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Contribution of Chromosome 9p21-22 Deletion to the Progression of Human Renal Cell Carcinoma

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To investigate the possible role of genomic aberrations of chromosome 9p21-22 in the tumorigenesis of human renal cell carcinoma (RCC), 10 RCC cell lines, 55 primary RCCs and 5 metastatic lesions were studied by Southern blotting and polymerase chain reaction-based analysis. Nine of 10 RCC cell lines showed a homozygous deletion of *MTS1/CDKN2/(p16)*, while only 1 in 55 primary tumors had this deletion. Loss of heterozygosity on 9p21-22 was observed in 5 of 10 informative primary RCCs from patients with metastasis, but in only 4 of 31 informative tumors (13%) without metastasis ($P=0.025$). Furthermore, 3 of 5 metastatic tumors (60%) showed hemi- or homozygous deletion of *MTS1/CDKN2*. These results indicate that the 9p21-22 deletion may be a relatively late event in RCC tumorigenesis and could be associated with RCC metastasis.

Key words: Renal cell carcinoma — Chromosome 9p21-22 — Loss of heterozygosity — Homozygous deletion — *MTS1/CDKN2/(p16)*

Inactivation of tumor suppressor genes has been considered a critical event in the etiology of many human tumors.[1] Several tumors have presented reproducible aberrations of specific chromosome regions within which tumor suppressor genes were found.[2,3] Recently, a putative tumor suppressor gene located on chromosome 9p21 was identified as *MTS1/CDKN2/(p16)*, encoding the protein p16.[4,5] This protein is one of the cyclin-dependent kinase inhibitors, and regulates the G1/S transition of the cell cycle by binding to cyclinD/CDK4.[6] Deletion of chromosome 9p21-22 has been reported in a variety of human cancers such as melanoma,[7] leukemia,[8] lung cancer,[9] glioma,[10] malignant mesothelioma,[11] bladder cancer,[12] esophageal cancer,[13,14] and gastric cancer.[15]

Homozygous deletions or mutations of the *MTS1/CDKN2* gene have been demonstrated with very high frequency in cell lines derived from a variety of different tumors including melanoma and renal cell carcinoma.[4,5] However, some reports have shown that the *MTS1/CDKN2* alteration was less common in primary tumors than in cell lines,[16–21] suggesting that the *MTS1/CDKN2* alteration might provide some advantage associated with the growth of tumor cells *in vitro*.

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[4] Abbreviations: RCC, renal cell carcinoma; LOH, loss of heterozygosity; MTS1, multiple tumor suppressor gene 1; CDKN2, cyclin-dependent kinase 4 inhibitor; PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism.

The analysis of an animal renal cancer model, the Eker rat model, has provided useful information regarding RCC[4] genetics. In addition to the critical locus for Eker rat RCC on chromosome 10q, the deletion of chromosome 5q31-33 was observed with high frequency, and was found to be a relatively late event associated with tumor progression.[22] This locus in the rat model coincides with the *ifna* locus which is syntenic with 9p22 in humans.[23] Therefore, the alteration of 9p21-22 is thought to be crucial in the development of human RCCs as well. In the present study, we analyzed the allelic status of 9p21-22, including *MTS1/CDKN2*, in 55 primary RCCs obtained from surgical specimens, 5 metastatic lesions obtained from surgical specimens or autopsy, and 10 cell lines, in order to clarify whether deletion of this region would play an important role in the tumorigenesis of RCC. Each clinical sample was snap-frozen in liquid nitrogen immediately after removal, and then stored at −80°C. DNA was extracted as described previously.[24] The following 10 cell lines were examined in this study; A-704, ACHN, Caki-1, Caki-2 (purchased from American Type Culture Collection (ATCC)), VMRC-RCZ (from Japanese Cancer Research Resources Bank (JCRB)), OS-RC-1, CCF-RC-1 (kindly provided by Dr. T. Kinouchi, The Center for Adult Diseases, Osaka and Dr. T. Hashimura, Kyoto University, Kyoto, respectively), NC-65,[25] RPMI-SE,[26] and KY-RC-1 (established in our laboratory).

