RB1CC1 activates the p16 promoter through the interaction with hSNF5

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Abstract. RB1-inducible coiled-coil 1 (RBIC1, also known as FIP200) is involved in dephosphorylation and increase of retinoblastoma tumor suppressor protein (RB1), but the RBIC1 molecular mechanism in the dephosphorylation of RB1 is not fully understood. We determined that RBIC1 activates the expression of p16 (also called INK4a/cDKN2a) through the activation of its promoter, using chromatin immunoprecipitation (ChIP) and p16 promoter-luciferase reporter assays. In addition, RBIC1 essentially requires binding with hSNF5 (also known as BAF47/IN1, a chromatin-remodeling factor) to activate the p16 promoter, in order to enhance the RB1 pathway and acts as a tumor suppressor. Evaluation of the RBIC1 mechanism of action is expected to provide useful information for clinical practice and future therapeutic strategies in human cancers.

Introduction

INK4a/CDKN2a is generally known as p16, a tumor suppressor protein that functions as an inhibitor of CDK4 and CDK6, which initiate phosphorylation of retinoblastoma tumor suppressor protein, RB1. p16 induces G1 cell cycle arrest, and plays a physiological role in the implementation of irreversible growth arrest (1). Thus, p16 plays a key role in cellular senescence in human cells, and is important in resistance to oncogenic transformation (1). It is known that p16 transcription is activated by hSNF5 (2-4) and Ets1/2 (5,6), and is inhibited by Id1 and Bmi-1 (7-9). Genetic mutation and deletion of these p16 regulators have not been widely reported, while hSNF5 is mutated with loss of functionality in malignant rhabdoid tumor (MRT), an extremely rare malignancy. Indeed, the functional relationship between p16 and RB is frequently deregulated in many types of human cancer. However, the mutations and deletions in p16, RB and upstream regulators of this pathway are not frequent (10). There could presumably be other factors than the p16 and RB that regulate the pathway relating to progression or prevention of tumorigenesis.

RB1-inducible coiled-coil 1 [RBIC1; the symbol referred to here and approved by the Human Genome Organization (HUGO) Gene Nomenclature Committee; also known as FIP200] was identified as an RB1 regulator. In our previous report, we described that RBIC1 activates the RB1 promoter through the 201 bp upstream GC-rich region (11). We have also reported that RBIC1 forms a complex with hSNF5 and/or p53, and coordinates with it to activate the p16 promoter (12).

In the present study, we initially performed an immunohistochemical analysis, and noted that nuclear RBIC1 expression was significantly correlated with p16 expression. In addition, the labeling indices of hSNF5 were constantly high (~80%) in all cases of breast cancer, and there was no correlation between hSNF5 and p16. We hypothesized that RBIC1 regulated the quantitative activation of the p16 promoter. Therefore, we analyzed the mechanism of how RBIC1 regulated p16 transcription enhancing its amount.

Here, we discovered that RBIC1 controlled the transcription level of p16 in the presence of hSNF5, and that RBIC1 was an upstream regulator of p16 transcription in many different types of cancer, except for MRT, an extremely rare malignancy.

Materials and methods

Antibodies. Rabbit antisera against RBIC1 (aa. 25-271 as epitope) were generated as previously reported (13), and the purified IgG fractions were used in the experiments. Anti-p16 (G175-405) and anti-BAF47 (25) were purchased from BD Bioscience (NJ, USA). Anti-HA (3F10) and anti-Flag (1e6) were from Roche (CT, USA) and Wako (Japan, Osaka), respectively.

