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Functional analysis of *OCIAD2* in hepatoblastoma

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ABSTRACT. Hepatoblastoma (HB) is the most common malignant liver tumor in children; however, the molecular mechanisms responsible for its progression remain unclear. We previously identified hypermethylation of the ovarian cancer immunoreactive antigen domain containing 2 (*OCIAD2*) as a poor prognostic factor for HB in a genome-wide methylation analysis of HB excision specimens. As it has been already reported that such hypermethylation of *OCIAD2* dysregulates the expression of *OCIAD2*, to elucidate the function of *OCIAD2* in HB, we evaluated altered cellular functions such as cell growth, invasion, and migration abilities using *OCIAD2* overexpression cell lines of HB. In the *in vitro* analysis with *OCIAD2* overexpression, no significant difference was observed in cell proliferation between the groups. However, migration and invasion abilities were significantly lower in *OCIAD2* overexpressed cell lines. Second, overexpression of *OCIAD2* reduced matrix metalloproteinase 2 (MMP2) levels in each cell line. These results suggest that *OCIAD2* suppresses HB invasion and migration in relation to MMP2.

Key words: Hepatoblastoma; Epigenomics; OCIA domain containing ; DNA methylation; Oncology

INTRODUCTION

Hepatoblastoma (HB) is the most common malignant tumor of the liver in children. Advances in therapy have led to satisfactory survival among children with non-metastatic HB; however, the outcomes of high-risk patients with extrahepatic tumors, macroscopic invasion of large vessels, and distant or lymph node metastases remain poor (Hishiki et al., 2011; von Schweinitz 2012; Hiyama et al., 2020). Therefore, new approaches based on innovative treatments and potent prognostic markers for better therapy planning are needed to improve the mortality of HB patients in advanced stages.

It is well known that beta-catenin mutation is a hallmark of HB, although results from recent whole-exome sequencing analyses have shown that other mutations are rarely seen in HB (Jia et al., 2014; Sumazin et al., 2016; Nagae et al., 2020; Carrillo-Reixach et al., 2021). This paucity of mutations has been reported in many pediatric tumors and may be correlated with their early age of onset (Vogelstein et al., 2013), indicating that epigenetic aberrations may be an important mechanism involved in the pathogenesis of HB.

We first reported that *RASSF1A* methylation was independently correlated with poor outcomes in HB patients (Honda et al., 2008; Honda et al., 2013). Furthermore, by using genome-wide analysis of DNA methylation, we successively identified four methylated genes, *OCIAD2, GPR180, PARP6*, and *MST1R,* which correlated with a poor prognosis in patients with HB tumors. The methylation status of the four genes correlated significantly with age at diagnosis and the presence of metastatic tumors or hepatic vein invasion (Honda et al., 2016).

Among these four genes, *OCIAD2* has recently been shown to be a tumor suppressor gene in ovarian mucinous tumors (Nagata et al., 2012) and hepatocellular carcinoma (Wu et al., 2017). Besides, in terms of the effect of hypermethylation of *OCIAD2* on expression, it has been reported that *OCIAD2* hypermethylation reduces the expression of *OCIAD2* (Wu et al., 2017). Consequently, these results suggest that decreased expression of *OCIAD2* might have a poor prognosis. Therefore, we investigated the molecular functions and pathways of *OCIAD2* in HB using constructed *OCIAD2* overexpression cell lines.

MATERIAL AND METHODS

Cell lines

We used HepG2 and HUH6 as HB cell lines and HLF as the poorly differentiated hepatocellular carcinoma (HCC) cell line. These cell lines were obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB, Osaka, Japan).

Expression analysis

Total RNA was isolated from cell lines using the QIAamp RNA Blood Mini kit (QIAGEN) and cDNA was synthesized using the First Strand cDNA Synthesis Kit and ReverTra Ace alpha (TOYOBO). Quantitative real-time PCR was performed using TaqMan Probes for *OCIAD2* and self-designed primers for *OCIAD2*, *GAPDH*, *OCIAD2*-F, TTGGTCCACAGCATAACAGG, *OCIAD2*-R, CCATGCTTTATTTTGCATTCC,

GAPDH-F, TTGGTCCACAGCATAACAGG, GAPDH-R, CCATGCTTTATTTTGCATTCC. Relative expression levels were calculated using the ΔCt method with the housekeeping gene *GAPDH*.

