Enhanced Expression of RAD51 Associated Protein-1 Is Involved in the Growth of Intrahepatic Cholangiocarcinoma Cells

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Abstract

Purpose: Intrahepatic cholangiocarcinoma (ICC) is the second most common primary cancer in the liver, and its incidence is increasing in developed countries.

Experimental Design: To discover novel molecular targets for the diagnosis and treatment of ICCs, we earlier analyzed expression profiles of 25 ICCs using a cDNA microarray containing 27,648 genes. In this study, we focused on the RAD51 associating protein-1 (RAD51AP1) gene because its expression was frequently elevated in our microarray data.

Results: Quantitative PCR confirmed that RAD51AP1 expression was elevated in the great majority of the ICCs examined. Immunohistochemical analysis with anti-RAD51AP1 antibody further corroborated its accumulation in 14 of 23 ICC tissues (61%). Notably, suppression of RAD51AP1 by short interfering RNA resulted in growth suppression of cholangiocarcinoma cells, suggesting its involvement in the development and/or progression of ICC. Because RAD51AP1 interacts with RAD51, a molecule involved in DNA repair, we investigated whether RAD51AP1 is implicated in DNA strand breaks using γ-irradiation. As a result, γ-irradiation augmented RAD51AP1 protein expression and brought a focus formation in the nuclei, where accumulated RAD51AP1 colocalized with phosphorylated histone 2AX (γ-H2AX) and RAD51. These data suggest that RAD51AP1 may play a role in cell proliferation as well as DNA repair.

Conclusion: Our findings may contribute to the better understanding of cholangiocarcinogenesis and open a new avenue to the development of novel therapeutic and/or diagnostic approach to this type of tumor.

Intrahepatic cholangiocarcinoma (ICC), arising from the epithelium of intrahepatic bile duct, is the second most common primary malignant tumor of the liver in the world. Epidemiologic studies have revealed that the incidence of ICCs is very high in Northeastern Thailand and is increasing in developed countries, such as England (1–3). One of the major causes of ICCs in Thailand is infection to liver fluke, including Opisthorhis viverrini, which is endemic in this area (3). However, the etiology of ICCs in developed countries remains unclear. It is hard to diagnose patients with ICC in early stages because of the lack of information of high-risk population for ICC in the countries that have no prevalence of liver fluke. What is worse, most of the ICCs are highly invasive and no effective chemotherapeutic drugs are currently available (4). The combination of these issues results in poor prognosis of patients with ICC. Hence, discovery of sensitive and specific diagnostic tumor markers for the detection of this tumor in early stages and the development of more effective drugs are matters of pressing concern.

Genetic alterations are involved in the carcinogenesis of ICC, which includes mutations in K-ras and TP53 and loss of heterozygosity at loci on chromosomes 1p, 6q, 9p, 16q, and 17p (5). In addition, deregulated expression of a number of genes has been reported in ICC; overexpression of cyclooxygenase 2 (6, 7), c-erbB-2 (7–9), vascular endothelial growth factor (10), hepatocyte growth factor/c-Met (11), interleukin-6 (9), and survivin (12). In our previous study, we carried out a genome-wide analysis of gene expression profiles of 25 ICCs by means of a cDNA microarray representing 27,648 genes (13). These efforts identified 52 genes that were commonly up-regulated in the tumors compared with epithelia of noncancerous intrahepatic bile duct. The panel of commonly up-regulated genes included genes that were reported to be involved in carcinogenesis. Although it is unclear how the remnants play a role in carcinogenesis, these genes may serve for novel diagnostic and/or therapeutic targets of ICC.

In this paper, we reveal that RAD51 associating protein-1 (RAD51AP1) is commonly overexpressed in ICCs and that its expression is associated with proliferation of cancer cells. We further show evidence that RAD51AP1 is involved in DNA...
damage by γ-irradiation. These results may be helpful for profound comprehension of cholangiocarcinogenesis and the development of new anticancer drugs targeting to RAD51AP1.