Histology and immunohistochemistry. To evaluate the correlation between RBIC1, p16 and hSNF5 in human breast cancer
tissue, surgical specimens from 59 cases were embedded in paraffin after overnight fixation in 10% buffered formalin, and processed for a conventional histopathological diagnosis according to the World Health Organization (WHO) guidelines. Then, serially sliced 4-µm sections from a specimen of each case were deparaffinized and incubated overnight at 4°C with primary antibodies to RB1CC1, p16 and hSNF5 after immersion in 0.3% H2O2 and autoclaving at 120°C for 1 min. The sections were rinsed with 1X PBS and incubated with the secondary antibody (Simple Stain MAX-PO; Nichirei, Japan) at room temperature for 1 h. The sections were then stained with 3,3'-diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin.

Microscopic evaluation and statistical analysis of correlation between RB1CC1 staining and the labeling index of p16 or hSNF5. The extent of RB1CC1 expression was classified into three categories: I, negative stain in both cytoplasm and nuclei; II, positive stain only in cytoplasm and negative in nuclei; III, positive stain in nuclei and/or cytoplasm. For p16 and hSNF5, a nuclear staining reaction that was brownish in color was labeled as positive. The p16 and hSNF5 labeling indices were determined as follows: positive tumor cells/total tumor cells x 100.

Luciferase-reporter assay. The p16 wild-type- or mutant-, RB1-, p21- or Tp53Aip1- luciferase reporter plasmid was co-transfected using FuGene HD (Roche) into the HeLa cells one day after seeding into 35-mm-well plates at the concentration of 4X10^4 cells. Twenty-four hours later, luciferase activity was analyzed using a luciferase assay kit (Toyo Inc., Japan) according to the supplier's recommendations.

Chromatin immunoprecipitation (ChIP) assay. ChIP assay of the endogenous p16 promoter regions in HeLa or TCC642 cells was done by the ChIP-IT™ control and ChIP-IT™ Express kits (Active Motif, CA, USA) according to the manufacturer's instructions using anti-RB1CC1 IgG, anti-hSNF5 IgG and control IgG (Active Motif). The amount of co-immunoprecipitated DNA was evaluated by the quantitative polymerase chain reaction (qPCR). The amplified region of p16 promoter was from -711 to -529 (the initiation ATG was defined as +1). The sequences of the PCR primers were 5'-AGGAAATCTTTGAACACTAGG-3' and 5'-GGGAAAGATATGGGTTTCTTC-3'. The PCR conditions were multiple cycles of 3 steps (98°C, 10 sec at 58°C, 30 sec at 68°C, 30 sec).

Immunoprecipitation and Western blotting. Before immunoprecipitation, cells were lysed in 1X TNE buffer (20 mM Tris-HCl containing 150 mM NaCl, 5 mM EDTA, 1% NP-40 and 1 mM Na3VO4 and a mixture of protease inhibitors). After cleaning lysed material by centrifugation at 15,000 rpm for 10 min, the supernatant was rotary-incipitated
with anti-HA (Roche) immobilized beads for 2 h at 4°C. The beads were washed five times with 1X TNE buffer and boiled in SDS sample buffer. Proteins resolved by SDS-PAGE were transferred to polyvinylidene difluoride (PVDF) membranes and immunoblotted with the indicated antibodies.

Results

**RB1CC1 and p16 expressions correlate in cancer tissues.** RB1CC1 and p16 were immunohistochemically analyzed in 59 cases of breast cancers. Nuclear RB1CC1 expression correlated with high p16 expression (Fig. 1A). p16 labeling indices (mean ± standard deviation) were 5.94±4.11%, 2.37±3.44% and 55.60±29.56% in grade I (8 cases), II (6 cases), and III (45 cases) RB1CC1 expression categories, respectively. p16 labeling index was significantly high in the case with nuclear RB1CC1 expression (grade III). One-way factorial ANOVA and multiple comparison tests accompanied by Scheffe’s significance were used to evaluate the relationship (**p<0.01**). These results suggested that RB1CC1 nuclear expression correlated with the p16 expression level.