Lysates were extracted from cell lines. Twenty micrograms of the lysate were loaded onto each lane, subjected to electrophoresis, and transferred to PVDF membranes. TBS with 3% BSA and 0.1% Tween-20 was used for blocking. Primary antibodies against *OCIAD2* (1:1000, Sigma), E-cadherin (1:100, Abcam), vimentin (1:1000, CST), MMP2 (1:1000, CST), GAPDH (1:5000, CST), and rabbit secondary antibodies (1:5000, CST) were used in this study. Blots were detected using enhanced chemiluminescence (ChemiDOC, BIO-RAD).

Construction of overexpression cell lines

Full-length *OCIAD2* cDNA (465 bp) was cloned by RT-PCR and recombined into a pLVSIN-IRES-ZsGreen1 Vector (TaKaRa Clontech). We obtained a recombinant lentiviral supernatant using the Lenti-X 293T and Lenti-X HTX Packaging system (TaKaRa Clontech). Transfection was performed using the Xfect transfection reagent (TaKaRa Clontech). Cells were collected and sorted using BD FACS Aria III (BD Biosciences).

Evaluation of cell function

We performed a proliferation assay to evaluate the cell growth. Each cell line was dispensed in 96-well plates at a density of 2,000 cells/well. The cell population was measured on days 0-2 using a Cell Titer 96 Aqueous One Kit (Promega) with a VARIOSCAN FLASH microplate reader (Thermo Fisher Scientific). This experiment was performed three times, and the results were analyzed.

We used the BD Matrigel Invasion Chamber (24-well plate 8.0 microns, BD) for the invasion assay. The seeding concentration was 150,000 cells per 500 μL of serum-free DMEM. After a 48-h incubation period, the penetrating cells were stained and counted in the whole area. This experiment was performed three times, and the results were analyzed.

We used Transwell (24-well plate 8.0 microns, Corning) for the migration assay. The seeding concentration was 50,000 cells per 100 μL of serum-free DMEM. After a 48-h incubation period, the penetrating cells were stained and counted in the whole area. This experiment was performed three times, and the results were analyzed.

Statistical analysis

Data are presented as mean ± SE. Statistical analyses were performed using Fisher's exact test or Student's *t*-test. Statistical analyses were conducted using the statistical package R (version 3.2.2; available as a free download from http://www.r-project.org); $P < 0.05$ was considered statistically significant.

RESULTS

Effects of *OCIAD2* overexpression on cell growth, invasion, and migration

To assess the function of the *OCIAD2* protein in HB, we constructed *OCIAD2*-overexpressing HepG2 and HUH6 cells derived from HB. *OCIAD2* mRNA expression in these cell lines was confirmed using quantitative real-time PCR. *OCIAD2* mRNA levels were several hundred-fold higher in HepG2 or HUH6 cells transfected with the *OCIAD2* vector than in those transfected with the empty vector (Figure 1a). The expression of *OCIAD2* protein was confirmed by Western blotting. HepG2 and HUH6 cells transfected with the *OCIAD2* vector expressed the *OCIAD2* protein, whereas cell lines transfected with the empty vector did not express the protein (Figure 1b).

