Genomic profiling of the genes on chromosome 3p in sporadic clear cell renal cell carcinoma

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Abstract. Somatic mutations of the BRCA1 associated protein-1 (*BAP1*) gene, which maps to 3p21, have been found in several tumors including malignant mesothelioma, uveal melanoma, and renal cell carcinoma (RCC). The role of BAP1 inactivation in tumor development remains unclear. It has been reported that *Vhl* knock-out mice did not develop RCC, but *Vhl* knock-out mice with single allele loss of *Bap1* in nephron progenitor cells developed RCC, indicating that Bap1 inactivation may be essential in murine renal tumorigenesis. To clarify the role of BAP1 in human RCC development, we performed mutation analyses, including copy number detection of *BAP1* and assessment of allelic imbalance using microsatellite polymorphisms on 3p, in 45 RCC samples derived from 45 patients without *VHL* or *BAP1* germline mutation. Additionally, we analyzed the sequences of the *VHL, PBRM1*, and *SETD2* genes, and examined promoter methylation of *VHL*. Using immunostaining, we also checked for expression of BAP1 protein, which is normally located in the nuclei. None of the RCCs had biallelic deletion of *BAP1*, but five (11.1%) showed a biallelic mutation (four with a sequence-level mutation with monoallelic loss and one with a biallelic sequence-level mutation); these cells were negative for nuclear BAP1 staining. These patients had worse recurrence-free survival than the patients without a biallelic mutation (p=0.046). However, there were no significant differences in worse outcome by multivariate analysis combined with age, T stage, histological subtype, infiltration and vascular invasion. In 35 RCCs (77.8%), monoallelic loss of *BAP1* was accompanied by *VHL* biallelic mutation or *VHL* promoter hypermethylation. In five RCCs (11.1%), we detected 3p loss-of-heterozygosity, but the copy number of *BAP1* was normal. Surprisingly, nuclear staining of BAP1 was negative in 10 out of 31 tumors (32.3%) with hemizygous normal *BAP1*, suggesting that haploinsufficiency may relate to RCC development.

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Introduction

Loss of chromosome 3p in many tumors is well established and it has been supposed for a long time that this chromosomal region might carry several tumor suppressor genes (1,2). In particular, sporadic clear-cell renal cell carcinoma (ccRCC) is characterized by a high frequency of allelic deletion or loss of heterozygosity (LOH) on chromosome 3p (>90%), causing biallelic inactivation of von Hippel-Lindau tumor suppressor (*VHL*) gene (3-5). It was reported that deletion of fragile histidine triad (*FHIT*) gene and flanking loci might occur as an initiating event followed by deletions at 3p12.2, 3p21.31-3p21.32, and 3p24.2-3p26.1 in the early stages, then continuous large deletions of 3p21.3-3p26.1 and 3p14.1-3p26.1 (6). Next-generation sequencing studies have identified frequent mutations in genes involved in chromatin modification such as polybromo 1 (*PBRM1*), SET domain containing 2 (*SETD2*), and BRCA1 associated protein-1 (*BAP1*) (7-10), all of which are located on 3p21. Moreover, *BAP1* mutations were mutually exclusive with *PBRM1* mutations (9,11).

Germline *BAP1* mutations are associated with an increased risk of malignant mesothelioma (MM), atypical melanocytic tumors, and other neoplasms (12-15). They also predispose to RCC (16,17). Moreover, somatic *BAP1* mutation was reported in ~10% of ccRCC patients of any ethnic background (9-11,18). Recently, Wang *et al* reported that mice deficient for Vhl together with one allele of Bap1 in nephron progenitor cells developed ccRCC, but mice deficient in Vhl did not (19). Wang *et al* suggested that *BAP1* is a more potent tumor suppressor gene than VHL, which they assessed as a weak tumor suppressor gene in the kidney. The question remains as to why VHL mutations occur at a much higher frequency than *BAP1* mutations in both familial and sporadic ccRCC (20).

The BAP1 protein is a nuclear protein that has been considered to function as a deubiquitinase of histone H2A (21). Two types of BAP1 inactivation have been reported: sequence-

level mutation of BAP1 combined with monoallelic loss of 3p, and biallelic deletion comprising broad deletions of 3p21 and narrow deletions of several exons or the entire *BAP1* gene. In metastasized uveal melanoma, the former mutation type occurs frequently (22,23). In MM, we reported a high frequency of biallelic loss of *BAP1* (24,25), in addition to sequence-level mutations in the *BAP1* gene (26). However, the observed frequency of BAP1 inactivation in MM has been variable among studies: low frequencies of mutations found by sequencing alone (12,26,27), and high frequencies of loss of nuclear staining, or genomic mutations identified by sequencing and copy number analysis (24,28,29). Nasu *et al* suggested immunostaining as the most accessible and reliable technique to detect BAP1 inactivation in MM biopsies (29). It was also reported that BAP1 expression in the nucleus was reduced in 44% of esophageal squamous carcinoma, although the mutation rate of the gene was ~2% (30). It is increasingly clear that BAP1 protein nuclear translocation may play a key role in tumorigenesis (31,32).

To clarify the status of inactivation of the *BAP1* gene in ccRCC development, we performed genomic analysis of the *BAP1* gene, assessed for 3p rearrangement, and conducted immunostaining for the BAP1 protein in RCC patients.

