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High-level Expression of the CD44 Variant Sharing Exon v10 in Renal Cancer

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To examine whether renal cell carcinoma displays altered CD44 expression we performed reverse transcription-polymerase chain reaction (RT-PCR) analysis of CD44 in 38 specimens from renal cancer, normal kidney and metastases of 19 patients and 6 renal cancer cell lines. To detect the CD44 variants, we utilized the RT-PCR Southern blot method. One out of 19 (5.3%) renal cancer specimens expressed a larger molecular weight band than 1 kb by RT-PCR analysis, in contrast to previous findings in colon and breast cancer. The band patterns in RT-PCR were different in 14/17 (82.4%) cases between normal kidney and tumors, and a band of about 700 bp was especially marked in 12/17 (70.6%) tumor specimens and 4/6 (66.7%) cell lines. By cloning and sequencing of the 700 bp band, we found that this variant is identical to the CD44 variant sharing only exon v10. Examination by Northern blot analysis has revealed that all tumors express a higher level of CD44 mRNA than paired normal kidneys. These findings suggested that the CD44 variants sharing exon v10 play some role in renal cancer.

Key words: CD44 — Exon v10 — Renal cancer — RT-PCR

Tumor metastasis consists of multiple steps, including detachment from the primary tumor, migration into the extracellular matrix, invasion into the vessels, transportation in the vessels, adhesion to the endothelium, reentry and metastatic growth. Even if cancer cells can enter the vessels, most can not metastasize.[1)] The details of metastasis remain unknown, but various adhesion molecules are considered to be critical.

CD44 is a widely distributed transmembrane glycoprotein which plays an important role in lymphocyte homing,[2,3)] lymphocyte activation[4,5)] and cell-cell adhesion, including tumor metastasis.[6,7)] Many CD44 variants are generated by the alternative splicing mechanism and a series of alternative exons are inserted at the membrane proximal region of the extracellular domain.[8–10)] Recently, qualitative and quantitative changes of CD44 expression were reported in various cancers.[11–15)] In particular, CD44 variants sharing exon v6 are considered to be related to lymph node metastasis.[16–18)] The quantitative increase of CD44 variants has also been considered to increase the metastatic potential of cancer cells.[19)] A relation between the different expressions of the CD44 variants and the growth types of the tumor has also been reported.[20)]

In this study, we examined the expression of CD44 variants on renal cancer by various methods and compared the findings with the clinico-pathological features. We also discuss the difference between renal cancer and other cancers and the characteristics of the CD44 variants in renal cancer.

MATERIALS AND METHODS

Tissue samples Thirty-eight fresh tissue samples (19 primary renal cancers, 3 metastatic cancers, 16 normal kidney tissues) were obtained from surgically resected specimens of 19 renal cancer patients showing various clinico-pathological features (Table I).

Cultured cell lines Six cell lines established from human renal cancer were used for analysis. Caki-1, Caki-2, ACHN and A704 were obtained from the American Type Culture Collection. KPK-1 and KPK-13 was kindly provided by Dr. S. Naitou, Kyushu University, Fukuoka.

RNA isolation and purification Total RNA was prepared from tissue specimens and cell lines by the guanidium thiocyanate/cesium chloride method.

RT-PCR-Southern blot analysis The reverse transcription-polymerase chain reaction-Southern blot (RT-PCR-Southern blot) was performed as described by Matsumura and Tarin.[11)] The synthetic oligonucleotides P1 and P2 were used as PCR primers, S1, K7, K8 , D3 and K10S were used as ^{32}P-labeled CD44 probes. K7 (5′-GCTTGTAGAATGTGGGGTCTCTT-3′), K8 (5′-GAATGGGAGTCTTCTCTGGGTGTT-3′) and K10S (5′-ACAGGTGGAAGAAGAGACCC-3′) were generated in our department. The probe S1 theoretically corresponds to exon 4 and detects the total number of different variants. The probe K7 corresponds to exon v5, K8

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Table I. Patients' Characteristics and Results of RT-PCR Analysis