**Materials and Methods**

**Cell lines and tissue specimens.** TFK-1 and HuH28 (two human ICC cell lines) were obtained from Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University; OZ (another ICC line) was from Japan Health Science Foundation; and ETK-1, RBE, and SSP-23 (three ICC lines) were from RIKEN Bioresource Center. HCT116 colon cancer cells were gifts from Dr. B. Vogelstein. All lines were grown in monolayers in appropriate media: RPMI 1640 (Sigma) for ETK-1, RBE, SSP-25, and TFK-1; MEM (Sigma) for Huh28; William's medium E (Sigma) for OZ; and McCoy's 5a for HCT116, supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution (Sigma). Cells were maintained at 37°C in an atmosphere of humidified air containing 5% CO2. We obtained ICC tissues from 21 patients who underwent hepatectomy due to metastatic liver tumors in Kyoto University Hospital with informed consent. Each tumor was diagnosed histopathologically according to the general rules for the clinical and pathologic study of primary liver cancer (4th edition, Liver Cancer Study Group of Japan). The mean age of the patients was 59 years, and the 21 tumors were composed of 4 well-differentiated, 12 moderately differentiated, and 5 poorly differentiated adenocarcinomas. The detailed clinicopathologic data are described in our earlier report (13).

**RNA extraction and quantitative reverse transcription–PCR.** Total RNA was extracted from each sample and ICC cell lines using the QIAGEN RNeasy Mini kit (QIAGEN) according to the manufacturer's protocol. Subsequent cDNA synthesis was done as described previously (14). The TaqMan assay was carried out according to the manufacturer's protocol (Applied Biosystems) with the same aRNAs used for array analysis. We used a mixture of noncancerous intrahepatic biliary epithelial cells for the normal control because some of the ICC cases involved intraductal invasion of cancer cells and the liver tissues were accompanied with different levels of inflammation. β-Actin gene served as an internal control. The sequences and probes were as follows: RAD51AP1 forward primer 5′-GCCCTTGCTGCTCCAGAT-3′ and reverse 5′-GGTCCTAGTGCTTGGCATGT-3′, probe 5′-FAM-AGCAC-GCTTAAACCT-MGB-3′ and β-actin forward primer 5′-GCCCAAGCACAATGAG-3′ and reverse 5′-ACACGGAGTACTTGCGCTCA-3′, probe 5′-FAM-TCAGATCTGCTCTCC-MGB-3′.

**Northern blot analysis.** Human multiple-tissue blots from (Clontech) were used for the analysis. We prepared additional blots as described elsewhere (15). Poly(A) RNA from normal organs was purchased from BD Bioscience Clontech, and that from cell lines was purified from total RNA by mRNA purification kit (Amersham Biosciences). The probe was prepared by reverse transcription–PCR using a set of primers 5′-AAACATGATCAAATCTACCCG-3′ and 5′-GGCTAGAGGTCTTATAGCAGGG-3′. Prehybridization, hybridization, and washing were carried out according to the supplier's recommendations. The blots were autoradiographed for 72 h.

**Preparation of polyclonal antibody against RAD51AP1.** The entire coding region of RAD51AP1 was amplified using a set of primers, 5′-ATTGATATCCATGCTGGCCTGTGACACATAG-3′ and 5′-ATCTCGAGGCTGTCGTTTAGCTAGGCGAAG-3′, and cloned into an appropriate enzyme site of pET28b vector (Novagen). The recombinant protein was expressed in Escherichia coli, BL21 codon plus strain (Stratagene), and purified using TALON resin (BD Bioscience) according to the manufacturer's recommendations. The recombinant RAD51AP1 protein was inoculated into rabbits, and antisera were purified from the rabbits on affinity columns according to the standard methodology.