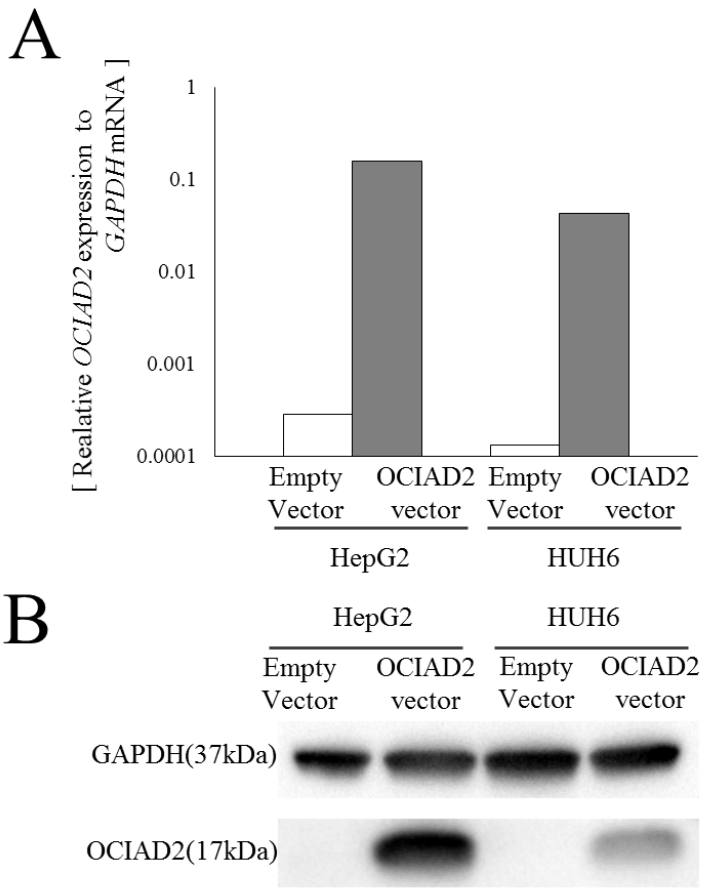

Figure 1. a Relative *OCIAD2* mRNA expression levels in the vector and empty vector groups by quantitative real-time PCR. **b** Relative *OCIAD2* protein expression levels in the *OCIAD2* vector and empty vector groups by Western blotting.

We evaluated altered cell functions such as proliferation, invasion, and migration abilities using the *OCIAD2* overexpression cell lines HepG2 and HUH6. As shown in Figure 3a, overexpression of *OCIAD2* did not affect cell growth. Cell growth was enhanced

in HepG2 by approximately three-fold and in HUH6 by approximately six-fold for 48 h, and no significant difference in cell growth was observed between cell lines transfected with the *OCIAD2* vector and empty vector (Figure 2a). The results of the cell invasion assay showed that the number of invaded HepG2 cells was 134±40/well in the empty vector group and 22±11/well in the *OCIAD2* vector group ($P < 0.05$). Similarly, the number of invaded HUH6 cells was 355±37/well in the empty vector group and 105±31/well in the *OCIAD2* vector group ($P < 0.02$). These results demonstrated that overexpression of *OCIAD2* significantly reduced the number of invading cells in HepG2 and HUH6 cells (Figure 2b). The migration assay revealed that the number of migrated HepG2 cells was 191±9/well in the empty vector group and 14±5/well in the *OCIAD2* vector group ($P < 0.01$). The number of migrated HUH6 cells was 1672±110/well in the empty vector group and 449±175/well in the *OCIAD2* vector group ($P < 0.02$). These results showed that overexpression of *OCIAD2* significantly reduced the number of migrated HepG2 and HUH6 cells (Figure 2c).