Materials and methods

Tumor specimens. We obtained 45 RCC samples from 45 patients who had radical nephrectomy at the Hospital of Hyogo College of Medicine. The patient summary is presented in Table I. Fresh frozen tumor tissues dissected from the surgical specimen and unaffected kidney tissue adjacent to the tumor regions for most patients (ID: RCC01-56), or peripheral blood for 13 patients (ID: RCC57-69), as a matched control were analyzed. Pathological examination showed that 42 tumors were diagnosed as ccRCC and three as chromophobe RCC (chRCC). Our study was approved by the Ethics Committee of Hyogo College of Medicine (permission no.: RINHI277), and performed in accordance with the Declaration of Helsinki (1995) of the World Medical Association (as revised in Fortalez, 2013). All patients provided written informed consent.

Microsatellite (MS) genotyping of chromosome 3p. We analyzed 13 polymorphic MS markers, identified from the 'Heterozygosity of MS Markers in the Japanese Population' database (https://dbarchive.biosciencedbc.jp/jp/heterozygosity-jp/desc.html), using a modified version of the method described by Dietrich *et al* (33). We examined the following markers: D3S1263 (3p25.3); D3S1266 (3p24.1); D3S1611 (3p22.2); D3S3687 (3p22.1); D3S3678 (3p22.1); D3S2420 (3p21.31); D3S1568 (3p21.31); D3S1573 (3p21.31); D3S3561 (3p21.1); D3S3648 (3p21.1); D3S1289 (3p14.3); D3S1300 (3p14.2); and D3S1285 (3p14.1). When the locus was heterozygous in the matched control sample, we calculated the ratio of the peak area of allele 1 compared with that of allele 2, and determined the allelic imbalance between the allele ratio of the tumor and that of the matched control sample. We judged LOH and deduced the rate of contaminating non-tumor cells included in RCC tumors based on the value of allelic imbalance. The rates of tumor content were lower than the rates estimated by pathological examination.

Multiplex ligation probe amplification (MLPA) analysis. We carried out MLPA analysis of genomic DNA for each of the 17 exons of the *BAP1* gene using a SALSA MLPA BAP1 kit according to the manufacturer's instructions (MRC Holland, Amsterdam, The Netherlands). This kit detects copy number changes in each exon of *BAP1* plus an additional ten genes on chromosome 3: *MLH1, RBM5, RASSF1, ZMYND10, HESX1, FHIT, MITF, ROBO1, PROS1* and *CPOX*. The peak area obtained using gene-specific probes in each sample was first normalized to the average peak areas obtained using control probes specific for other chromosomal locations. A final ratio was then calculated by dividing by the value for genomic DNA isolated from the matched control. The sample was scored to have loss or gain of one copy number (monoallelic loss or amplification) by comparing the MLPA ratio and the rate of tumor content in the RCC sample. For example, if the rate of tumor content calculated by MS analysis and the MLPA ratio were 0.5 and 0.75, respectively, this would indicate monoallelic loss of *BAP1* in an RCC sample that consisted of ≤50% non-tumor cells.

Target sequencing. We isolated DNA using an AllPrep DNA/RNA mini kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions, and sequenced the coding regions of *VHL, BAP1, SETD2* and *PBRM1*. For *VHL*, direct sequencing was conducted using a BigDye Terminator v3.1 kit on an Applied Biosystems 3130xl Genetic Analyzer (Foster City, CA, USA); primer sequences are presented in Table II. By consulting the contamination rate of non-tumor cells in each tumor sample, a barely imperceptible variant in the tumor sample could be detected by sequencing on both strands. For the other genes, next-generation sequencing was performed on an Illumina MiSeq (San Diego, CA, USA) using paired-end 250-bp runs. Libraries were prepared from 250 ng of genomic DNA from 45 independent RCC tumors using a Haloplex Custom kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. Illumina paired-end reads were each aligned to the human NCBI Build 37 reference sequence using bwa software (biobwa.sourceforge.net/, version 0.6.0). The aligned sequence files were sorted and merged using SAMtools (samtools.sourceforge.net/, version 0.1.18). GATK (http://www.broadinstitute.org/gatk/) was used for realignment, base quality score recalibration, single nucleotide variant or indel (small insertions and deletions) variant calling, and variant quality recalibration. SnpEff was used to categorize the effects of variants by impact (34). Functional annotation of genetic variants provided by ANNOVAR (www.openbioinformatics.org/annovar/, version 2013, Feb 11) and dbNSFP (35) were used to identify protein-damaging mutations. Somatic mutations or germline variants were judged by comparison between Sanger sequence data from the ccRCC tumor and its matched control.

Promoter methylation of VHL. Bisulfite sequencing was performed for the patients who did not have a mutation in the *VHL* gene: ID 5, 6, 7, 21, 23, 36, 44, 55 and 67. After bisulfite modification of 100 ng tumor or normal DNA using an EpiTect Fast DNA Bisulfite kit (Qiagen), the regions containing both methylated and unmethylated alleles across 26 CpG dinucleotides in the *VHL* promoter were amplified using a touchdown

Table I. Summary of genomic alterations detected in RCC tumors.

