High Expression of the *Cap43* Gene in Infiltrating Macrophages of Human Renal Cell Carcinomas[1]

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ABSTRACT

We used suppression subtractive hybridization to identify highly expressed genes in the cancerous region of human renal cell carcinoma (RCC) compared with noncancerous tissue. Nine genes were identified to show increased expression in the cancerous region compared with the noncancerous region. The nine genes included *thymosin* β*4*, secreted protein acidic and rich in cysteine (*SPARC*), *Cap43*, *ceruloplasmin*, *serum amyloid A*, *osteopontin*, heat shock protein 90 (*HSP90*), *LOT1*, and *casein kinase I*. Of these 9 genes, *in situ* hybridization with 10 clinical samples consistently showed a strong expression of Cap43 mRNA in infiltrating macrophages in RCCs, but not in cancer cells proliferating in an alveolar pattern. However, Cap43 mRNA was also apparently detected in epithelial cells of the renal proximal tubuli in noncancerous tissue. The higher expression of the *Cap43* gene in the cancerous region of RCCs appears to depend on macrophage infiltration. Moreover, treatment with phorbol ester resulted in enhanced expression of the *Cap43* gene in human monocytic cells *in vitro*. The expression of the *Cap43* gene in infiltrating macrophages is discussed in association with the differentiated or activated status of monocyte/macrophage.

INTRODUCTION

RCC,[3] a common urogenital malignancy, is known as one of hypervascular and invasive tumors and often shows resistance to various chemotherapeutic treatments. Alteration of the *VHL* or *TSC* tumor suppressor genes and of the *MET* oncogene is thought to be associated with the formation of renal tumors (1–4). However, how the occurrence and progression of RCCs are induced at the molecular level remains unclear, for the most part. Moreover, both angiogenic and invasive phenotypes of RCC are thought to be associated with production of various angiogenic factors including vascular endothelial growth factor or basic fibroblast growth factor and altered expression of adhesion molecules and/or metalloproteinases (5–8). The isolation and determination of highly expressed genes in cancerous regions of RCCs are expected to be of particular importance. To understand the malignant phenotype of RCCs, various experimental methods have been established to compare patterns of gene expression, including differential display, serial analysis of gene expression, cDNA microarray, and SSH. In our present study, we isolated nine genes highly expressed in the cancerous region of human RCC tissue compared with the noncancerous region by SSH (9). Of the nine genes, we further examined the expression of one gene named *Cap43* in RCCs because *in situ* hybridization with RCC clinical samples consistently showed a strong expression of only Cap43 mRNA in infiltrating macrophages. The expression of the *Cap43* gene in human RCCs is discussed in association with malignant status of RCC and infiltrating macrophages.

MATERIALS AND METHODS

Samples. Noncancerous and cancerous tissue from the same case with a RCC were obtained from a 64-year-old Japanese male. Resected specimens from 10 cases (age range, 42–74 years) with RCCs who underwent operations in the Department of Urology at Kyusyu University Hospital between 1997 and 2000 were examined for dot blot analysis and Northern blot analysis. All surgical specimens were snap-frozen immediately after removal and stored at −80°C. Tumor tissue samples were obtained under an institutional review board-approved protocol, with subjects providing informed consent. Paraffin-embedded sections from 10 RCC cases including the above-mentioned 10 cases were obtained to perform *in situ* hybridization for the *Cap43* gene. Histological confirmation of the diagnosis was obtained in all cases.

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[3] The abbreviations used are: RCC, renal cell carcinoma; SSH, suppression subtractive hybridization; FBS, fetal bovine serum; PMA, phorbol 12-myristate 13-acetate; HSP, heat shock protein; SPARC, secreted protein acidic and rich in cysteine; TAM, tumor-associated macrophage; DIG, digoxigenin.

mRNA Isolation, cDNA Synthesis, and SSH. Total RNA from cancerous and noncancerous tissue was isolated by the guanidium isothiocyanate method. We purified mRNA using oligotex beads (Qiagen). cDNA was synthesized using SMART PCR cDNA synthesis (Clontech). SSH was performed between the noncancerous region (driver) and the cancerous tissue (tester) using the PCR select cDNA subtraction kit (Clontech) according to the manufacturer's recommendations.