PCR-based LOH analysis was performed at two microsatellite markers, *IFNA* and *D9S171* as described

elsewhere.[11,27] LOH was considered to have occurred when the signal intensity of one allele was reduced by more than 40%, in comparison with the corresponding allele in normal DNA. The allelic status of the *MTS1/CDKN2* or *MTS2/p15* loci was examined by Southern blot analysis and/or by PCR-based analysis. Southern blotting and hybridization were performed as described previously.[24] The PCR product of *MTS1/CDKN2* exon 2 was used as the Southern blotting probe. In clinical material, homozygous and hemizygous deletions were considered present when the hybridization signal intensity was reduced by more than 60% and 35%, respectively, in comparison with the corresponding signal intensity in normal kidney tissue after standardizing the amount of DNA loaded based on the signal intensity of reprobed β-actin. In the cell lines, a complete absence of signal was considered a homozygous deletion. Amplification of exons 1 and 2 of *MTS1/CDKN2* was performed as described elsewhere.[4] In the samples suspected of homozygous deletions, multiplex PCR was performed using a fragment of another chromosomal locus, *D10S216* (sequences obtained from Dr. M. R. Eccles, University of Otago, Dunedin, New Zealand).

Table I shows the loci of the homozygous deletions on 9p21-22 in 10 RCC cell lines. Nine of the 10 cell lines showed homozygous deletions at the *MTS1/CDKN2* locus, and 3 of them exhibited homozygous deletions exclusively at this locus. Considering that the physical distance between *MTS2/p15* and *MTS1/CDKN2* is about 20 kb,[4] it is of interest that this centromeric breakpoint resided on this short region in 4 RCC cell lines. The smallest overlapping region was restricted between the *MTS2/p15* and *IFNA* loci. Kamb *et al.* showed that 67% of RCC cell lines demonstrated homozygous deletions of *MTS1/CDKN2*.[4] These observations, together with ours, suggest that the tumor suppressor gene for RCC, possibly *MTS1/CDKN2*, could be located within this region.

Table I. Homozygous Deletion of *MTS1/CDKN2* in RCC Cell Lines

Cell line	*D9S171*	*MTS2/p15*	*MTS1/CDKN2*	*IFNA*
ACHN	+	+	−	−
NC-65	+	+	+	+
A-704	+	+	−	+
Caki-1	+	−	−	+
Caki-2	+	−	−	+
RPMI-SE	+	+[a]	−	+
CCF-RC-1	+	+	−	+
VMRC-RCZ	+	−	−	+
OS-RC-1	+	−	−	+
KY-RC-1	−	−	−	−

a) Rearrangement.
+, retention of at least one allele; −, homozygous deletion.

In this study, however, a homozygous deletion of *MTS1/CDKN2* was only observed in 1 of 55 primary RCCs (Fig. 1). In view of the fact that homozygous deletions of *MTS1/CDKN2* have been observed more frequently in cultured cells than primary tumors,[16–19] the exact role of the *MTS1/CDKN2* gene in human tumorigenesis remains controversial. Table II summarizes the results of PCR-based LOH analysis and Fig. 2A illustrates representative autoradiograms. Nine (22%) of 41 informative cases for either the *IFNA* or *D9S171* loci presented with LOH on this region. When these 41 cases were classified into 2 groups according to metastatic status at the time of nephrectomy, LOH at either *IFNA* or *D9S171* was observed in 5 of 10 cases with metastasis (50%), but in only 4 of 31 cases without metastasis (13%). This difference was statistically significant ($P=0.025$, Fisher's exact probability test). In addition, we showed that hemi- or homozygous deletions of *MTS1/*

