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Original Articles

Stabilization of MCRS1 by BAP1 prevents chromosome instability in renal cell carcinoma

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ABSTRACT

Characterization of the exome and genome of carcinoma (ccRCC) by next-generation sequencing identified numerous genetic alternations. BRCA1-associated protein-1 (BAP1) was identified as one of the most frequently mutated genes in ccRCC, suggesting that BAP1 is a potential key driver for ccRCC cancer initiation and progression. However, how BAP1 mutations contribute to ccRCC remains to be elucidated. BAP1 is a nuclear de-ubiquitinating enzyme and cleaves the ubiquitin chain from the substrates. Here, we identified MCRS1 as a *bona fide* substrate for BAP1. MCRS1 is a component of the centrosome proteins, and plays an essential role in spindle assembly. BAP1 binds to MCRS1 and stabilizes MCRS1 by de-ubiquitination. BAP1 contributes to chromosome stability partially via MCRS1. A positive correlation was identified between BAP1 and MCRS1 expression in ccRCC tissues. Both BAP1 loss and MCRS1 down-regulation in ccRCC were associated with adverse clinicopathological features. This study revealed a novel mechanism for BAP1 involved in MCRS1 stability regulation, and provided insight in understanding the relationship between BAP1 mutations and chromosome instability in ccRCC.

Introduction

Renal cell carcinoma (RCC) is the most lethal of the common urologic cancers and constitutes 2%–3% of all adult malignant neoplasms [1]. Approximately 271,000 new diagnoses of RCC are made each year worldwide, and 116,000 patients die of the disease [2]. Clear cell renal cell carcinoma (ccRCC) is the most common type of RCC, accounting for 70%–80% of all kidney cancers. Although complete surgical resection can achieve a cure for localized ccRCC, a high proportion of patients with metastases or relapse have a poor outcome despite recent advances in systemic therapy [3]. Recently, whole-genome and exome sequencing identified BRCA1-associated protein-1 (BAP1) as one of the most frequently mutated genes in ccRCC [4–6]. BAP1 inactivated mutations cause BAP1 protein loss in ccRCC, which is related with worse clinical outcomes and aggressive clinicopathological features [5]. Study using a genetically manipulated mouse model demonstrated that BAP1 mutation, cooperating with the intragenic mutations in VHL, is responsible for initiating ccRCC development [7]. However, how BAP1 mutation contributes to the initiation and progression of ccRCC remains poorly understood.

Posttranslational modification of proteins by covalent attachment of ubiquitin via E1-E2-E3 ubiquitin ligation enzyme cascades controls many essential cellular processes [8]. The ubiquitin ligases select substrates for ubiquitin conjugation, which is reversed by the action of de-ubiquitination enzymes (DUB) [9]. BAP1 is a nuclear DUB that was originally identified as a BRCA1-binding protein in a yeast two-hybrid screen [10,11]. BAP1 has been linked to the de-ubiquitination of several substrates in cells, including the transcriptional regulator host cell factor 1 (HCF1), histone H2Aub, Ino80, and γ-tubulin [12–16]. However, very limited numbers of BAP1 targets were identified and functionally explored in ccRCC.

The microspherule protein 1 (MCRS1) was originally identified as the interaction partner of the p120 nucleolar protein [17]. MCRS1 has been implicated in regulating various biological processes, such as transcription, mitosis, cell proliferation, and senescence [18–21]. Moreover, studies showed that MCRS1 comprises a component of the centrosome, essential for spindle assembly and cell division. Loss of MCRS1 caused chromosome instability and aneuploidy [22,23].

Abbreviations: BAP1, BRCA1-associated protein-1; MCRS1, microspherule protein 1; ccRCC, clear cell renal cell carcinoma; DUB, de-ubiquitination enzymes; Co-IP, co-immunoprecipitation.

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In the present study, MCRS1 was identified as a novel BAP1 interactor. BAP1 loss correlates with MCRS1 down-regulation in ccRCC. Moreover, de-ubiquitination of MCRS1 by BAP1 plays important roles in regulating proper mitotic progression and prevents chromosome instability.