**RB1CC1 enhances the activity of p16 promoter.** In order to identify the critical region for RB1CC1 to activate the p16 promoter, the luciferase reporter assay was used. Wild-type (p16wt) and the various deletion mutants (A, B, C, AB and BC) of the p16 promoter reporter plasmid were co-transfected into HeLa cells with the RB1CC1-expression vector (RB1CC1) or empty (pcDNA) plasmid (Fig. 2). While the luciferase activity of the p16 mutant A (-870 to -624 bp) was approximately equal to that co-transfected with pcDNA, the promoters, which contained the region of B (-624 to -315 bp) and/or C (-326 to -22 bp), were ~3-5-fold enhanced by RB1CC1. Importantly, the promoter containing both regions (BC, -624 to -22 bp) has been reported that hSNF5 largely contributes to p16 expression (2-4). To evaluate the contribution of hSNF5 to p16 expression in vivo, we immunohistochemically analyzed the labeling indices of hSNF5 and p16 in similar cases of breast cancers. The labeling indices of hSNF5 were >80% in most of the breast cancer cases, and there was no correlation between hSNF5 and p16 labeling indices (Fig. 1C).
was additively stimulated by RB1CC1, and reached similar levels to that of the wild-type promoter (p16wt). These results suggested that RB1CC1 required the limited domain (-624 to -22 bp) of the p16 promoter to activate the promoter in a dose
(DNA-length) -dependent manner. Additionally, to identify the critical narrow domain of the p16 promoter activated by RB1CC1, we modified the p16 wild-type reporter plasmid and screened each activity co-transfected with RB1CC1. We made 8 (p16mut-Ebox1, -GT, -SpW, -sGC, -iGC, -Sp1, -Sp2, -Ebox2) and 8 (p16mut- NFkB, -GATA, -Ets1GATA, -Ets1, -Ets3, -Ets2Ets3, -Ets1Ets3 and -Ets1Ets2Ets3) kinds of the promoter mutants in the regions of B (-624 to -315 bp) and C (-326 to -22 bp), respectively. The p16 promoter activity was maintained irrespective of any other mutations (Fig. 2B and C). Altogether, while the critical narrow domain of p16 promoter was not required for the activation caused by RB1CC1 expression, the region BC (-624 to -22 bp) was required in a DNA-length-dependent manner.

RB1CC1 requires hSNF5 for binding to the p16 promoter.
To examine whether RB1CC1 binds to the p16 promoter, the ChIP experiments were performed using HeLa and TTC642 (hSNF5-/-) cells. Immunoprecipitated DNA fragments were amplified by PCR using the primer pairs specific to the p16 promoter (Fig. 3). In HeLa cells, the immunoprecipitates containing a p16 promoter fragment were significantly enriched by antibodies against RB1CC1 and hSNF5, in contrast to precipitates of normal IgG control. In TTC642 cells, which are genetically null for hSNF5, antibody against RB1CC1 failed to enrich the immunoprecipitates of p16 promoter fragments. Therefore, we considered that RB1CC1 binds to the -711 to -529 bp region of the p16 promoter, and that the binding essentially requires hSNF5 to activate the p16 promoter. RB1CC1 could not interact with the p16 promoter in the hSNF5-null cells such as TTC642.