	Patient information						3p		VHL		BAP1			PBRM1	SETD2
RCC_ID	Sex	Age	Histological type	TMN	Recurrence[a]	Survival[b]	LOH[c]	Monoallelic loss[d]	Somatic mutation	Promoter methylation[e]	Monoallelic loss[f]	Somatic mutation	Nuclear staining	Somatic mutation	Somatic mutation
2	M	56	ccRCC	3a	-	+	+	+	p.E70X	ND	+	Wild	(-)	p.I571V	Wild
4	M	45	ccRCC	2a	-	+	+	+	p.N78S	ND	+	Wild	(-)	Wild	Wild
5	M	52	ccRCC	2a	-	+	+	+	Wild	+	+	Wild	(-)	Wild	Wild
6	M	77	ccRCC	1b	-	+	-	-	Wild	+	-	Wild	(+)	Wild	Wild
7	M	45	ccRCC	1a	-	+	-	-	Wild	-	-	Wild	(+)	Wild	Wild
8	M	52	ccRCC	1a	-	+	+	+	p.D126fs	ND	+	Wild	(+)	p.H24fs	Wild
9	M	73	ccRCC	3a	-	-[g]	+	+	p.F136fs	ND	+	Wild	(+)	p.S987X	p.T220fs
10	M	55	ccRCC	1b	-	+	+	+	p.L188P	ND	+	Wild	(-)	Wild	Wild
11	M	54	ccRCC[h]	3a	+	-	+	+	p.L158fs	ND	+	Wild	(-)	p.I1345fs	Wild
13	M	58	ccRCC	3a	-	+	+	+	p.F119L	ND	+	p.K286fs	(-)	Wild	Wild
14	F	59	ccRCC	1a	-	+	+	+	Splicing acceptor	ND	+	Wild	(+)	Wild	Wild
15	M	73	ccRCC	1a	-	+	+	+	p.Y185X	ND	+	Wild	(+)	p.S1253fs	Wild
17	F	66	ccRCC	3a	-	+	+	+	p.E70X	ND	+	Wild	(-)	p.P703L	Wild
18	F	58	ccRCC	1a	-	+	+	+	p.S65X	ND	+	Wild	(-)	p.F684Y	Wild
20	M	56	ccRCC	1a	+	+	+	+	p.T133fs	ND	+	p.W202X	(-)	Wild	Wild
21	M	81	ccRCC	1a	-	+	+	+	Wild	+	+	Wild	(+)	Wild	Wild
24	M	74	ccRCC	3a	+	+	+	+	p.D179fs	ND	+	Wild	(+)	Wild	Wild
25	M	71	ccRCC	1b	-	+	+	+	p.L135fs	ND	-	Wild	(+)	p.K553X	p.V1820E
26	M	74	ccRCC	1a	-	+	+	+	p.S80R	ND	+	Wild	(+)	Splicing donor	Wild
30	M	68	ccRCC	4	+	+	+	+	p.N131X	ND	+	Wild	(+)	p.H712P	p.S204X
31	F	67	ccRCC	3a	-	+	+	+	p.Q96fs	ND	+	Wild	ND	p.K1282fs	Wild
32	M	71	ccRCC	2a	+	+	+	+	p.C162W	ND	+	Wild	ND	Wild	Wild
33	M	50	ccRCC	2a	-	+	+	+	p.L169P	ND	+	Wild	(+)	Wild	Wild
34	M	60	ccRCC	3a	+	+	+	+	p.L178fs	ND	+	Wild	(+)	p.E1501fs	Splicing donor
38	M	75	ccRCC	1a	-	+	+	+	p.F136fs	ND	+	Wild	(-)	p.Y998X	Wild
41	M	78	ccRCC	1a	-	+	+	+	p.V74D	ND	+	Wild	(-)	p.H573fs	Wild

Table I. Continued.

	Patient information						3p		VHL		BAP1			PBRM1	SETD2
RCC_ID	Sex	Age	Histological type	TMN	Recurrence[a]	Survival[b]	LOH[c]	Monoallelic loss[d]	Somatic mutation	Promoter methylation[e]	Monoallelic loss[f]	Somatic mutation	Nuclear staining	Somatic mutation	Somatic mutation
43	F	63	ccRCC	2b	-	+	+	+	Splicing donor	ND	+	Wild	(-)	Wild	Wild
50	M	63	ccRCC	3a	+	+	+	+	p.G144fs	ND	-	p.C91F	(-)	Wild	p.K2525X
52	F	46	ccRCC	1b	-	+	+	+	p.L163P	ND	+	Wild	(+)	p.E532X	Wild
53	M	71	ccRCC	1b	-	+	+	+	p.G114fs	ND	+	Wild	(+)	p.S1253fs	Wild
55	F	72	ccRCC	3a	+	+	-	-	Wild	-	-	Wild	ND	Wild	Wild
57	M	84	ccRCC	3b	-	+	+	-	p.R120fs	ND	-	Wild	(+)	Wild	Wild
58	F	57	ccRCC	3a	+	+	+	+	p.A149fs	ND	+	Wild	ND	Splicing acceptor	Wild
61	M	72	ccRCC	1a	-	+	+	+	p.R161P	ND	+	Wild	(+)	p.K1373fs	Wild
62	M	63	ccRCC	1a	-	+	+	+	p.W88X	ND	+	p.I586fs	(-)	Wild	Wild
63	F	53	ccRCC	1b	-	+	+	+	p.N90Y	ND	+	Wild	(+)	Wild	p.T1761fs
64	F	62	ccRCC	3a	+	+	+	+	p.L135fs	ND	+	p.R701fs	(-)	Wild	Wild
65	M	74	ccRCC	1b	-	+	+	+	Splicing acceptor	ND	+	Wild	(+)	p.V964del	Wild
66	M	83	ccRCC	3a	-	+	+	+	p.S111R	ND	+	Wild	(+)	Wild	p.A1591fs
67	F	77	ccRCC	1a	-	+	+	-	Wild	+	-	Wild	(+)	Wild	Wild
68	F	72	ccRCC	3a	-	+	+	+	p.S111R	ND	+	Wild	(+)	Splicing donor	Wild
69	M	44	ccRCC	1b	-	+	+	+	p.T157fs	ND	+	Wild	(+)	p.Q238X	Wild
23	F	65	chRCC	1a	-	+	-	-	Wild	-	-	Wild	(-)	Wild	Wild
36	F	73	chRCC	1b	-	+	+	-	Wild	-	-	Wild	ND	Wild	Wild
44	F	46	chRCC	2b	-	+	+	+	Wild	-	-	Wild	(-)	Wild	Wild