Cloning and Sequencing of the Subtracted cDNA and Reverse Northern Dot Blot Analysis. The products from the secondary PCR (subtracted cDNA) were inserted into the PCRII-TOPO vector (TOPO TA cloning kit; Invitrogen), and ligated DNA was transfected into competent cells with selection for ampicillin resistance. One hundred clones were randomly selected and added to Luria-Bertani medium with ampicillin and grown at 37°C. Each plasmid DNA was prepared, and both strands of the isolated cDNA clones were sequenced using a Big-Dye Terminator Cycle Sequencing kit (Applied Biosystems) and a DNA sequencing system (model 377; Applied Biosystems) with M13 universal primers. For sequence analysis, the BLAST program at the National Center for Biotechnology Information was used. We designed specific primers for 32 genes that were identified independently, and a sufficient amount of their PCR products was spotted onto a nylon Hybond-N membrane (Amersham) and hybridized to ^{32}P-labeled cDNA probes from both noncancerous and cancerous tissue. Radioactivity was detected using a Fujix Bas 2000 bio-image analyzer, and the expression level of each gene was normalized to β-actin expression.

Cell Culture. Human monocytic U937 cells were cultured in RPMI 1640 supplemented with 10% FBS. These cells were incubated in culture medium supplemented with 1% FBS for 16 h and treated with or without 10 μg/ml PMA, 100 ng/ml lipopolysaccharide, 1000 IU/ml IFN-α, and 50 ng/ml IFN-γ for 6 h.

RNA Dot Blot and Northern Blot Analyses. For dot blot analysis, 1 μg of total RNA obtained from both noncancerous and cancerous tissue of 10 RCC cases was spotted onto a nylon membrane (Hybond N+; Amersham), respectively. For Northern blot analysis, total RNA (10 μg) was electrophoresed on a 1% agarose gel containing 2.2 μM formaldehyde and transferred onto a nylon membrane. Each membrane was cross-linked with UV light at 0.25 J/cm^2 with FLUO-LINK (Viler Lourmat, Marne-La-Vallee, France). The probes used were fragments amplified by PCR using specific primers. The primers for each gene were as follows: (*a*) *Cap43,* coding strand primer 5′-GCTACAACCCCCTCTTCAAC-3′ and non-coding strand primer 5′-GGGTTCACGTTGATAAGGAC-3′; (*b*) *thymosin β4,* coding strand primer 5′-GCTTCGCTTTTCCTCCGCTA-3′ and non-coding strand primer 5′-CGCACGCCTCATTAC-GATTC-3′; (*c*) *SPARC,* coding strand primer 5′-TCTTTGCCA-CAAAGTGCACC-3′ and non-coding strand primer 5′-CCAGTGTACAGGGAAGATG-3′; (*d*) *osteopontin,* coding strand primer 5′-CAGTCTGATGAGTCTCACCA-3′ and non-coding strand primer 5′-ATGCACCATTCAACTCCTCG-3′; and (*e*) β-*actin,* coding strand primer 5′-TCCTGTGGCATC-CACGAAAC-3′ and non-coding strand primer 5′-GAAG-CATTTGCGGTGGACGA-3′. For RNA dot blot and Northern blot analysis, the procedure after hybridization was performed as described previously (10).

***In Situ* Hybridization.** Fragments amplified by PCR using specific primers of *Cap43* were cloned into pCR2.1 (Invitrogen) in two orientations, and DIG-labeled sense and antisense RNA probes were synthesized with T7 RNA polymerase using a DIG-RNA Labeling Kit (Boehringer Mannheim) according to the manufacturer's instructions. The procedure for *in situ* hybridization was as described previously (10).