Case	Age	Sex	Grade	INF	Stage[a)]	Growth type[b)]	Histological type[c)]	Emphasis of 700 bp band (T>N)	Ratio of CD44mRNA (T/N)
1	47	F	1	α	I	I	clear cell	+	ND
2	75	M	1	α	II	S	clear cell	−	ND
3	58	F	1	β	I	S	clear cell	+	ND
4	61	F	1	α	II	S	clear cell	+	ND
5	65	M	2	γ	IV	R	granular cell	−	ND
6	58	M	2	α	IV	R	clear cell	+	ND
7	75	F	2	α	II	S	granular cell	−	ND
8	70	M	1	α	III	R	clear cell	+	3.3
9	38	M	1	α	II	S	clear cell	+	ND
10	57	M	1	α	II	S	clear cell	−	ND
11	55	M	2	α	III	I	mixed cell	+	ND
12	74	M	1	α	III	R	clear cell	+	ND
13	68	M	1	α	III	S	clear cell	+	2.6
14	83	M	2	α	IV	R	clear cell	+	2.8
15	50	M	1	α	II	S	clear cell	ND	ND
16	79	F	1	α	III	I	clear cell	ND	ND
17	71	F	3	γ	IV	R	mixed cell	+	5.7
18	52	M	3	γ	IV	R	mixed cell	+	13.5
19	63	F	1	α	I	S	clear cell	−	2.9

a) Robson's classification.
b) Reported previously (Ref. 24).
c) Japanese Urological Association and Pathological Society.
R, rapid type; I, intermediate type; S, slow type; T, tumor; N, normal kidney; ND, not done.

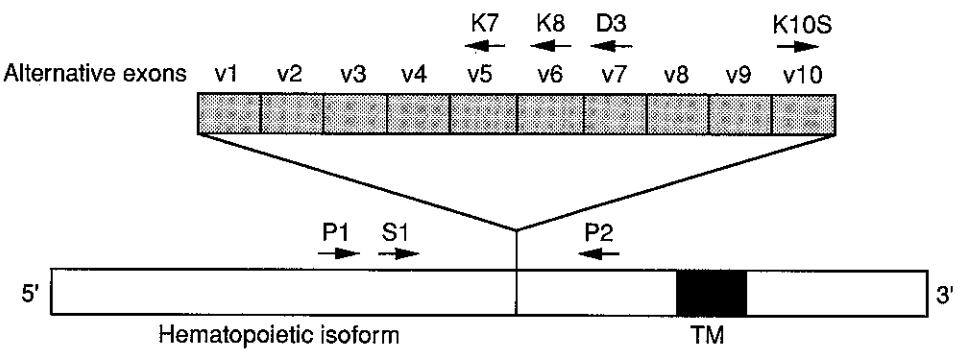

Fig. 1. Schematic representation of the hematopoietic isoform and alternative exons as well as of the oligonucleotides used in the present study. Open box, constitutive exons; crosshatched box, alternative exons; solid box, transmembrane domain (TM); oligonucleotides P1 and P2, PCR primers; S1, K7, K8 and K10S, probes.

corresponds to exon v6 , D3 corresponds to exon v7 and K10S corresponds to exon v10; each probe can detect variants sharing their transcripts (Fig. 1).

Northern blot analysis Six renal cancer cell lines and 15 surgical specimens from 6 patients (6 primary, 3 metastatic, 6 normal) were used for analysis. Five μg of total RNA was loaded per lane on 1% agarose gels containing 2.2 *M* formaldehyde, transferred to nitrocellulose filters and hybridized with a ^{32}P-labeled CD44 fragment which was generated by RT-PCR and in agreement with a hematopoietic variant (482 base pairs (bp)). The glyceraldehyde 3-phosphate dehydrogenase (G3PDH) probe and 28S rRNA oligonucleotide probe were used to check the intactness of the RNA samples.