[a]+, had a recurrence. The sites of metastasis were lung, bone, adrenal gland, brain, liver, mediastinal lymph node and local recurrence. Currently, one patient died of cancer, two patients are alive without disease, and others are receiving targeted therapy (n=5) or interferon (n=2). -, without disease. [b]+, alive; -, died. [c]+, with LOH; -, without LOH. [d]+, with monoallelic loss; -, without allelic loss. [e]+, with promoter methylation; -, without promoter methylation; ND, not done. [f](+), positive staining; (-), negative staining, ND, not determined. [g]Had distant metastasis at the nephrectomy were receiving interferon, but died of cancer two months after the nephrectomy. [h]Having partially sarcomatous area.

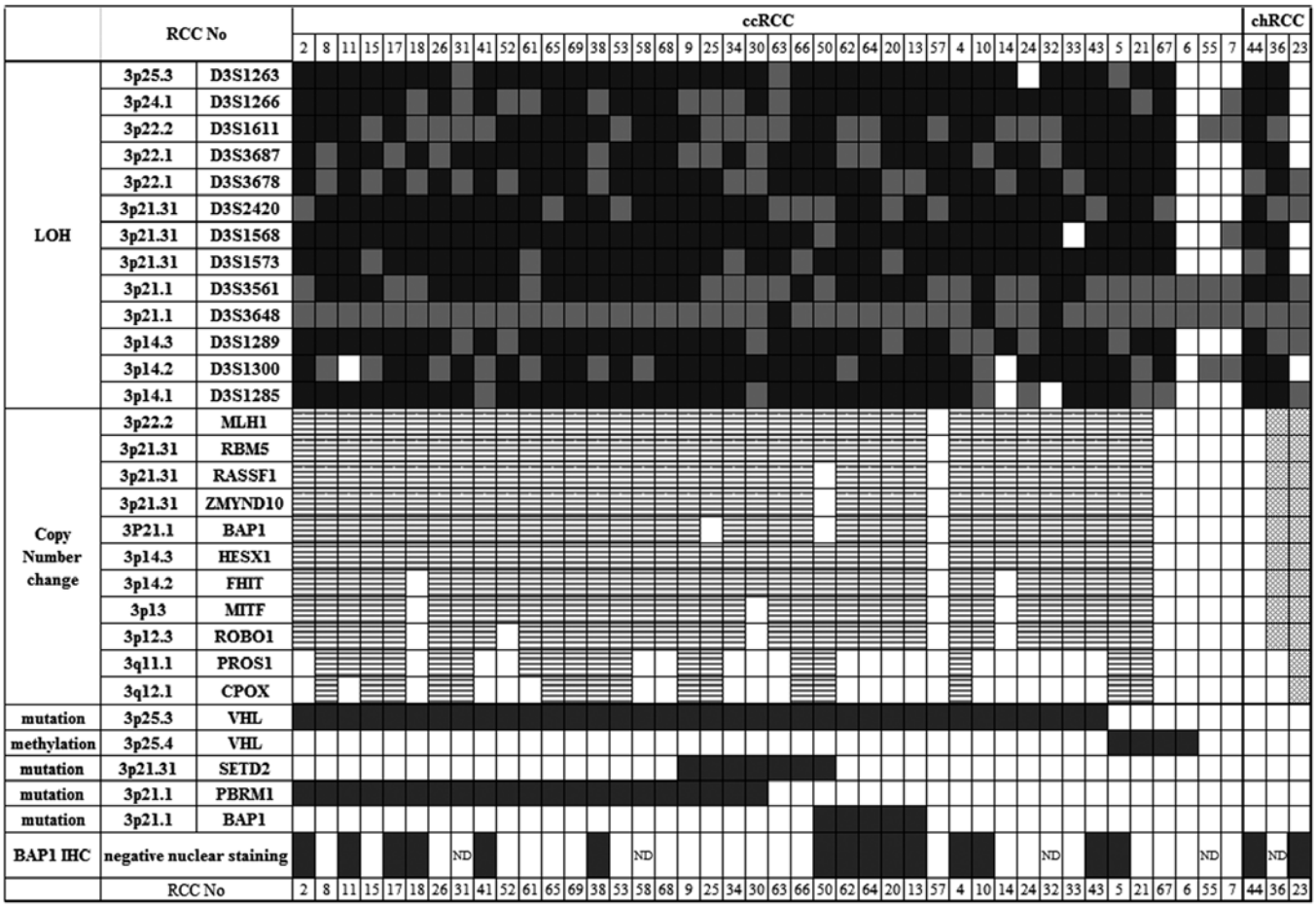

Figure 1. Summary of genome alterations and BAP1 protein expression in renal cell carcinomas. A solid fill indicates that the tumor had the corresponding alteration with respect to the matched control. Loss of heterozygosity (LOH), black fill indicates LOH, gray fill indicates a homozygous genotype in germline DNA, in which case LOH could not be judged in the tumor. Copy number change, horizontal lines indicate loss of one copy and hatched lines indicate gain of one copy. BAP1 immunohistochemistry, a solid fill indicates negative staining for nuclear BAP1 protein. 'ND' in the nuclear staining column indicates that a call could not be made because the tumors had focal negative or weak positive expression.