Immunohistochemistry. Resected specimens of RCCs were fixed in 10% formalin solution, processed routinely, and embedded in paraffin. Six-μm-thick sections were stained immunohistochemically using an avidin-biotinylated peroxidase complex method with a mouse monoclonal antibody against the macrophage marker CD68 (Dako Glostrup, Denmark). The sections were counterstained lightly with hematoxylin (10).

Quantification of Macrophages. In each case, macrophage infiltration was assessed microscopically in the three hottest after a brief scan of the entire section at low power, and the number of macrophages/microscopic field (×400 magnification) was recorded (10).

RESULTS

Differential Expression of Identified cDNA between the Cancerous and Noncancerous Regions in Human RCC. By SSH, we isolated clones that were relatively highly expressed in the cancerous region compared with the noncancerous region from a surgically resected sample of one case with RCC (see "Materials and Methods"). One hundred clones inserted into the PCRII-TOPO vector were randomly selected, and then they were sequenced. Thirty-two genes identified independently were further tested by reverse Northern dot blot hybridization. Of the 32 genes, the expression of 9 genes was higher in the cancerous region than in the noncancerous region of this case (Fig. 1). These nine genes included *LOT1, HSP90, casein kinase I, thymosin β4, serum amyloid A, ceruloplasmin, SPARC, Cap43,* and *osteopontin*. The expression level of each gene was normalized to β-actin expression. The expression of these nine genes was 1.4–4.3-fold higher in the cancerous region than in the noncancerous region (Table 1).

Specificity of Highly Expressed Genes in RCCs. To examine whether the nine genes are also highly expressed in the cancerous region compared with the noncancerous region in other cases with RCCs, RNA dot blot analysis was performed with 10 RCC cases. Fig. 2*A* shows an example of experimental data for five genes. Of 10 cases, a higher expression of thymosin β4 (8 cases), SPARC (7 cases), Cap43 (6 cases), and ceruloplasmin (6 cases) was seen in the cancerous region as compared with the noncancerous region. The expression levels of Cap43, thymosin β4, SPARC, ceruloplasmin, serum amyloid A, HSP90, and osteopontin mRNA in the cancerous and noncancerous regions were normalized to β-actin levels in 10 cases (Table 2). The expression levels of serum amyloid A, osteopontin, and HSP90 mRNA appeared to be similar between the cancerous and noncancerous regions in 5–6 of 10 cases with RCCs (Table 2). The expression of LOT1 mRNA was not observed by dot blot analysis, and the expression of casein kinase I appeared at similar levels in the cancerous and noncan-

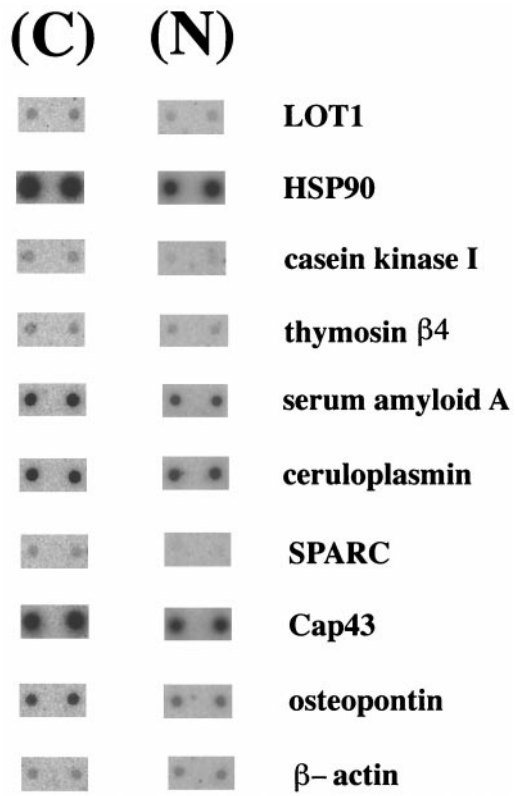

Fig. 1 Reverse Northern dot blot analysis for one case with RCC in which SSH was performed. Of 32 genes identified by SSH and sequence analysis, 9 genes showed altered expression between the noncancerous (*N*) and cancerous region (*C*) in RCCs. Specific primers of those 32 genes were designed, and a sufficient amount of those PCR products was double-spotted onto a nylon Hybond-N membrane and hybridized to ^{32}P-labeled cDNA probes synthesized using gene-specific primers from both the noncancerous and the cancerous tissue. β-Actin expression was the control. Radioactivity was detected and normalized to β-actin expression (see also Table 1).