Flow-cytometric analysis by FACScan Six cell lines were incubated with a monoclonal antibody (mAb) J-173 (Cosmo Bio., Tokyo) or VFF-7 (Bender Medsystems, Vienna, Austria), both of which are specific for human CD44, for 45 min at 4°C, washed in phosphate-buffered saline, incubated with affinity purified fluorescein-

conjugated rabbit anti-mouse antibody for 30 min at 4°C, then washed and analyzed on a FACScan (Becton-Dickinson and Co., Mountain View, CA). The mAb J-173 was considered to correspond to a common region of CD44 variants and the mAb VFF-7, to exon v6 of CD44.

Sequencing of an about 700 bp fragment of RT-PCR product A band of about 700 bp, which is the emphasized band in RT-PCR of renal cancer tissues, was obtained from the RT-PCR product of Caki-1. The fragment was excised from the 1.5% of agarose gel after separation by electrophoresis, and cloned into the pBluescript II SK− vector (Stratagene Cloning Systems, La Jolla, CA). The fragment was sequenced with an AutoRead sequencing kit (Pharmacia P-L Biochemical Inc., Milwaukee, WI) and an autosequencer (A.L.F. DNA sequencer II, Pharmacia P-L Biochemical Inc.).

RESULTS

Expression of CD44 variant in surgical specimens and cultured cell lines and comparison with clinico-pathological features of renal cancer patients In RT-PCR-Southern blot analysis, we used 38 surgical specimens (19 primary, 3 metastatic, 16 normal kidney) and established 6 cell lines. The hematopoietic isoform (482 bp) and numerous additional variants were detectable in all surgical specimens. By RT-PCR analysis (ethidium bromide fluorescence instead of Southern blot analysis probed with S1, because the bands on Southern blot analysis look dimer than those in the case of ethidium bromide fluorescence of the gel), larger molecular weight bands of more than 1 kb were expressed in only 1 of 19 cancer specimens (5.3%) and 1 of 6 cell lines (16.7%). Fourteen out of 17 cases (82.4%) revealed some difference in the band pattern between normal and tumor specimens. In particular, a band of about 700 bp was emphasized in 12 of 17 tumor specimens (70.6%) and 4 of 6 cell lines (66.7%) (Fig. 2). However there was no correlation between the band patterns of CD44 (the expression of a large molecular band of more than 1 kb and the emphasis of the 700 bp band) and the clinico-pathological features (histological type, grade, INF, growth type, clinical stage) (Table I).

Two out of 6 cell lines (33.3%) (KPK-1, A704) showed larger molecular weight bands by RT-PCR-Southern blot analysis. A704 showed several faint bands, and the quantity of CD44 mRNA was considered slight or negligible (Fig. 2).

Some bands of surgical specimens and cell lines correspond to probes K8 and D3, which indicate exon v6 and v7, but the 700 bp band did not (Fig. 3).

Sequence of the fragment of about 700 bp We cloned the 700 bp band into Bluescript II SK− and sequenced it with an autosequencer. The nucleotide sequence of a part of this fragment was identical to exon v10 (195 bp). In the RT-PCR-Southern blot analysis, probe K10S, which corresponds to exon v10, also hybridized intensely to the 700 bp band (Fig. 4).