RB1CC1 requires hSNF5 to activate the p16 promoter.
Recently, we have identified that RB1CC1 requires the involvement with hSNF5 and/or p53 to enhance the RB1 pathway (12). To evaluate the requirement of hSNF5 and p53 to activate p16, we utilized HeLa, TTC642 (hSNF5-/-) and H1299 (p53-/-) cells. Luciferase plasmid containing p16 promoter was transfected into these cells together with pcDNA or RB1CC1 (Fig. 4). RB1CC1 could not activate p16 promoter adequately in TTC642 and H1299 cells, while the activation increased by about 7-fold in HeLa cells. The data suggested that RB1CC1 required the involvement of hSNF5 and p53 to activate the p16 promoter. Similarly, each luciferase-reporter containing RB1, p21 or TP53AIP1 was applied into these cells, and we validated the molecular requirement of hSNF5 or p53 for the promoter activation caused by RB1CC1. RB1CC1 could activate neither the RB1 promoter in TTC642 cells, nor the p21 or TP53AIP1 promoters in H1299 cells. These results suggested that RB1CC1 required hSNF5 to activate the RB1 promoter, and p53 to enhance the promoters of p21 and TP53AIP1 (Fig. 4).
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hSNF5 binds to C-terminus of RB1CC1, which is largely required to activate the p16 promoter. To confirm that RB1CC1 requires hSNF5 to activate the p16 promoter, the binding between RB1CC1 and hSNF5 was evaluated, and it was additionally analyzed whether an RB1CC1 mutant that was unable to bind to hSNF5 could activate the p16 promoter. We generated RB1CC1 deletion-mutants (dCCB: aa1078-1363 deleted; and dLZ: aa1357-1594 deleted) (Fig. 5A). HA-tagged hSNF5 was cotransfected with the plasmids encoding flag-tagged RB1CC1 wild-type or mutants in HEK293 cells. In this co-immunoprecipitation assay, hSNF5 could not bind the dLZ mutant, which had lost the C-terminal region of RB1CC1, but could bind the dCCB mutant and RB1CC1wt (Fig. 3B). In addition, p16 promoter activity caused by RB1CC1wt or the mutants was validated using the luciferase assay. The RB1CC1 dLZ mutant that was unable to bind to hSNF5, could

Figure 3. RB1CC1 requires hSNF5 for the binding to the p16 promoter. The ChIP PCR analysis was performed in HeLa and TTC642 (hSNF5-/-) cells using anti-RB1CC1 and anti-hSNF5 antibodies. The immunoprecipitated DNA fragments were amplified by PCR using the primer-pairs specific to p16 promoter (-711 to -529 bp). The values indicated the means ± standard errors from more than quadruplicate experiments. Student’s t-test; asterisks (* p<0.05) indicate statistically significant differences with negative IgG.

Figure 4. RB1CC1 requires the involvement with hSNF5 to activate the p16 promoter. RB1CC1 also requires hSNF5 for RBl promoter activation, and needs p53 for the activations of p21 and TP53AIP1. Luciferase plasmid containing RB1, p16, p21 or TP53AIP promoter was transfected into HeLa, TTC642 (hSNF-/-) or H1299 (p53-/-) cells together with RB1CC1. Luciferase activity was calculated as the relative fold induction of each versus the value of co-transfection with pcDNA. The values indicate the means ± standard errors from quadruplicate experiments.
inadequately activate the \textit{p16} promoter, while RB1CC1wt or the dCCB mutant activated it adequately. In summary, RB1CC1 could activate the \textit{p16} promoter, depending to a large extent on the interaction with hsnF5.

**Discussion**

INK4a/CDKN2a/p16 functions as an inhibitor of D-type cyclin-dependent kinases (CDK4 and CDK6) that initiate phosphorylation of RB1, so p16 has the capacity to arrest cells in G1 phase of the cell cycle (1). Transcription of p16 is activated by Ets1/2 (5,6) and hsnF5, a chromatin-remodeling factor (2-4). Reciprocally, BMI-1, a polycomb protein, and Id1, a bHLH protein, are known as negative regulators of p16 (7-9).

RB1CC1 enhances the transcription of RB1 (11), and forms a complex with hsnF5 and/or p53 (12). Nuclear RB1CC1 expression was correlated with a high p16 expression (Fig. 1A-B), so it was suggested that RB1CC1 activated the p16 promoter. In the present study, hsnF5 was expressed in all of the breast cancer cases, and there was no correlation between hsnF5 and p16 expressions (Fig. 1C). Following-up on these immunohistochemical results; we analyzed the mechanism of how RB1CC1 controls p16 expression.