cerous regions in all 10 cases (data not shown). We further examined mRNA levels of Cap43, thymosin β4, and SPARC in four cases by Northern blot analysis (Fig. 2*B*). Both dot blot and Northern blot analyses consistently showed an increased expression of Cap43 in the cancerous region compared with the noncancerous region in cases 7 and 8 (Fig. 2, *A* and *B*). By contrast, the expression of Cap43 was higher in the noncancerous region than in the cancerous region in cases 6 and 9. Similar results were obtained when the expressions of thymosin β4 and SPARC were compared between dot blot and Northern blot analysis (Fig. 2, *A* and *B*).

Detection of Cap43 mRNA in Infiltrating Macrophages of RCC Tissues. We first examined whether these nine genes were expressed in cancer cells themselves or in other stroma cells in RCCs. The localization of mRNA of these nine genes in RCC tissue was assessed by *in situ* hybridization with each antisense RNA probe in two cases. Of the nine genes, we observed a positive expression of only the *Cap43* gene in infiltrating macrophage-like cells in the cancerous regions (data not shown). Therefore, we further examined the expression of Cap43 mRNA in eight other RCC cases.

Table 1 Genes that were highly expressed in the cancerous region of RCC using a BLAST search of the public databases

Genes	C:N ratio[a]	Appearance[b]	Accession no.[c]
LOT1	3.0	1	U72621
HSP90	1.9	1	X15183
Casein kinase I γ 3L	3.1	1	AF049090
Thymosin β4	2.1	1	E01650
Serum amyloid A	2.4	1	NM 000331
Ceruloplasmin	1.4	1	NM 000096
SPARC	4.3	1	NM 003118
Cap43	2.0	2	AF004162
Osteopontin	2.4	1	J04765

[a] The increase in the signal obtained with the cDNA probe from the cancerous tissue (C) compared with the cDNA probe from the noncancerous tissue (N) by reverse Northern dot blot analysis (see Fig. 1).

[b] The number of times that the same gene appears in the analysis of 100 clones.

[c] The accession number in the public databases.

The expression of the *Cap43* gene was determined in both noncancerous regions and cancerous regions by *in situ* hybridization with an antisense Cap43 RNA probe in 10 RCC cases. Fig. 3 shows one example of *in situ* hybridization. Expression of the *Cap43* gene was detected mainly in epithelial cells of the proximal tubuli (Fig. 3*A*) in the noncancerous regions of all 10 cases. In contrast, a positive staining for the *Cap43* gene was observed only in large and round cells that have a rich cytoplasm, especially near the capsule in the cancerous region of 6 of 10 cases (Fig. 3*C*), and not within cancer cells proliferating in an alveolar pattern in the cancerous regions (Fig. 3*B*). Positive staining for the *Cap43* gene was also found in large, round cells scattered within the mass of cancer cells (data not shown).

In the cancerous regions of RCCs and other human tumors, macrophages are often observed in the stroma (11). The appearance of macrophages was determined by immunostaining with anti-CD68 antibody, which is an antibody specific for macrophages. No apparent infiltration of macrophages is observed in the noncancerous region of RCCs, whereas many CD68-positive cells are observed, mainly near the capsule in the cancerous regions. When examined using a series of specimens, most cells stained for Cap43 mRNA were also positive for anti-CD68 antibody (Fig. 3*D*). We also examined immunostaining with anti-CD3 antibody, which is an antibody for T lymphocytes. However, the distribution of cells stained for Cap43 mRNA was different from that of CD3-positive cells.[4] Cells that stained positively for Cap43 mRNA in the stroma could be predominantly macrophages infiltrating the cancerous regions.