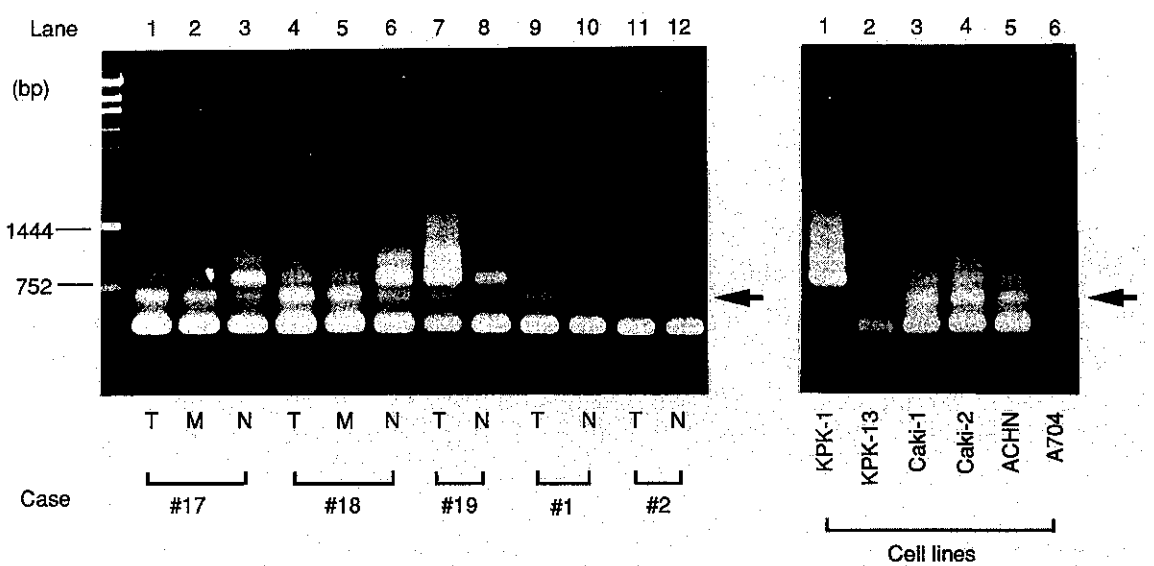

Fig. 2. Photographs of the ethidium bromide fluorescence of the gel (1.5% agarose) after 30 amplification cycles of cDNA with primers P1 and P2 from (left panel) surgical specimens and (right panel) cell lines. The hematopoietic isoform corresponds to the lowest band (482 bp). The arrow shows a band of about 700 bp which corresponds to the CD44 variants sharing only exon v10. Abbreviations used: T, primary renal cancer; M, metastatic renal cancer; N, normal kidney.