Materials and methods

Yeast two-hybrid screening

The yeast two-hybrid screen was performed with full length BAP1 cloned in-frame with the GAL4 DNA binding domain in vector PGBKT7 (Clontech). The yeast cells were transformed with PGBKT7-BAP1 and the human HeLa cDNA library. A total of 2×10^7 independent clones were screened by growth in deficient medium and X-gal staining. The positive clones were subsequently retested in fresh yeast cells, and the identities of prey were determined with interaction sequence tags (ISTs) obtained by DNA sequencing. The reading frame was verified.

Cell culture and transfection

786-O, HK-2, and 293T cells were obtained from the American Type Culture Collection. 786-O cells were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS). 293T cells were cultured in DMEM with 10% FBS. HK-2 cells were cultured in DMEM/F12 (GIBCO) supplemented with 10% FBS. Cells were transiently transfected with plasmids or siRNAs using Lipofectamine 3000 or RNAiMax Transfection Reagent (Invitrogen) according to the manufacturer's instructions.

Expression constructs

The BAP1 and MCRS1 cDNAs were purchased from Genechem, and subcloned into pCIN4-FLAG-HA and pCMV-Myc expression vectors. The cDNA for MCRS1 was also subcloned into PCIN4-mCherry vectors to create a mCherry-MCRS1 fusion protein. BAP1 and MCRS1 mutants were generated by the KOD-Plus Mutagenesis Kit (TOYOBO). All the constructs were verified by DNA sequencing.

RNA interference

The negative control and specific siRNAs for BAP1 and MCRS1 were purchased from GenePharma. Transfection of siRNAs was performed following the manufacturer's instructions. siRNA sequence information is provided in Table S1.

Immunoprecipitation

For immunoprecipitation of the FLAG-tagged proteins, transfected cells were lysed 24 hr after transfection with BC100 buffer. The whole-cell lysates were immunoprecipitated by overnight incubation with monoclonal anti-FLAG antibody-conjugated M2 agarose beads (Sigma). After three washes with FLAG lysis buffer, followed by two washes with BC100 buffer, the bound proteins were eluted from the beads with FLAG-Peptide (Sigma)/BC100 and were subjected to Western blotting. For immunoprecipitation of the endogenous proteins, cells were lysed with cell lysis buffer (Cell Signaling), and the lysates were centrifuged. The supernatant was precleared with protein A/G beads (Sigma) and incubated with the indicated antibody overnight at 4 °C. The immunocomplexes were then incubated for 2 hr at 4 °C with protein A/G beads. After centrifugation, the pellets were collected and washed five times with lysis buffer, resuspended in sample buffer, and further analyzed by SDS–PAGE.

Western blotting

Cell lysates or immunoprecipitates were subjected to SDS–PAGE, and then proteins were transferred onto nitrocellulose membranes (GE Healthcare). The membranes were blocked in Tris-buffered saline (TBS; pH 7.4) containing 5% nonfat milk and 0.1% Tween-20, washed three times in TBS containing 0.1% Tween-20, and incubated with the primary antibody overnight at 4 °C, followed by the secondary antibody for 1 h at room temperature. Antibody binding was visualized using the ECL Chemiluminescence System (Santa Cruz). Information of primary antibodies used in this study is provided in Table S2.

Quantitative RT-PCR

Total RNA from transiently transfected cells was extracted using the TRIzol reagent (Invitrogen), and cDNA was reverse transcribed using the Superscript RT Kit (TOYOBO), according to the manufacturer's instructions. Primer sequence information is provided in Table S1. PCR amplification was performed using the SYBR Green PCR Master Mix Kit (TOYOBO). Endogenous GAPDH was used for normalization.