Cap43 Expression and Macrophage Infiltration in the Cancerous Region of RCC Cases. We next examined whether expression of the *Cap43* gene in the cancerous region was correlated with macrophage infiltration. As shown in Fig. 2, dot blot analysis in 10 cases demonstrated Cap43 mRNA expression at various levels. The correlation between Cap43 mRNA levels in the cancerous region and average number of macrophages infiltrating the cancerous region was assessed. We

[4] A. Nishie and M. Kuwano, unpublished data.

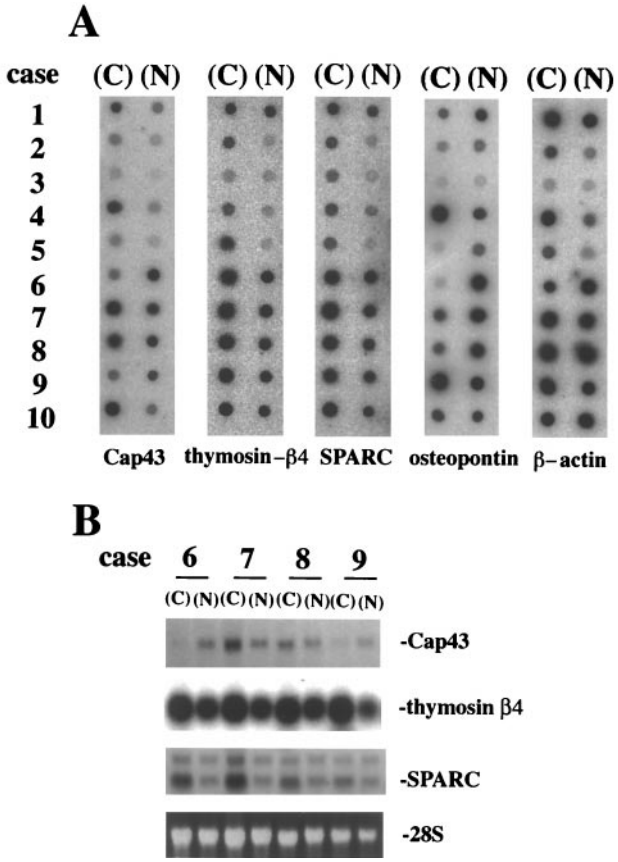

Fig. 2 *A*, RNA dot blot analysis of *Cap43*, *thymosin* β*4*, *SPARC*, and *osteopontin* genes in 10 RCC cases. One μg of total RNA obtained from both noncancerous (*N*) and cancerous tissue (*C*) of 10 RCC cases was spotted onto a nylon membrane. The membrane was hybridized to ^{32}P-labeled double-stranded DNA probes produced by the Klenow fragment, and radioactivity was detected and normalized to β-actin expression. *B*, Northern blot analysis of four RCC cases including cases 6–9. Ten μg of total RNA obtained from both noncancerous (*N*) and cancerous (*C*) tissue of four RCC cases were transferred onto a nylon membrane. The membrane was hybridized to ^{32}P-labeled double-stranded DNA probes produced by the Klenow fragment. The radioactivity was normalized to each rRNA.

observed a significant correlation ($r = 0.640$) between Cap43 mRNA levels and macrophage infiltration (Fig. 4).

Up-Regulation of the *Cap43* Gene in Human Monocytic Cells by a Differentiating Agent. *In situ* hybridization analysis demonstrated an apparent expression of the *Cap43* gene in macrophages in the cancerous region of six RCC cases examined. We further examined whether the expression of the *Cap43* gene was induced in human monocytic cells by various activating agents. The expression of Cap43 mRNA was specifically increased three times by PMA (Fig. 5), consistent with the findings of a previous study (12). No apparent change in Cap43 mRNA levels was observed using lipopolysaccharide, IFN-α, and IFN-γ.