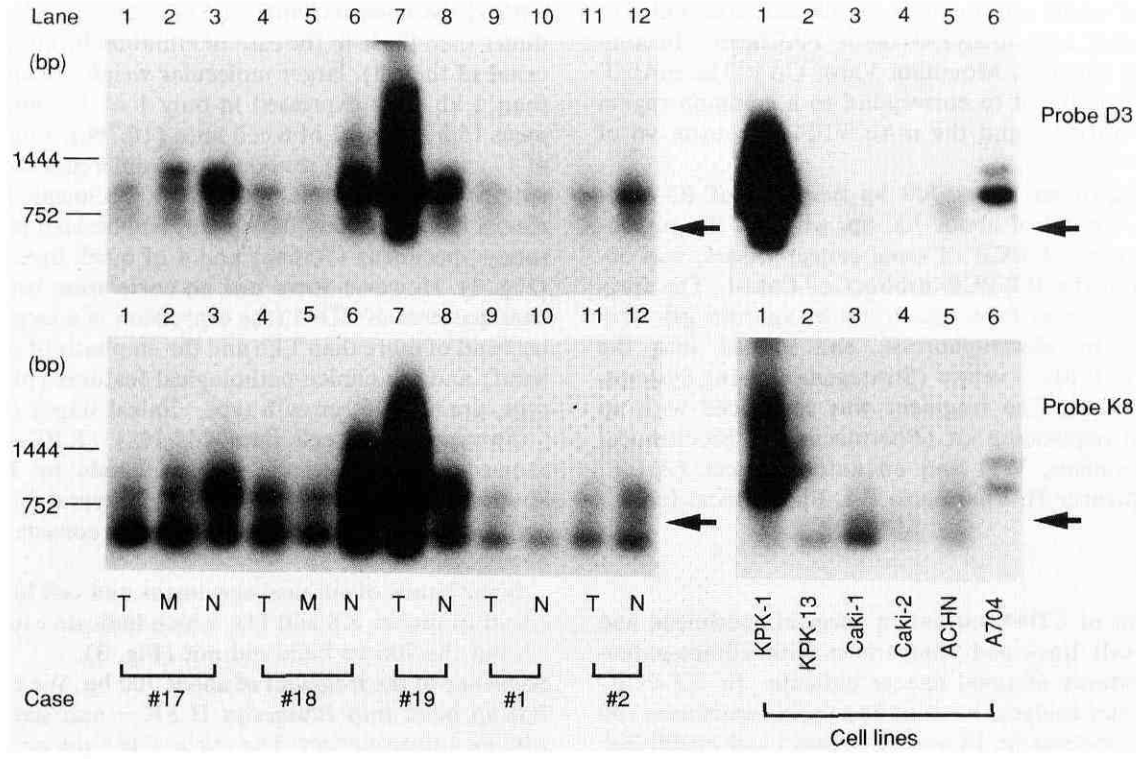

Fig. 3. RT-PCR-Southern blot analysis of surgical specimens and cell lines probed with (upper panel) oligonucleotide D3 and (lower panel) K8. K8 reacts to CD44 variants sharing exon v6 and D3 reacts to CD44 variants sharing exon v7. The arrow shows a band of about 700 bp, to which K8 and D3 did not hybridize. Abbreviations used: T, renal cancer; N, normal kidney.

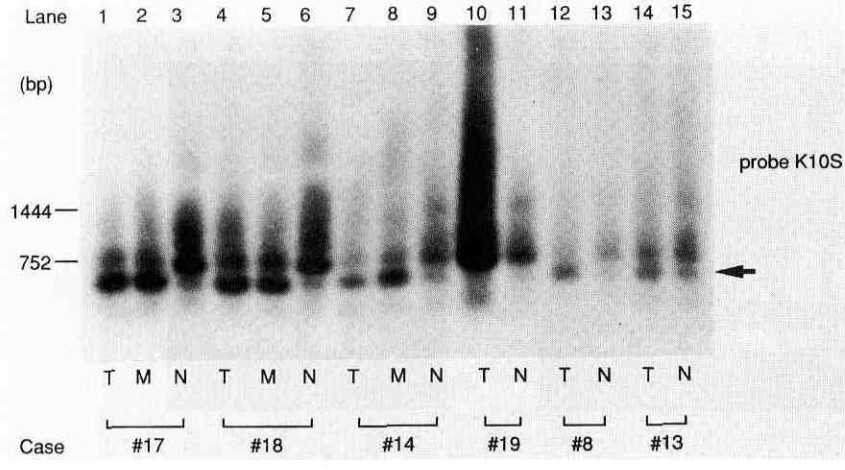

Fig. 4. RT-PCR-Southern blot analysis of surgical specimens probed with oligonucleotide K10S. K10S reacts to CD44 variants sharing exon v10. The arrow shows a band of about 700 bp, to which K10S hybridizes intensely. Abbreviations used: T, renal cancer; N, normal kidney.

Quantitative analysis of CD44 molecules In Northern blot analysis we used 6 cell lines and 15 surgical specimens. In the surgical specimens, the expression of CD44 mRNA was higher in every cancer tissue than in the paired normal kidney tissue. The increase of the ratio of CD44 mRNA/28S rRNA was 2.6- to 13.5-fold as evaluated with the Bio-Image Analyzer BAS2000 (Fuji Film Institution, Tokyo) (Table I). There were no differences between primary and metastatic cancers in the levels and the patterns of expression of CD44 mRNA (Fig. 5).