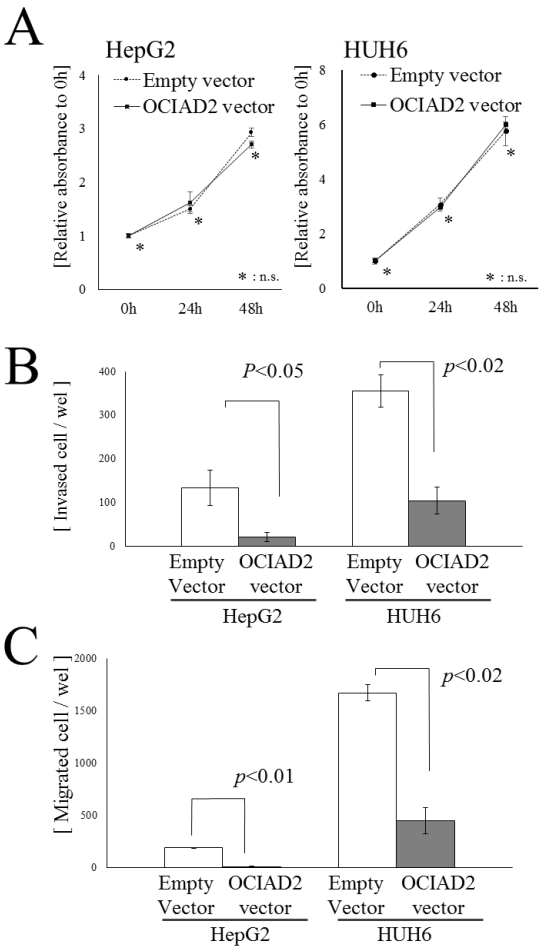

Figure 2 **a** Proliferation curves of the *OCIAD2* vector and empty vector groups. **b** Invasion assay results of the *OCIAD2* vector and empty vector groups. **c** Migration assay results of the *OCIAD2* vector and empty vector groups.

Effects of *OCIAD2* overexpression on protein expression

To clarify the mechanisms by which *OCIAD2* reduces migration and invasion abilities, we investigated the altered expression of proteins such as E-cadherin, vimentin, and MMP2 using Western blotting. In this analysis, we added HLF as a poorly differentiated hepatocellular carcinoma cell line exhibiting the typical morphological phenotypes of epithelial-to-mesenchymal transition (EMT). Overexpression of *OCIAD2* did not affect the expression of E-cadherin or vimentin; however, it reduced the expression of MMP2 in each cell line (Figure 3a, Figure 3 b).

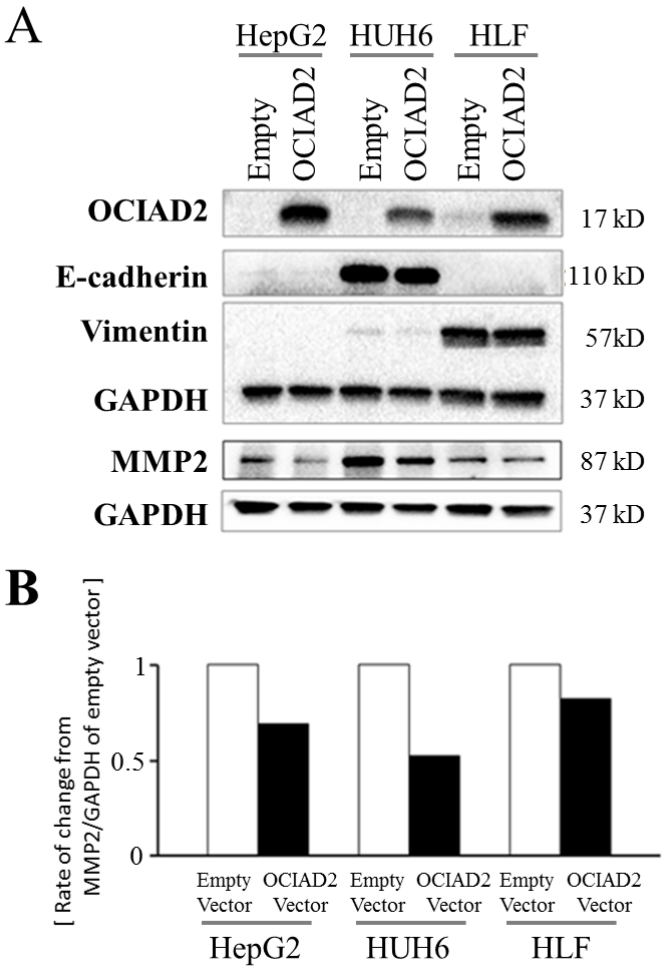

Figure 3. a Expression of *OCIAD2*, E-cadherin, vimentin, GAPDH, and MMP2 proteins in HUH6, HepG2, and HLF in the *OCIAD2* vector and empty vector groups detected by Western blotting. **b** Relative MMP2 protein expression levels in the six liver tumor cell lines by Western blotting.