DISCUSSION

In this study, we showed a number of differentially expressed genes in human RCC and described the usefulness of SSH with material obtained from primary cancer tissue. Reverse Northern dot blot analysis was performed to reduce the number of false positive clones. Nine of 32 genes identified by sequencing were highly expressed in the cancerous region compared with the noncancerous region in RCC. Sequencing analysis of the nine clones showed a high degree of homology with already known genes. Of the nine genes, the expressions of *SPARC*, *thymosin* β*4*, and *ceruloplasmin* were higher in the cancerous region than in the noncancerous region in more than 6 of 10 cases when surgically resected specimens were analyzed. *SPARC*, *thymosin* β*4*, and *ceruloplasmin* genes were reported to be closely associated with malignancy or angiogenesis in various kinds of tumors (13–22).

The *Cap43* gene was isolated as a gene induced with nickel compounds in human bronchoalveolar epithelial cells using the differential display technique (23). This gene expresses a 3.0-kb mRNA encoding a 43-kDa protein and has three human homologues (RTP, Drg-1, and rit42; Ref. 23). The Cap43 protein contains a new motif consisting of 10 amino acids repeated three times in the COOH terminus (23). The expression of the *Cap43* gene is relatively low in the cancerous region compared with the noncancerous region in colon, breast, and prostate cancers (24, 25). Two studies have shown that Cap43 might be involved in growth, differentiation, and metastasis of tumor cells (25, 26). Contrary to these previous studies, the expression of Cap43 mRNA was higher in the cancerous region than in the noncancerous region in 6 of 10 cases with RCCs. *In situ* hybridization showed that a strong staining for the *Cap43* gene was observed mainly in the stroma cells, especially near the capsule within the cancerous region, and that most of the Cap43-positive stromal cells were macrophages infiltrating the RCCs. Moreover, we observed a significant correlation between Cap43 mRNA levels and macrophage infiltration in the cancerous region of RCCs (Fig. 4). Expression levels of the *Cap43* gene in the cancerous regions of RCCs thus appear to be due to the number of Cap43-positive macrophages.

Infiltration of TAMs is often observed in RCCs (11). Macrophage infiltration is closely associated with angiogenesis and malignancy in various human tumor types, such as breast cancer, colon cancer, brain tumor, melanoma, and non-Hodgkin's lymphoma (10, 27–30). Furthermore, TAMs infiltrating human breast cancers (27) and malignant melanomas

Table 2 Expression patterns of identified genes between the cancerous (C) and their noncancerous regions (N) in 10 RCC cases

Genes	C > N[a]	C = N[b]	C < N[c]
Thymosin β4	8	0	2
SPARC	7	2	1
Ceruroplasmin	6	3	1
Cap43	6	2	2
Serum amyloid A	3	5	2
Osteopontin	2	6	2
HSP90	1	5	4

[a] The number of cases in which each gene is highly expressed in the cancerous region compared with the noncancerous region.

[b] The number of cases in which each gene is equally expressed between the cancerous region and the noncancerous region.

[c] The number of cases in which each gene is highly expressed in the noncancerous region compared with the cancerous region.