PCR program with an annealing temperature of 50˚C: 1st PCR primers: 5'-TTAYGGAGGTYGATTYGGGAG-3' and 5'-ACRATTACAAAAAATAACCTA-3' (amplicon: 335 bp); nested PCR primers: 5'-YGGGTGGTTTGGATYG-3' and 5'-AATTCACCRAACRCAACA-3' (amplicon: 226 bp), as previously reported (36). The cytosine positions in CpGs were inspected for thymine or cytosine signals on the chromatograms. Promoter methylation was not detected with normal samples; tumor samples with ≥30% methylated CpGs were judged as methylated.

Nuclear staining. RCC tissues embedded in paraffin were cut into 3-μm-thick sections and heated in antigen retrieval solution (Bond Epitope Retrieval Solution 2, pH 9.0; Leica Biosystems, Nussloch, Germany) at 98˚C for 10 min. Then, the sections were incubated with mouse monoclonal antibody against human BAP1 (C-4; sc-28383; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:150 dilution). Bond Polymer Refine Detection (Leica Biosystems) was used for visualization with 3, 3'-diaminobenzidine tetrahydrochloride. Substantial tumors had heterogeneous nuclear staining for BAP1, with some cells staining positively and some cells staining negatively. Negativity was evaluated when the majority of tumor cell nuclei were stained negatively while there was obvious staining in the nuclei of normal renal tubule cells on the same slide. A pathologist reviewed all slides and categorized tumors as positive, negative, or weak-positive. After samples with weak-positive expression were excluded, 40 samples were available for analysis (Table I).

Statistical analysis. Survival curves were calculated by the Kaplan-Meier method and compared by log-rank test. Two patients died of cancer, therefore the analysis for overall survival was not done. All p-values were two-tailed, and differences were considered significant at $p<0.05$.

Results

Ninety-five percent of ccRCC tumors had either biallelic mutation or promoter methylation of VHL, while chRCCs did not. A high frequency, 36/45 (80%), of *VHL* mutation was detected in RCC tumors by Sanger sequencing. The mutation type was: nonsense in six, frameshift in 15, splice site in three, and missense in 12 (Table I). The RCC tumors with *VHL* mutation showed LOH on 3p (Fig. 1). No patients had germline mutation of this gene (data not shown). Promoter methylation

Table II. Sequences of the primers used for the direct sequencing of the *VHL* gene.

Exon	Uses for PCR or sequencing	Forward or reverse	Sequence
1	PCR and sequencing	Forward	TGGAAATACAGTAACGAGTTGGC
	PCR and sequencing	Reverse	GCTTCAGACCGTGCTATCGT
2	PCR	Forward	GGAGAAAATAGGTGCCCTGAC
	PCR and sequencing	Reverse	GGCAAAAATTGAGAACTGGGCT
	Sequencing	Forward	CCAAAGTGCTGGGATTACAGG
3	PCR	Forward	GGGGGCCATCAGCATAACAC
	PCR and sequencing	Reverse	TACTTCTCTAATGGGCAGGCA
	Sequencing	Forward	GTAGTTGTTGGCAAAGCCTC