**Immunoblotting and immunohistochemistry.** Cell extracts prepared as described elsewhere (16) were separated by 15% SDS-PAGE and immunoblotted with anti-RAD51AP1 polyclonal antibody or anti–β-actin antibody (Sigma). Horseradish peroxidase–conjugated donkey anti-rabbit or sheep anti-mouse IgG (Amersham Biosciences) served as the secondary antibody for the ECL Detection System (Amersham Biosciences). Immunohistochemical staining was carried out using affinity-purified polyclonal antibody against human RAD51AP1. Paraffin-embedded tissue sections of cholangiocarcinomas and corresponding normal portions of the liver were subjected to the SAB-PO peroxidase immunostaining system (Nichirei) according to the manufacturer's instructions. For antigen retrieval of the RAD51AP1 protein, the specimens were immersed in a 10 mmol/L citrate buffer (pH 6.0) and pressure microwaved for 10 min at 100°C.

**Foci formation assay.** ETK-1 cells were grown and treated in chamber slides and subjected to specified doses of γ-irradiation. At specified times after irradiation, cells were fixed in 4% paraformaldehyde for 15 min at room temperature followed by a treatment of 0.1% Triton-X for 3 min. After blocking with 3% bovine serum albumin for 1 h, the primary antibodies were added in 1% bovine serum albumin overnight at 4°C; rabbit anti-RADSAP1 polyclonal antibody and mouse anti–γH2AX monoclonal antibody (Upstate) were used for the primary antibodies. Regarding RAD51, we constructed a plasmid expressing RAD51 by PCR amplification using primers 5′-ATTGGATTCATGCTGGCCTGTCGAC-3′ (forward) and 5′-AAATCTAGATCTGGCTCTCCGCAATATCC-3′ (reverse), and the PCR product was cloned into BamHI-XhoI enzyme site of the pcDNA3.1-myc/His expression vector (Invitrogen). Then, we transiently transfected ETK-1 cells with pcDNA3.1-myc/His-RAD51 24 h before γ-irradiation. Mouse anti-myc (9E10) monoclonal antibody was used as primary antibody for detection of RAD51. The reactions were visualized after incubation with Alexa Fluor 488 goat anti-mouse IgG or Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes) for 1 h at room temperature. Fluorescence images were obtained with a TCS SP2 AOBS Spectral Confocal Scanning System (Leica). Nuclei were classified as positive for RAD51AP1 or γ-H2AX foci formation when more than five foci were detected per nuclei. At least 100 cells selected at random were counted per experiment point.

**Gene silencing effect of RAD51AP1.** A plasmid vector (psiH1B3X.0) expressing double-stranded short interfering RNA (siRNA) with a hairpin loop was generated as described previously (14). Plasmids expressing siRNAs were prepared by cloning double-stranded oligonucleotides into the BsiI site of the psiH1B3X.0 vector. The oligonucleotides used for RAD51AP1 and EGFP siRNAs were as follows: 5′-TCCCGAATCTACGTGCGAAGATGTTCAGCTCTCCACAGTAATC-3′ and 5′-AAAACATGATCAAATCTACCCGG-3′, for siRAD51AP1-si1; 5′-TCCCGAATCTACGTGCGAAGATGTTCAGCTCTCCACAGTAATC-3′ and 5′-AAAACATGATCAAATCTACCCGG-3′, for siRAD51AP1-si2. Plasmids expressing antisense of RAD51AP1 (psiH1B3X.0-RAD51AP1-mis1, psiH1B3X.0-RAD51AP1-mis2), and EGFP (psiH1B3X.0-EGFP), or control plasmid (mock) were transfected into ETK-1 or SSP25 cholangiocarcinoma cells using FuGENE6 reagent according to the manufacturer’s instructions (Roche). The cells were maintained for 12 days after transfection in appropriate culture media supplemented with 0.3 µg/µL (for ETK-1) or 0.4 µg/µL (for SSP25) of Geneticin (Life Technologies). The number of cells was counted per experiment point.

