and positive control(HT-29 cells lines), 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 present in cancer samples corresponds to the expected region of CD44v amplification product. (B) Southern blot analysis of gel shown in panel A with internal probe P2.

ing exon 6 to 11 at the detectable level, whereas it was not detected in any of the 15 normal samples. Thus, the CD44v containing exon 6 to 11 seems to be uniquely related to the bladder cancers. Three cases of tumor sample did not express CD44v containing exon 6 to 11, but it is more likely that it is caused by RNA degradation during RNA extraction because the CD44s also were not observed in these cases. The strong association between altered CD44 gene expression and neoplasia reported by us as well as by others<sup>5,8,11~16)</sup> establishes that CD44v will be a useful marker of the malignancy. Although we did not titrate the detection limit of tumor cells in this investigation, we found that the de-

tection of CD44v(containing exon 6 to 11) was quite specific in tumor samples. RT-PCR system is a rapid and reliable method. Taken together, detection of CD44v in urine samples by RT-PCR may be useful for screening of bladder cancers. The mechanism for increased expression of the CD44v transcripts containing exon 6 to 11 in cancerous tissue remains to be determined. However, we found that two samples of bladder cancer patients which expressed CD44s transcripts did not express CD44v transcripts. This result means that tumor always does not express CD44v.

Picker et al<sup>11)</sup> and a number of research groups<sup>2,5,6,11~15)</sup> indicated that lymphocytes may migrate

through activated high endothelial venules presumably using CD44 surface glycoprotein as one of the homing device and found that alternatively spliced transcripts of CD44 have a role in metastasis of certain human tumor cell lines and a rat pancreatic adenocarcinoma lines. And then, Herrlich et al<sup>15</sup> proposed that, through CD44 variant glycoprotein, metastasizing tumor cells may bind a specific ligand in the lymph nodes like lymphocytes. However, our data could not establish the correlation between expressions of CD44v and metastatic potential of the cancer and currently experiments to clarify the relationship between the overexpression of CD44v and metastatic activity in bladder cancer are underway in our laboratory.

### CONCLUSION

CD44 cell surface glycoprotein has been known as a lymphocyte homing receptor. Recently, some specific alternatively spliced variants of CD44 transcripts have been found to be overexpressed in human tumors, and have been proposed as a diagnostic marker of various tumors. We analysed the CD44s and CD44v in urine samples of bladder cancer patients and of normal person with no evidence of neoplasia. Total RNA were isolated from urine samples and RT-PCR were run using primers against cDNA of CD44s and a splice variant region covering exon 6 to 11. RT-PCR products of CD44s were detected in 15(100%) of 15 normal samples and in 12 of the 15 cancer specimens, and PCR amplification products of CD44v was detected in 10(sensitivity=83%) of the 12 cancer specimens which were confirmed the presence of CD44s transcripts in urine, but none of the normal samples expressed CD44v transcripts(specificity=100%). Southern blot analysis using internal probes also verified the same results of

RT-PCR. These results suggest that expression of CD44v covering exon 6 to 11 is uniquely related to the cancer progression, thus detection of CD44v covering exon 6 to 11 may be helpful for the screening and early diagnosis of bladder cancer.

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