DISCUSSION

We elucidated two novel mechanisms by which *OCIAD2* functions as a tumor suppressor using *in vitro* assays: *OCIAD2* inhibited cell invasion and migration in HB cell lines. Furthermore, overexpression of *OCIAD2* appeared to reduce the expression of MMP2, which was associated with cell invasion and migration. As shown in our previous study, the methylation status of *OCIAD2* was significantly associated with several clinical parameters, including poor outcomes in HB (Honda et al., 2016). These results taken together suggest that epigenetic dysregulation of *OCIAD2* plays a critical role in the progression of HB.

OCIAD2 was first reported by Strausberg et al. (2002) through the National Institute of Health Mammalian Gene Collection Project. Further studies on *OCIAD2* have been published. In the lung adenocarcinoma mixed subtype with the bronchoalveolar carcinoma component, Ishiyama et al. (2007) showed that expression of OCIAD2 begins during progression from *in situ* to invasive carcinoma; however, it is associated with a favorable prognosis. *OCIAD2* was identified as a biomarker of malignant ovarian mucinous tumors, with diagnosability being more specific than that of *OCIAD1* and more sensitive than CEA (Nagata et al., 2012). These findings indicated that *OCIAD2* had a tumor-suppressive function. Regulation of *OCIAD2* has been unclear; however, we previously showed that expression of *OCIAD2* is up-regulated by treatment with 5-aza-deoxcytidine in both HUH6 and HepG2. After 5-aza-2-deoxycytidine was incorporated into the DNA strand, it irreversibly bound to methyltransferase and inhibited its activity, resulting in accelerated demethylation of DNA (Honda et al., 2016). Moreover, it had been reported earlier that expression of *OCIAD2* is suppressed by hypermethylation of *OCIAD2* (Wu et al., 2017).

We analyzed *OCIAD2* functions in constructed *OCIAD2* overexpression cell lines and showed that the overexpression of *OCIAD2* reduced invasion and migration abilities. Additionally, it has been reported that *OCIAD2* suppresses tumor invasion and growth in HCC (Wu et al., 2017). These results indicate that *OCIAD2* functions as a tumor-suppressive gene. Zhang et al. (2014) demonstrated that TGF signaling induces the expression of *OCIAD2* through Smad2/3 and Smad4 and showed its relationship with EMT. These findings also indicate that *OCIAD2* plays an important role in carcinogenesis and tumor progression. On the other hand, in experiments with HEK293, a fetal kidney cell line, it was reported that overexpression of *OCIAD2* enhanced cell proliferation and migration via JACK/STAT3 signaling (Sinha et al., 2018). However, overexpression of *OCIAD2* inhibited MMP2 expression in the present study. MMP2 is an important protein for each step of cancer progression, such as degradation of the extracellular matrix till its invasion through the basement membrane (Noel et al., 1997). *OCIAD2* overexpression did not induce variations in E-cadherin or vimentin levels, which are also known to be associated with EMT. The pathway connecting *OCIAD2* and MMP2 has not yet been elucidated. It has been reported that *OCIAD2* suppresses HCC invasion via the AKT pathway and that activation of the AKT pathway leads to MMP2 expression in HCC (Wu et al., 2017). These findings can be the key to unraveling the pathway. Further functional investigations are required to obtain more knowledge on their associations. Thus, our in vitro studies indicated that *OCIAD2* acts as a tumor suppressor in HB, partly contributing to EMT control.

In conclusion, we demonstrated that overexpression of *OCIAD2* reduced cell invasion and migration, as well as MMP2 expression in HB cell lines. These findings offer

opportunities to understand the mechanism of migration and invasion in HB, as well as to identify potential therapeutic targets for HB.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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