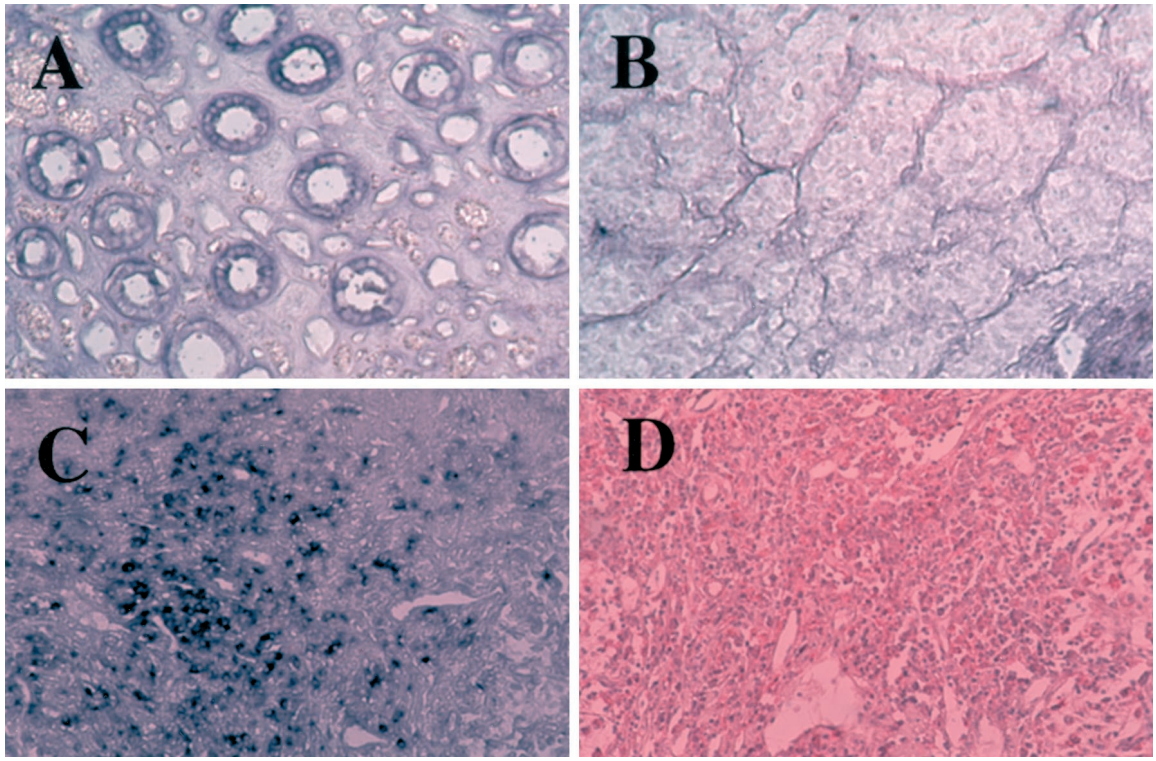

Fig. 3 Detection of Cap43 mRNA by *in situ* hybridization and immunohistochemical staining for CD68 in RCCs. DIG-labeled sense and antisense RNA probes of Cap43 were synthesized and hybridized to the RCC section of case 7. *A* shows the noncancerous tissue using an antisense RNA probe. *B* and *C* show the cancerous tissue using an antisense RNA probe. Cap43 mRNA was partially detected in the epithelial cells of the proximal tubuli (*A*). A strong staining for the *Cap43* gene was observed particularly in large, round cells with a rich cytoplasm, especially near the capsule (*C*), and not within cancer cells proliferating in an alveolar pattern (*B*). No significant signal was observed using a sense RNA probe. When the sections were treated with RNase in the process of washing, the result was the same, alhough the entire signal was reduced. Sections were stained with anti-CD68 antibody using an avidin-biotinylated peroxidase complex method. *D* illustrates the result of case 7. No apparent macrophage infiltration was observed in the noncancerous region, whereas prominent infiltration was observed in the cancerous region, especially near the capsule. The distribution of macrophages was almost consistent with that of cells stained for Cap43 mRNA. Magnification: ×200, *A* and *B*; ×100, *C* and *D*.

(31) showed high expression of thymidine phosphorylase, and those cells infiltrating human gliomas showed high expression of heme oxygenase-1 (10). These studies suggested that the appearance of thymidine phosphorylase-positive or heme oxygenase-1-positive macrophages is somehow associated with malignant and/or angiogenic status in the above human tumors. Piquemal *et al.* (12) has shown that differentiation of human monocytic cells by phorbol ester results in increased expression of the *Cap43* gene. We also confirmed that monocytic cells enhanced expression of the *Cap43* gene in response to PMA (Fig. 4). Differentiation of monocytic cells thus appeared to accompany enhanced expression of the *Cap43* gene. The role of the *Cap43* gene in macrophages remains unclear, but this gene is expected to be a marker of mature form during differentiation of the mononuclear phagocyte system and also of TAMs. From our present study, the appearance of Cap43-positive macrophages might have an important role in the malignant or angiogenic status of RCCs. Further study is needed to investigate the biological role of the *Cap43* gene in macrophages.