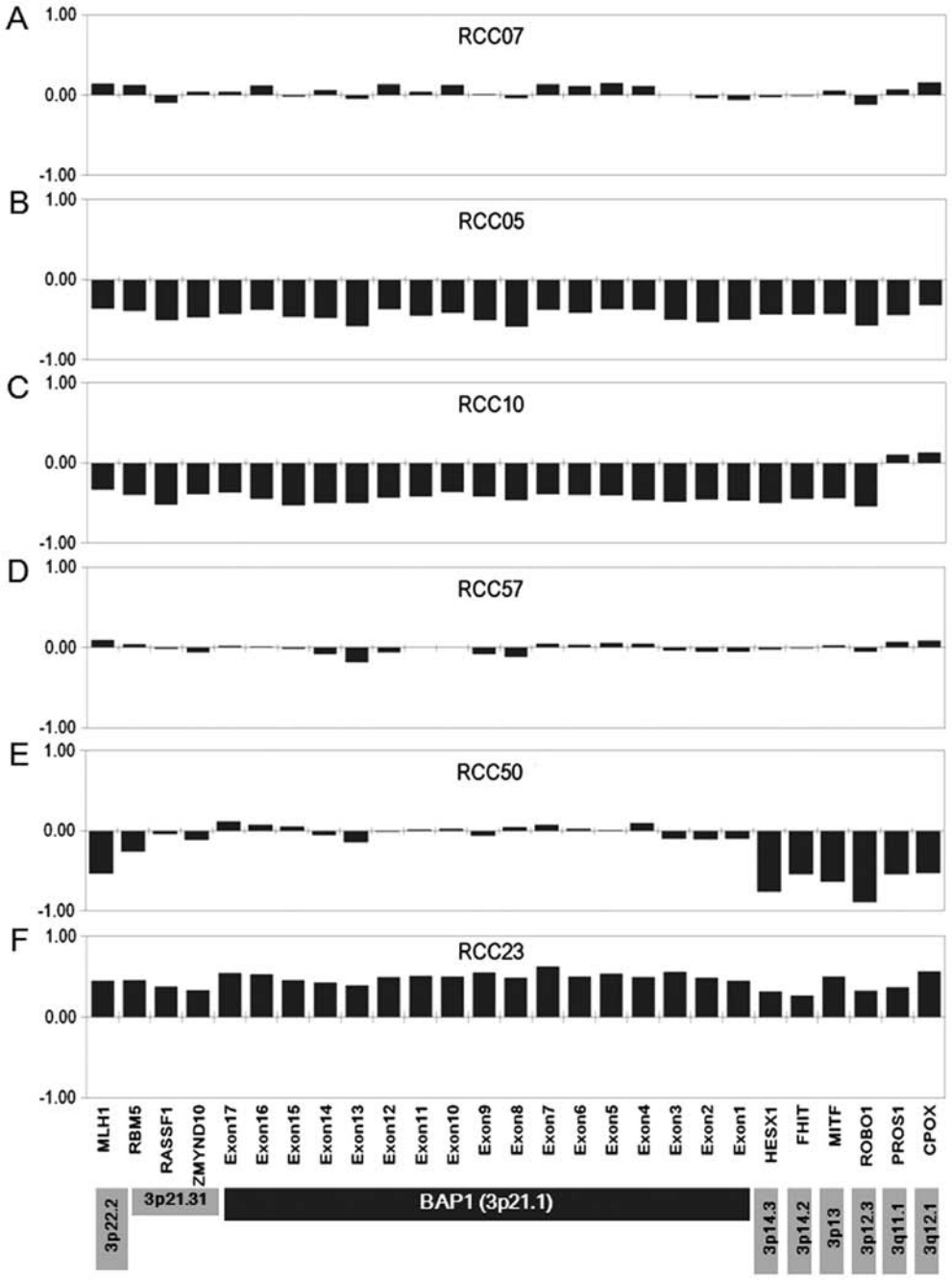

Figure 2. MLPA data for all 17 exons of the *BAP1* gene on 3p21.1. The data of BAP1, as well as for 10 genes on chromosome 3p and chromosome 3q, are displayed on the x-axis. The $\log_2$ ratio of MLPA data for each probe is indicated on the y-axis. From top to bottom, (A) RCC07 without 3p LOH; (B and C) RCC05 and 10, respectively, with LOH and monoallelic loss of 3p; (D) RCC57 with LOH but not monoallelic loss of 3p; (E) RCC50 with 3p LOH but not monoallelic loss of the *BAP1* locus; (F) RCC23 showing amplification of 3p.