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measured with Cell Counting Kit-8 (Dojindo). The cells were also fixed with 100% methanol and stained with Giemsa’s solution.

Statistical analysis. Statistical significance was determined by Mann-Whitney U test using a commercially available software, Statview 5.0 (SAS Institute). Difference of P < 0.01 was considered statistically significant.

Results

Overexpression of RAD51AP1 in ICC. We had previously analyzed expression profiles of 25 ICCs by a cDNA microarray containing 27,648 genes and identified 52 commonly up-regulated genes (13). Among the 52 genes, RAD51AP1 expression was elevated >5-fold in all 13 tumors that passed our cutoff criteria (data not shown). By real-time reverse transcription–PCR analysis, we subsequently examined RAD51AP1 expression levels using the RNA samples that had served for the microarray analysis. We analyzed 21 ICC tissues, but could not analyze the remaining four because the RNAs were used up in the microarray analysis. As a result, we observed elevated RAD51AP1 expression (>5-fold) in 17 of 21 tumors compared with a mixture of normal biliary epithelia (Fig. 1A), which corroborated the enhanced RAD51AP1 expression in ICC.

Expression of RAD51AP1 in normal organs. To investigate RAD51AP1 expression in human normal tissues, we carried out multiple-tissue Northern blot analysis using RAD51AP1 cDNA as a probe. A 2.3-kb RAD51AP1 transcript was expressed abundantly in testis, and slightly in thymus, placenta, small intestine, stomach, and bone marrow, but not in any other 17 tissues examined (Fig. 1B). We additionally carried out Northern blot analysis using six ICC cell lines, all of which expressed high levels of RAD51AP1 (Fig. 1C), whereas its expression was not detected in important normal organs, such as heart, liver, lung, and kidney.

Accumulation of RAD51AP1 protein in tumor cells. To analyze expression of RAD51AP1 protein and its function, we raised an anti-RAD51AP1 rabbit polyclonal antibody. Exogenous myc-tagged RAD51AP1 was stained in the nuclei of COS7 cells transfected with plasmids expressing myc-tagged RAD51AP1 by immunocytochemical staining (Supplementary Fig. S1). Consistently, endogenous RAD51AP1 was localized in the nuclei. Next, we evaluated its detailed subcellular localization during cell cycle progression by synchronizing ETK-1 cells with aphidicolin and carried out immunocytochemical staining using anti-RAD51AP1 polyclonal antibody as a primary antibody. The protein was located mainly in the nucleus when the cells were at G1, S, and G2 phase; however, it distributed diffusely in cytoplasm, not associated with chromatin, at M phase, possibly due to the lack of nuclear membrane (Supplementary Fig. S2). Immunohistochemical staining with anti-RAD51AP1 polyclonal antibody using paraffin-embedded tissues revealed that accumulated RAD51AP1 was observed dominantly in the nucleus of cancer cells in 14 of 23 cases (61%) compared with corresponding bile duct epithelia and surrounding connective tissues (Fig. 2).
Effect of RAD51AP1 siRNAs on the growth of cholangiocarcinoma cells. To investigate the role of RAD51AP1 in biliary tumorigenesis, we constructed plasmids expressing siRNAs specific to RAD51AP1 (psiH1BX-RAD51AP1). Transfection with psiH1BX-RAD51AP1 suppressed RAD51AP1 expression in ETK-1 and SSP25 cholangiocarcinoma cells compared with a control plasmid, psiH1BX-EGFP (Fig. 3A). To determine sequence-specific suppression, we prepared mutant plasmids containing nucleotide substitutions (RAD51AP1-mis1 and RAD51AP1-mis2) of psiH1BX-RAD51AP1 and examined their gene silencing effect in ETK-1 and SSP25 cells. Although psiH1BX-RAD51AP1 markedly suppressed endogenous expression of RAD51AP1 protein, the mutant plasmids psiH1BX-RAD51AP1-mis1, or psiH1BX-RAD51AP1-mis2 had no suppressive effect on RAD51AP1 expression, showing almost similar levels of expression to the control (psiH1BX-EGFP; Fig. 3A; data not shown for SSP25). We further did colony formation assay using these plasmids to investigate the involvement of RAD51AP1 in proliferation of cancer cells. The number of viable ETK-1 cells transfected with psiH1BX-RAD51AP1 was significantly decreased compared with those transfected with psiH1BX-RAD51AP1-mis1, psiH1BX-RAD51AP1-mis2, or psiH1BX-EGFP control plasmids, suggesting that RAD51AP1 might play an essential role in the growth of cholangiocarcinoma cells (Fig. 3B).