To detect the RB1CC1-activated site of the p16 promoter, we performed the promoter mapping using the luciferase-reporter assay. A limited domain (-624 bp to -22 bp) of the p16 promoter was required in a dose-dependent manner for the transcriptional activation caused by RB1CC1 (Fig. 2A). We screened the p16 promoter from -624 bp to -22 bp, to identify the critical narrow domain of the promoter, but were unable to find it (Fig. 2B-C). These results made us hypothesize that another essential important factor might be a mediator, not in a sequence-specific but in a dose (DNA-length) -dependent manner, when the p16 transcription was activated by RB1CC1. Previously, we indicated that RB1CC1 interacted with hsnF5 (12) and suggested that RB1CC1 activated the p16 promoter through the interaction with hsnF5.
To identify the possible cooperation between RB1CC1 and hSNF5 in activating the p16 promoter, we evaluated whether RB1CC1 and hSNF5 bound to the promoter at -711 to -529 bp. It was revealed that both molecules bound to this region of the p16 promoter in HeLa cells, while neither could bind there in hSNF5-null TTC642 cells (Fig. 3). In addition, our preliminary ChIP-sequence data using the next-generation sequencer (Solid, Life Science Technology) suggested that RB1CC1 bound to the -621 to -575 bp region of the p16 promoter (data not shown) in HeLa cells. This region of the p16 promoter is consistent with the binding region of hSNF5 that has been reported previously (3,4). Together, it is strongly suggested that hSNF5 functions as an essential interacting molecule when RB1CC1 binds to the p16 promoter. hSNF5 is a chromatin-remodeling factor that can activate the promoters without requiring specific binding sequences. Therefore, RB1CC1 activated the p16 promoter not in a sequence-specific, but in a dose (DNA-length)-dependent manner in our luciferase-reporter assay.

RB1CC1 binds to hSNF5 (12) as well as p53 (15), so the promoter enhancements caused by RB1CC1 were compared among HeLa, TTC642 (hSNF5-null) and H1299 (p53-null) cells, in order to validate the requirement for hSNF5 or p53 when RB1CC1 activated RB1, p16, p21 and TP53AIP1. It was revealed that RB1CC1 required p53 as an essential molecule to activate p21 and TP53AIP1, which are known as p53-targeting genes. Considering the RB1 promoter, RB1CC1 required hSNF5 rather than p53. Reciprocally, in order to activate p16 promoter, RB1CC1 required not only hSNF5 but also p53 as the important partners (Fig. 4). These results suggested that RB1CC1 might importantly form a large transcriptional complex with hSNF5 as well as p53 for p16 promoter enhancement.

Finally, we examined the binding between RB1CC1 and hSNF5 and the activation of p16, because it had been reported that hSNF5 was the upstream transcriptional activator for p16 (2-4). It was revealed that hSNF5 bound to the C-terminal region of RB1CC1, and that an RB1CC1 mutant without this region reduced significantly the ability to activate the p16 promoter (Fig. 5). Together with the finding that the C-terminus of RB1CC1 is essential for its nuclear localization (11), it is suggested that RB1CC1 can bind to hSNF5 in cell nuclei and is recruited to the p16 promoter by hSNF5 in order to activate the promoter. hSNF5 is expressed in most cancer cells, while hSNF5 is specifically deleted only in Mrt, a childhood rare malignancy. In many different types of cancer, other factors, including RB1CC1, may play important roles in p16 expression, although hSNF5 can recruit them to the promoter region.

In conclusion, the present study revealed that RB1CC1 activates the p16 promoter and enhances p16 expression, and that hSNF5 is an essential interacting molecule that makes it possible for RB1CC1 to play this role. Further studies on RB1CC1 are needed in order to help clarify the way forward in research on oncogenesis and senescence. Clinically, the evaluation of RB1CC1 status is a possible biomarker for the prognostic indicator in patients with various types of cancer.

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