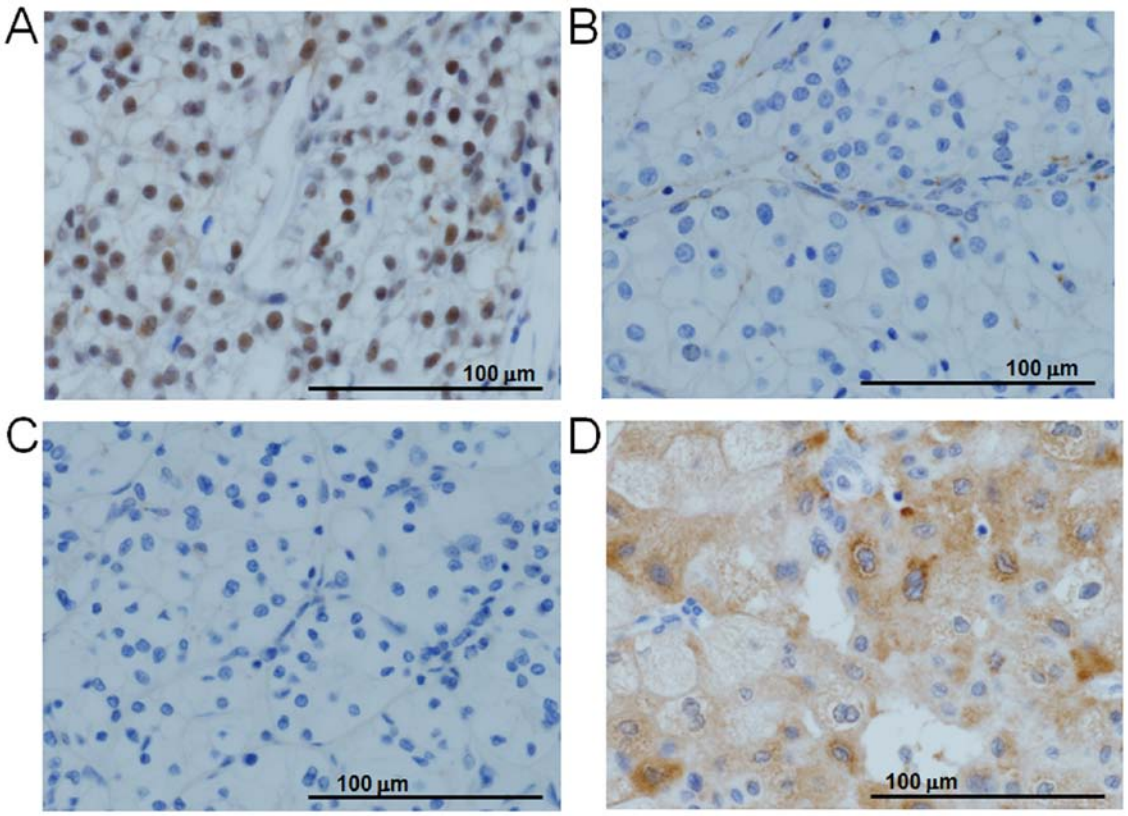

Figure 3. BAP1 nuclear staining by immunohistochemistry. (A) RCC09 showing positive nuclear staining; (B) RCC20 with biallelic mutation showing negative staining; (C) RCC02 with hemizygous normal *BAP1* showing negative staining; (D) chRCC23 showing negative nuclear staining but strong cytosolic staining. Scale bars represent 100 μm.

of *VHL* was detected in four tumors: three (RCC05, 21 and 67) with LOH and RCC06 without LOH. Especially prominent hypermethylation was detected in RCC06 and RCC67 (data not shown). By pathological examination, three (RCC23, 36, and 44) out of five tumors without *VHL* mutation were noted to be chRCC. All tumors with either somatic mutation or promoter methylation in *VHL* were from patients with ccRCC.

Seventy-eight percent of RCC tumors had monoallelic loss of BAP1, but none showed biallelic loss of BAP1. All selected MS markers had a high frequency of heterozygosity in the Japanese population except for D3S3648; because this marker is close to the *BAP1* locus, the homozygosity rate was high [42 of our 45 subjects (93.3%)], and LOH was mostly not determined for the D3S3648 region. Instead, we deduced LOH of the *BAP1* region by combining the LOH data of flanking loci with copy number data determined by comparison of RCC tumor and matched control samples.

We did not find biallelic loss of *BAP1* in RCC samples. LOH in 3p was detected at a frequency of 41/45 (91%; Table I and Fig. 1), and using MLPA analysis we identified monoallelic loss of all exons of *BAP1* together with genes on 3p22.2-3p14.3 in 35 of 45 tumors (77.8%; Figs. 1, 2B and 2C). Two ccRCCs (RCC14 and 18) demonstrated loss of *BAP1*, but not *FHIT* nor genes on 3p13-12. Although showing 3p LOH, three tumors that had no changes in copy number to any *BAP1* exons and any genes analyzed on 3p were judged to show uniparental disomy: RCC44, 57 and 67 (Figs. 1 and 2D). Two tumors (RCC25 and 50) retained two copies of *BAP1* although other genes on 3p had lost one copy (Fig. 2E). In two chRCCs (RCC23 and 36), MLPA indicated amplification of all exons of *BAP1* together with other genes on 3p (Fig. 2F), although gross copy number changes among the genome regions designed as normalization probes (13 control probes specific to chromosomes other than chromosome 3) were also detected (data not shown).

Biallelic inactivation of BAP1 was detected in five ccRCCs, but not combined mutation of both BAP1 and PBRM1. Somatic mutation in *BAP1* was detected in five ccRCCs with LOH (mutation frequency 11.1%); three ccRCCs had a frameshift mutation (RCC13, 62, and 64), one had a nonsense mutation (RCC20), and one had a missense change at amino acid Cys91, which is essential for ubiquitin C-terminal hydrolase activity (RCC50) (http://www.uniprot.org/uniprot/Q92560). Thus, all mutations detected in *BAP1* were predicted to cause loss-of-function. The mutation frequencies of *PBRM1* and *SETD2* in our RCCs were 21/45 (46.7%), and 7/45 (15.6%), respectively (Table I and Fig. 1). Only RCC50 had a mutation in both *BAP1* and *SETD2*. Somatic mutations in both *BAP1* and *PBRM1* were not detected; this combined mutation has been reported to be rare.

Approximately 1/3 of ccRCCs with hemizygous normal BAP1 showed negative nuclear staining. BAP1 nuclear staining was negative in the tumors with biallelic mutation of *BAP1* (Fig. 3B). In addition, BAP1 nuclear staining was negative in 10 ccRCCs with hemizygous normal *BAP1* (10/31, 32.3%)

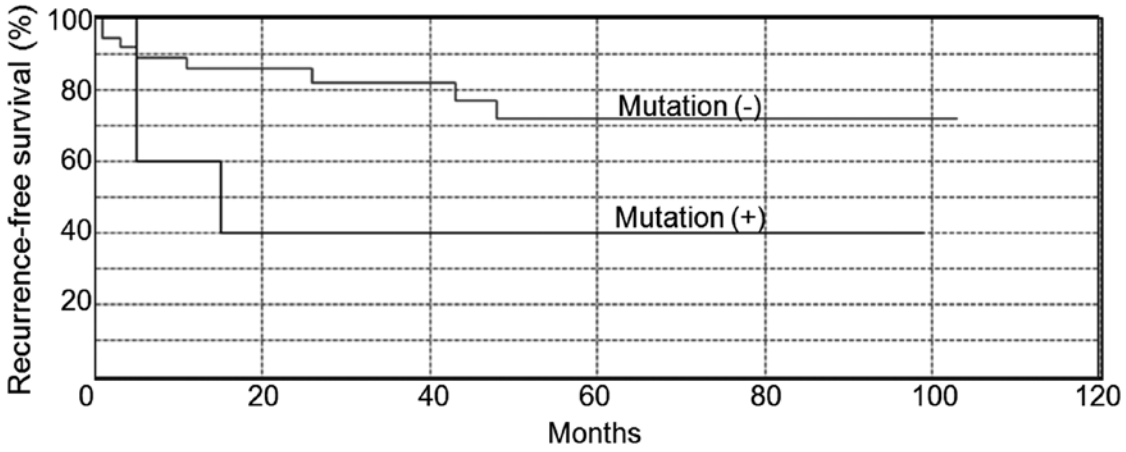

Figure 4. Impact of *BAP1* mutation on recurrence-free survival time. Survival curves were calculated by the Kaplan-Meier method and compared by log-rank test. All p-values were two-tailed, and differences between 'with' [mutation (+)] and 'without' [mutation (-)] *BAP1* mutation were considered significant at $p<0.05$ ($p=0.046$).