Involvement of RAD51AP1 in DNA repair caused by γ-irradiation. RAD51AP1 was identified as a Rad51-interacting protein by yeast two-hybrid system (17). Because Rad51 plays a role in DNA repair, especially in homologous recombination, we evaluated whether RAD51AP1 is involved in DNA damage. We investigated focus formation of γ-H2AX, a phosphorylated form of histone H2A, which is a hallmark of DNA double-strand break and DNA repair. Expectedly, a time course analysis of the focus formation depicted that the foci of γ-H2AX was visible as early as 4 h after the irradiation in ETK-1 and HeLa cells (Fig. 4A; data not shown in HeLa cells), the consistent observation with previous reports showing that γ-H2AX is phosphorylated rapidly in response to DNA damage (18). An assessment of the staining pattern of RAD51AP1 detected foci formation in the cells 24 h after irradiation (Fig. 4A; data not shown for HeLa cells), and the foci colocalized with those of γ-H2AX. The foci of RAD51AP1 were not clearly detected at 4 h, but started to be detectable at 8 h after γ-irradiation, showing different kinesis of focus formation of these two proteins (Fig. 4A and B). In addition, the foci of RAD51AP1 were colocalized with those of RAD51 (Fig. 4C), which provided supportive evidence of cooperative involvement of RAD51AP1 and RAD51 in DNA double-strand break repair. Western blot
analysis revealed that RAD51AP1 was significantly accumulated in the cells 12 h after exposure to 4 or 20 Gy of γ-irradiation (Fig. 4D; data of HeLa cells not shown). To investigate the role of p53 in RAD51AP1 induction, we examined its expression levels in HCT+/+ and HCT116-/- cells, expressing wild-type or null p53, respectively. Consequently, both cell lines accumulated similar levels of RAD51AP1 after γ-irradiation (Fig. 4E), suggesting that RAD51AP1 is enhanced in a p53-independent fashion.

**Discussion**

In the present study, we found that expression of RAD51AP1 was enhanced in the majority of cholangiocarcinoma tissues compared with normal bile duct epithelia. Several studies reported that RAD51AP1 was up-regulated in hepatocellular carcinomas (19), acute myeloid leukemia with complex karyotypic abnormalities (20), and aggressive mantle cell lymphoma (21). These data are in line with the view that its elevated expression is associated with cell proliferation. Consistently, our Northern blot analysis revealed that its expression was detected in placenta, small intestine, stomach, and bone marrow, organs containing cells with high replication, but not in brain or spinal cord, those with low levels of dividing cells. Furthermore, down-regulation of RAD51AP1 by gene-specific siRNA resulted in growth suppression of cholangiocarcinoma cells. This result indicates that RAD51AP1 expression is essential for the growth of tumor cells and that its inhibition may be a novel therapeutic option for treatment of cholangiocarcinoma cells. On the other hand, CpG methylation of its promoter region leads to the reduced expression of RAD51AP1 in prostate cancer cells (22). Therefore, RAD51AP1 may play a role in a tumor type–dependent manner. Alternatively, other factor(s) may take place the signaling pathway mediated by RAD51AP1.