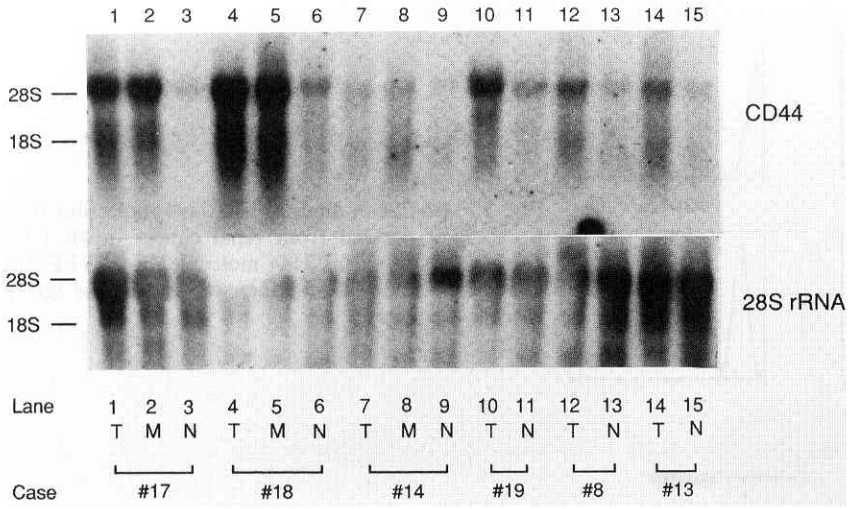

Fig. 5. Northern blot analysis of CD44 mRNA from 15 surgical specimens. The probe is a fragment (482 bp) of hematopoietic CD44. Abbreviations used: T, primary renal cancer; M, metastatic renal cancer; N, normal kidney.

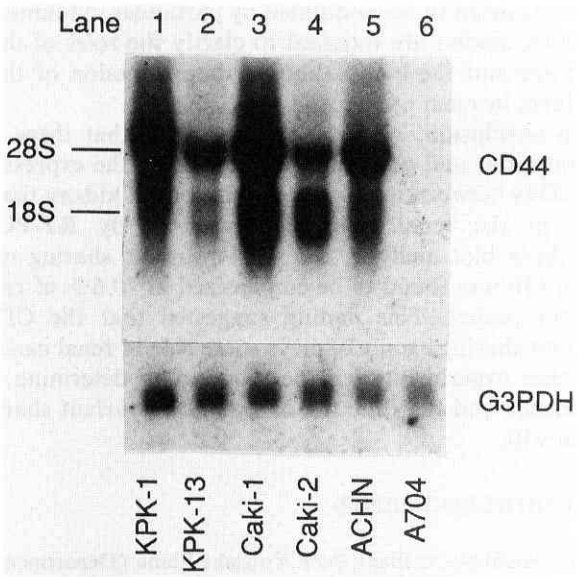

Fig. 6. Northern blot analysis of CD44 mRNA from 6 cell lines derived from renal cancer.

Northern blot analysis of cell lines exhibited two patterns. Although five cell lines (KPK-1, KPK-13, Caki-1, Caki-2, ACHN) expressed a large quantity of CD44 mRNA and three major bands, A704 did not reveal a detectable level of mRNA (Fig. 6). In FACScan analysis, 6 cell lines were examined for the expression of CD44 molecules using mAb J-173 and mAb VFF-7. Five cell lines expressed high levels of CD44 molecules detected with mAb J-173, but showed insignificant expression of molecules detected with mAb VFF-7. A704 expressed a negligible quantity of CD44 molecules (Fig. 7). Interestingly, KPK-1, which expressed a high level of CD44 mRNA, expressed lower levels (six- to thirty-fold decrease) of molecules detectable with mAb J-173 in terms of mean fluorescence intensity. Accordingly, the results of Northern blot and FACScan analyses showed that the two cell lines (KPK-1, A704) which showed large molecular weight fragments on RT-PCR-Southern blot analysis express smaller quantities of CD44 molecules than the other cell lines. The variants sharing exon v6 accounted for a small percentage of all CD44 molecules in all cell lines.

DISCUSSION

Previous reports showed that the expression of CD44 was different both quantitatively and qualitatively between cancer tissues and normal tissues in various cancers, such as colon cancer, gastric cancer and breast cancer.[11-15] The quantitative increase of CD44 molecules was related to the metastatic potential of cancer cells.[19] On the other hand, a qualitative change usually involves appearance of high-molecular-weight variants in the above-mentioned cancer tissues.[11,14] We also recognized similar changes in bladder cancer (unpublished results). The qualitative changes were considered to be caused by abnormality of the alternative splicing mechanism, but the reason for this and the mechanism of the changes in the cancer cells remain unknown.