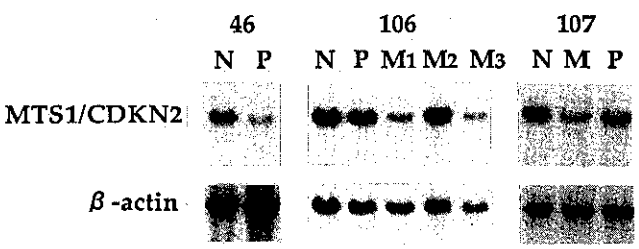

Fig. 1. Representative examples of Southern blotting. Approximately 7 μg of DNA from the primary tumor (P), metastatic tumor (M) or paired normal kidney (N) was loaded in each lane. The signal for 46P was scored as a homozygous deletion of *MTS1/CDKN2* with a 70% reduction in signal intensity when compared to that of corresponding normal kidney tissue (46N). The signals for 106M1 (lung metastasis) and 106M3 (metastasis to the contralateral kidney) were scored as hemizygous deletions with 35% and 40% signal reductions, respectively. However, in 106P and 106M2 (liver metastasis), the signal intensity was almost the same as that of the normal kidney (106N). The signal reduction rate was calculated as described in the text. 107P and 107M showed both hemi- and homozygous deletions with 35% and 67% signal reductions, respectively.

Table II. LOH on 9p21-22 in Primary RCCs and Metastatic Status

	No. of cases	*IFNA*	*D9S171*	Total (%)
Metastasis				
(+)	13	4/9	3/3	5/10 (50)[a]
(−)	42	4/31	0/9	4/31 (13)[a]
Overall	55	8/40	3/12	9/41 (22)

a) $P=0.025$ in Fisher's exact probability test.

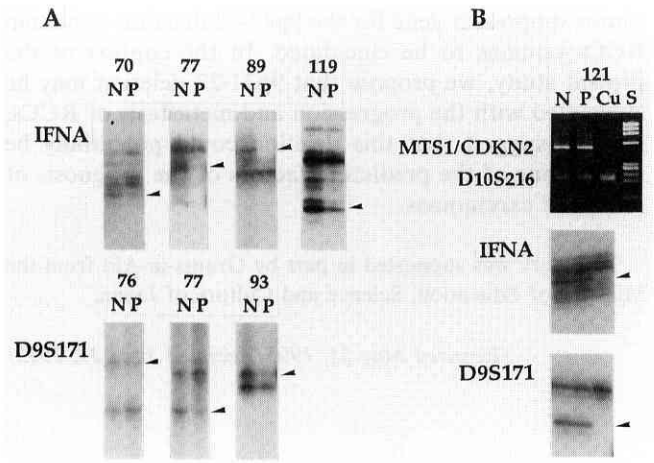

Fig. 2. PCR-based LOH and deletion analysis of 9p21-22 in primary RCCs. (A), Primary tumor (P) and paired normal kidney (N) DNA were amplified by PCR using 2 microsatellites, *D9S171* and *IFNA*. Arrowheads show the positions of the PCR products which indicated allelic loss. (B), Additional changes on the 9p21-22 loci in the low passage cultures when compared with the original tumors. In the primary tumor (P), heterozygosity was retained. However, low passage cultures (Cu) showed LOH at both *D9S171* and *IFNA*, and a homozygous deletion of *MTS1/CDKN2*. In the PCR-based deletion analysis, multiplex PCR was performed using the two primer sets, *MTS1/CDKN2* exon 2 and *D10S216*. S, size markers ($\phi \times 174$/*Hae*III digest).

CDKN2 occurred in 3 of 5 (60%) metastatic lesions (Fig. 1). These observations support the notion that the deletion of 9p21-22 is a relatively late event in tumorigenesis, possibly associated with the progression and metastasis of RCCs.