RAD51AP1 was first identified by yeast two-hybrid system as a protein interacting with human RAD51 (17). RAD51 functions as a key factor catalyzing homologous recombination process, one of the repair mechanisms involved in resolving stalled replication forks during DNA replication (23–26). Homologous recombination is induced in response to DNA damage by environmental insults, as well as spontaneous misreplication during cell cycle progression, to maintain genetic stability. Because RAD51AP1 associates with...
RAD51, we studied here the role of RAD51AP1 in DNA double-strand breaks. We, here, showed that RAD51AP1 colocalized with γ-H2AX after the exposure to γ-irradiation, suggesting that RAD51AP1 is associated with DNA double-strand breaks. Although we observed induction of both RAD51 and RAD51AP1 by 20 Gy of γ-irradiation, another study reported that RAD51AP1 did not respond to various DNA damage, such as γ-irradiation, doxorubicin, and UV irradiation in HCT116 colon cancer cells (21). In their data, suppression of RAD51AP1 by siRNA had little or no effect on homologous recombination repair. Therefore, induction of RAD51AP1 may be dependent on the dose of insults and condition of cells. In our study, a time course analysis of foci formation by γ-irradiation documented that foci formation of RAD51AP1 occurred in comparatively late phase of DNA repair process, whereas γ-H2AX was recruited rapidly at the site of double-strand breaks (18). Considering that other molecules associated with DNA damage, such as ATM, MRE11, NBS, and RAD50, accumulate at relatively early time points after double-strand breaks (27), RAD51AP1 may exert a distinctive role after DNA double-strand breaks. Interestingly, mitomycin C, a DNA cross-linking anticancer drug, increased RAD51AP1 in colon cancer cells, and knockdown of RAD51AP1 enhanced sensitivity of HeLa cells to mitomycin C (21). Therefore, RAD51AP1 may play an important role in cell survival after DNA cross-linking reaction. Further experiment using RAD51AP1-null cells will clarify its role against various genotoxic insults.

Recently, it was reported that expression of RAD51AP1 was augmented through transactivation of E2F1, E2F2, and E2F3 transcription factors (28). During cell cycle progression, RAD51AP1 started to accumulate in an early S phase and kept high levels of expression to G2-M phases (21). This is consistent with the recent finding that RAD51AP1 expression was transactivated by E2F1, E2F2, and E2F3 transcription factors (28). Accumulated evidence disclosed that DNA repair machinerys are closely related to DNA replication. In physiologic conditions, homologous recombination is increased in late S phase after the foci formation of RAD51 (25) in response to strand breaks during DNA replication. In cancer cells, a number of molecules associated with DNA repair are up-regulated in the early steps of carcinogenesis. DNA damage responses, such as histone H2AX and Chk2 phosphorylation, p53 accumulation, nuclear staining of p53BP1, and apoptosis, are activated in premalignant cells, as well as cancerous cells (29, 30). Highly proliferating cancer cells are likely to associate with double-strand breaks induced by increased DNA replication, which should be corrected by DNA repair machineries. Although enhanced expression of RAD51AP1 did not confer transforming activity to NIH3T3 cells (data not shown), its inhibition resulted in cell growth retardation in ICC cells. Therefore, cells with elevated expression of RAD51AP1 may have advantage for their survival.

In conclusion, we identified RAD51AP1 as a commonly up-regulated gene in ICCs and showed abundant accumulation of RAD51AP1 protein in a great majority of ICCs by immunohistochemistry. Our data clearly underscore the importance of elevated expression of RAD51AP1 in cholangiocarcinogenesis because knockdown of RAD51AP1 showed retarded growth of ICC cells. Although further investigations are of necessity to disclose the mechanisms of RAD51AP1 in cell proliferation and survival, RAD51AP1 may serve for a novel molecular target for the development of anticancer drugs.

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