(Figs. 1 and 3C). Three chRCCs had strong BAP1 staining in the cytosol (Fig. 3D), although two of them were negative and one was excluded from analysis with nuclear staining.

Biallelic inactivation of BAP1 led to worse outcome. We investigated the relationship between *BAP1* mutation and tumor recurrence. The ccRCC tumors with biallelic loss-of-function mutation in *BAP1* correlated with recurrence-free survival time ($p=0.046$, Fig. 4), but not by multivariate analysis combined with age, T stage, histological subtype, infiltration and vascular invasion (data not shown). *SETD2* mutations significantly correlated with recurrence-free survival time ($p=0.007$), but *PBRM1* mutations did not ($p=0.761$) (data not shown). The ccRCC tumors with negative BAP1 nuclear staining did not associate with tumor recurrence ($p=0.143$, data not shown).

Discussion

We confirmed that 95% of our ccRCCs had features of the VHL-dependent pathway of tumorigenesis. VHL inactivation (mutation by base substitution or indel, or promoter methylation) perfectly paralleled 3p LOH in ccRCC tumors. It is known that point mutations in *VHL*, and loss of 3p, which has been suggested to associate with the deletion of *FHIT* located on 3p14.2 (5,6), are early events during tumorigenesis and are thought to initiate ccRCC development. Tumors from two stage I ccRCC patients (RCC14 and 18) had monoallelic loss of *BAP1* together with genes on 3p22.2-3p14.3, but not *FHIT*. Deletion of *FHIT* might not be essential for a large loss of 3p, but mutations of several tumor suppressor genes on 3p might trigger a large loss of 3p by cooperating with *VHL* mutation.

Mutations in *BAP1, PBRM1* and *SETD2* were detected in ccRCCs with *VHL* mutation. The rate of hemizygous truncation mutation of *BAP1* in ccRCC was 4/42, resulting in negative nuclear expression of the encoded protein. RCC50, which showed uniparental disomy on chr3p21.31-3p21.1, had a biallelic *BAP1* missense mutation that would cause functional loss, and also a biallelic *SETD2* mutation. In addition, this tumor was negative for BAP1 nuclear staining, although the mutation did not occur in the nuclear-localization signal. Biallelic deletion of *BAP1* was not detected. The frequency of *BAP1* mutation (11.1%) is close to that in other studies (9-11,18). Even though the mutation rate of *BAP1* was low, 1/3 of our tumors showed negative nuclear staining of BAP1 by immunohistochemistry. Peña-Llopis *et al* have also reported this difference, but the difference was small because 25 out of 176 samples were negative for nuclear staining, and 22 out of 25 tumors without expression had a *BAP1* mutation (9). A subsequent multi-institutional cohort study using immunohistochemistry without genetic testing indicated that BAP1 protein was negative for nuclear staining in 82 of 559 ccRCC tumors (14.7%) (37). Because of the genetic heterogeneity of ccRCC (38), it was difficult to validate heterogeneous staining for BAP1 to obtain uniformity in scoring, without differences among institutions.

There are several ways to explain negative nuclear expression of BAP1 protein without biallelic mutation or deletion: epigenetic regulation of gene expression by DNA methylation or histone modification, excess degradation of mRNA or protein, repressed translation by miRNA, or dysregulation of nuclear localization. Promoter methylation of *BAP1* was not detected in ccRCC (39) nor in MM (29). Nuclear localization is important for the function of this protein (31). Recently a ubiquitin-conjugating enzyme, UBE2O, has been noted to multi-monoubiquitinate the nuclear localization signal of BAP1, resulting in its cytoplasmic sequestration. UBE2O activity is counteracted by BAP1 autodeubiquitination through intramolecular interactions (32).

Three chRCC tumors showed strong cytosolic staining of BAP1 that might result from copy number amplification of this gene or aberrant transportation by BAP1 ubiquitinated by UBE2O. We could not test the expression level of the *BAP1* gene because our RCC samples contained a substantial proportion of non-tumor cells. Because ~30% of ccRCC tumors with hemizygous normal *BAP1* showed negative nuclear staining (Fig. 1), haploinsufficiency might be related to RCC development.

BAP1 mutations have been tightly linked to worse clinical outcome in multiple studies (9,11,40). Our data showed that

patients with biallelic *BAP1* mutation had worse recurrence-free survival than the patients without biallelic mutation (p=0.046), although multivariate analyses did not show the association between *BAP1* mutation and tumor recurrence probably due to small sample size. We did not find an association between negative nuclear BAP1 staining and tumor recurrence. This shows that genomic analysis of *BAP1* gene is useful in interpreting the prognosis of RCC.

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