In the present study, only 1 renal cancer tissue (5.3%) revealed a band of over 1 kb and other renal cancer tissues did not express such large bands in RT-PCR analysis. These findings are different from those reported previously in colon and breast cancers,[11,14,21] but nevertheless, 82.4% of renal cancer tissues expressed a band pattern different from that of the paired normal kidney

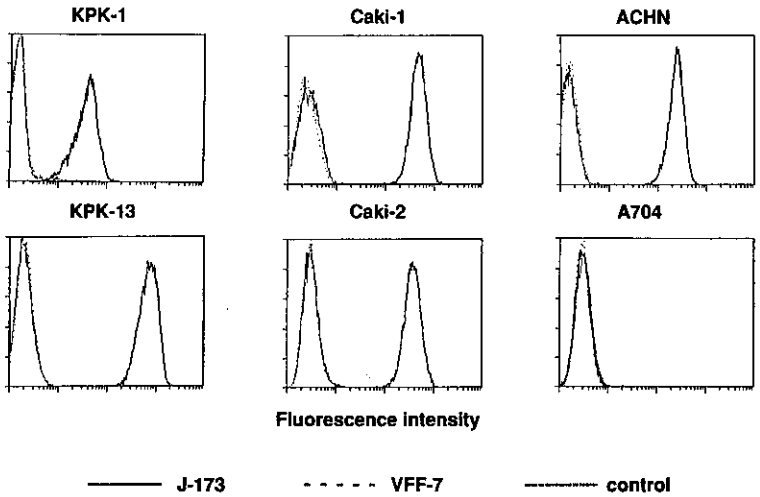

Fig. 7. FACScan analysis of CD44 molecules from 6 cell lines derived from renal cancer. mAb J-173 corresponds to each CD44 molecule, mAb VFF-7 to CD44 molecules sharing exon v6, and mouse IgG is used as a control.

tissue. In particular, a band of about 700 bp exhibited intensified expression in 70.6% of cancer tissues as compared with normal tissues. By cloning and sequencing, the variant was revealed to contain only exon v10 (65 amino acids). Although this variant is not specific to renal cancer tissues, a quantitative increase of this variant was estimated, based on the quantitative increase of CD44 mRNA (two- to thirteen-fold) seen in cancer tissues by Northern blot analysis. However, R1 (epithelial variant: 878 bp fragment in our RT-PCR method) is not a tumor-specific band, though an increase in the ratio of R1/H (hematopoietic isoform) was reported in colon cancer.[21] The relative increase of the CD44 variant sharing exon v10 in RT-PCR also appears to be a special characteristic of renal cancer.

Regarding CD44 variants sharing exon v6, which is thought to be related to metastatic potential, especially in lymph node metastasis,[16–18] some small-molecular-weight bands (about 500 bp–1 kb) were expressed in all surgical specimens and cell lines as determined by RT-PCR-Southern blot analysis. The amounts of these variants in renal cancer cell lines were extremely small by FACScan analysis. Previous reports showed that the expression of CD44 variants sharing exon v6 in normal kidney tissue is negative by immunohistochemistry.[22, 23] Our findings in renal cancer were similar to those reported. Recently, the expression of CD44 variants on some cell types has been reported to be modulated by particular cytokines.[23] Further studies are required to clarify the roles of these variants and the modulation of the expression of these variants in renal cancer.

In conclusion, we have demonstrated that there are quantitative and qualitative differences in the expression of CD44 between renal cancer and normal kidney tissue, and in the renal cancers themselves. By RT-PCR-Southern blot analysis, the CD44 variant sharing only exon v10 was found to be emphasized in 70.6% of renal cancer tissues. This finding suggested that the CD44 variant sharing exon v10 plays some role in renal cancer. Further experiments will be required to determine the incidence and the function of the CD44 variant sharing exon v10.

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