To clarify the meaning of the difference in the frequency of 9p21-22 deletions between primary tumors and cell lines, 5 low passage (<10) cultures, prepared from 8 RCC samples, were examined. Table III shows the allelic status of these 5 cultures and the original tumors. We showed that 3 of the 5 low passage cultures (60%) had additional allelic losses on 9p21-22, when compared with their original tumors. Unlike established cell lines, low passage cultures are considered to provide a reliable assessment of the genotype of the original tumor.[28] Furthermore, additional deletions of the *MTS1/CDKN2* locus were also observed in 3 metastatic lesions from 2 cases, 106M1, 106M3, and 107M, as compared with the primary tumors (Fig. 1). These observations suggest that tumor cells with deletions on 9p21-22 survive selective pressure such as culture or metastasis. In some cases, the proportion of these cells in the primary tumors may be so small that the deletions would be undetectable by the usual LOH studies (Fig. 2B). This heterogeneity of the tumors which were studied is another possible explanation for the observed deletion rate.

Table III. Allelic Status of Chromosome 9p21-22 in RCCs and Cultures[a]

Case	*IFNA* or *D9S171*		*MTS1/CDKN2*	
	Tumor	Culture	Tumor	Culture
70	LOH	HD	R	HD
108	NI	HD	R	HD
121	ROH	LOH	R	HD
119	LOH	LOH	R	R
120	ROH	ROH	R	R

a) Low passage (<10) cultures derived from surgical specimens; LOH, loss of heterozygosity; HD, homozygous deletion; R, retention of at least one allele; NI, non-informative; ROH, retention of heterozygosity.

According to Knudson's "two-hit hypothesis," however, both alleles of a tumor suppressor gene must be inactivated for malignant transformation to occur. In such instances, the first genetic events would probably be small genetic alterations such as point mutations. In the present study, mutations in exons 1 and 2 of *MTS1/CDKN2* were screened on all of the tissue samples by a standard PCR-SSCP analysis, as described previously.[29] Radiolabeled PCR products of exon 2 were split into 2 fragments by digestion with *Sma*I. Abnormal band shifts implying mutations were not detected (data not shown). In order to exclude the possibility of false-negative results in the PCR-SSCP analysis, direct sequencing of the exon 2 of *MTS1/CDKN2* was performed in those cases with a deletion of one *MTS1/CDKN2* allele, using the sequence primers described elsewhere.[30] The sequencing, however, revealed no mutation in any of the cases examined (data not shown). Our observation is consistent with the results of recent molecular studies, in which a low frequency of mutations in the *MTS1/CDKN2* gene was reported in various human cancers.[16, 17, 19–21] Although we could not exclude the possibility that mutations might have occurred in non-coding regions, the fact that homozygous deletion of the *MTS1/CDKN2* gene might be a common inactivation mechanism could explain the failure to detect mutations in this gene. Conversely, *MTS1/CDKN2* might be a tumor suppressor gene which confers a growth advantage on tumor cells, following the loss of only one allele.

An alternative explanation for the low frequency of *MTS1/CDKN2* mutations may be that another tumor suppressor gene near the *MTS1/CDKN2* locus may play an important role in RCC tumorigenesis. Cairns *et al.* reported a lack of mutations within the *MTS1/CDKN2* gene in primary RCCs, although the existence of a tumor suppressor gene located between *D9S171* and *IFNA* was

postulated.[31] In familial melanoma, mutations of *MTS1/CDKN2* have similarly been observed with low frequency, again suggesting an alternative target in the 9p21-22 deletion.[32]

The chromosome 3p deletion is the most frequent genetic event in RCC. Recently, a tumor suppressor gene for RCC, the von Hippel-Lindau gene, has been cloned,[33] and shown to be mutationally inactivated in almost all cases of clear cell subtype RCCs.[34] However, the chromosome 3p alteration is not considered to be associated with either invasiveness or metastasis.[34] Whether *MTS1/CDKN2* or possibly another gene is the responsible tumor suppressor gene for the 9p21-22 deletion in human RCCs remains to be elucidated. In the context of the present study, we propose that 9p21-22 deletion may be associated with the progression and metastasis of RCCs, and we suggest that this deletion could potentially be used as one of the predicting factors of the prognosis of renal cell carcinomas.

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