Immunofluorescence

Cells cultured on coverslips in 24-well plates were fixed in 4% paraformaldehyde for 10 min and permeabilized in 0.2% Triton X-100 solution for 5 min. The coverslips were blocked with 2% BSA plus 5% goat serum for 1 h, and subsequently incubated with primary antibodies against HA, α-tubulin, and γ-tubulin, which was followed by sequential incubation with fluorescent secondary antibodies (Alexa 488 goat anti-mouse, Alexa 488 goat anti-rabbit, or Alexa 546 goat anti-mouse; Invitrogen). Finally, cells were counterstained with DAPI to reveal the nuclei. Fluorescence images were captured and processed using a fluorescence microscope.

Clinical specimens and immunohistochemistry

Tissue samples were obtained from patients with previously untreated, nonmetastatic ccRCC who underwent radical nephrectomy at the Department of Urology, Shanghai General Hospital from August 2012 to December 2013. The specimens were collected from a normal region (at least 5 cm from the tumor) and from a tumor for each patient. The histological diagnosis was confirmed simultaneously by examining hematoxylin and eosin (H & E) stained sections by two pathologists. The pathological stage was determined according to the American Joint Committee on Cancer (AJCC), and the tumor grade was classified using the Fuhrman grading system [24]. All patients were informed, and consent was given.

All the specimens were fixed in formalin for up to 24 h immediately after surgery, and then dehydrated, paraffinized, and embedded in paraffin blocks. Tissue sections were cut at 3–4 μm and air-dried overnight. The sections were deparaffinized, rehydrated, and subjected to heat-induced antigen retrieval with sodium citrate buffer (10 mM sodium citrate, 0.05% Tween-20 (pH 6.0)), which was followed by incubation with 3% hydrogen peroxide for 5 min to block endogenous peroxidase activity. Sections were then incubated with the appropriate primary antibody, and were sequentially incubated with biotinylated goat anti-mouse IgG. For signal detection, the VECTASTAIN ABC kit (Vector Laboratories) was used according to the manufacturer's instructions. The slides were further counterstained with hematoxylin. Appropriate positive and negative controls were utilized for each immunostain run.

Statistical analysis

Experiments were carried out with three or more replicates unless otherwise stated. All statistical tests were two-sided and performed using GraphPad Prism (Graphpad Software). Statistical analyses were performed by Student's t-test for most studies. The relationship between BAP1 and MCRS1 expression was analyzed by the Spearman rank correlation. Differences between the expression of BAP1 or MCRS1 and clinicopathological features were assessed by Fisher's exact test. Values with $P < 0.05$ were considered statistically significant.

Results

BAP1 interacts with MCRS1

To identify molecular mediators of BAP1 function, we screened a human fetal brain cDNA library with a yeast two-hybrid approach using the full length BAP1 as bait. Seventeen positive clones were obtained and determined by DNA sequencing, representing five unique interactions (Table S3). Among them, ten clones correspond to BAP1 fragments. This result implied that BAP1 can interact with itself. In addition to BAP1, four clones correspond to MCRS1 fragments (Fig. S1). Since BAP1–MCRS1 interaction has not been reported in the literature, we aimed to investigate the potential functional relationship between BAP1 and MCRS1.

To verify that MCRS1 is a *bona fide* BAP1 interactor, we first examined whether BAP1 can interact with MCRS1 in cells. FLAG-HA (FH)-BAP1 and Myc-MCRS1 constructs were co-expressed in 293T cells. Cells were subsequently harvested for co-immunoprecipitation (Co-IP) with the anti-FLAG antibody. As shown in Fig. 1A, Myc-MCRS1 was immunoprecipitated by FH-BAP1, suggesting an exogenous interaction between these two proteins. In addition, a reciprocal Co-IP assay was performed using lysates of 293T cells co-transfected with FH-MCRS1 and Myc-BAP1 constructs. The results indicated that FH-MCRS1 was able to immunoprecipitate Myc-BAP1 (Fig. 1B). Next, we investigated whether endogenous BAP1 and MCRS1 can interact with each other. In this case, 786-O cells, a BAP1-wildtype ccRCC cell line [5], were chosen for subsequent study.