The expression of the *Cap43* gene was not observed in renal cancer cells themselves, whereas it was positively detected in epithelial cells of the proximal tubuli in the noncancerous region of all 10 cases (Fig. 3, *A* and *B*). Considering the fact that

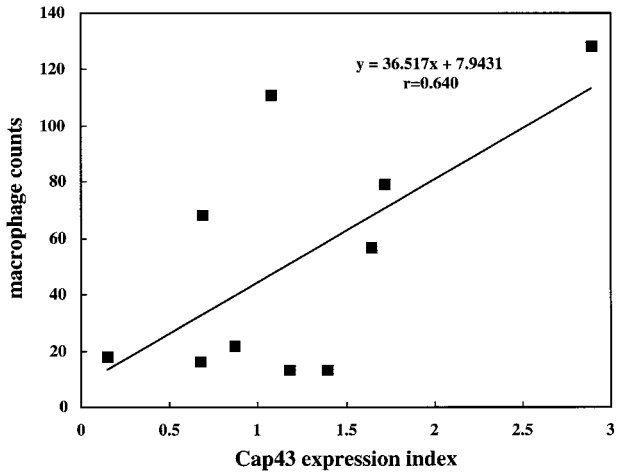

Fig. 4 Correlation between expression of Cap43 mRNA and macrophage infiltration. Cap43 mRNA levels in the cancerous region are based on the data in Fig. 2. Macrophage counts in the cancerous region were assessed microscopically in the three, and the average number of macrophages/microscopic field (×400 magnification) was recorded. Expression of Cap43 mRNA was closely correlated with macrophage infiltration in 10 RCC cases.

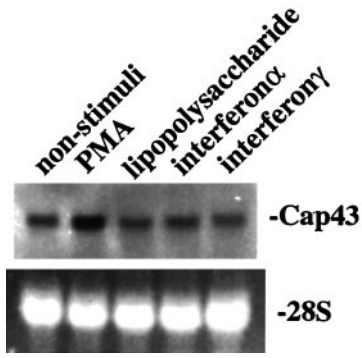

Fig. 5 Induction of Cap43 mRNA by stimulation with PMA. Human myelomonocytic cells were incubated in the culture medium with 1% FBS for 16 h and treated with or without 10 μg/ml PMA, 100 ng/ml lipopolysaccharide, 1000 IU/ml IFN-α, and 50 ng/ml IFN-γ for 6 h. Ten μg of total RNA obtained from treated cells were isolated and subjected to Northern blot analysis. The expression of Cap43 mRNA was increased 3-fold by stimulation with PMA.

RCCs originate from epithelial cells of the proximal tubuli, expression of the *Cap43* gene might disappear when malignant phenotypes are acquired in human RCCs. This study has also shown that Cap43 might be involved in growth and cell differentiation in RCCs as well as colon, breast, and prostate cancers (25, 26).

In conclusion, we isolated nine genes that were highly expressed in the cancerous region of RCC compared with noncancerous tissue. Of these nine genes, one gene named *Cap43* appeared to be somehow associated with the malignant characteristics of cancer cells because the *Cap43* gene was expressed in normal epithelial cells of the proximal tubuli but not in renal cancer cells. Moreover, Cap43 mRNA was highly expressed in infiltrating macrophages in the cancerous region of RCCs, suggesting that Cap43 is a novel biochemical marker for differentiation of renal cells or monocytes/macrophages. The expression of the *Cap43* gene in the cancerous region of RCCs could reflect macrophage infiltration. Further study is required to understand how the expression of the *Cap43* gene in infiltrating macrophages could be associated with the acquisition of malignant phenotypes of RCC.

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