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Original Article

Serum REIC/Dickkopf-3 Protein Level Predicts Disease-Free Survival in Patients with Hepatocellular Carcinoma

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The physiological role of the reduced expression of immortalized cells (REIC)/Dickkopf-3 (Dkk-3) protein in patients with hepatocellular carcinoma (HCC) remains unclear. In this study, we evaluated the effect of the REIC/Dkk-3 protein on HCC cell proliferation and assessed the relationship between the serum REIC/Dkk-3 protein level and the prognosis in patients with HCC. We evaluated the REIC/Dkk-3 protein-induced anticancer effects on Huh7 and Hep3B cells (HCC cell lines) in the presence of peripheral blood mononuclear cells (PBMCs), and found that combination treatment with REIC/Dkk-3 protein and PBMCs reduced the proliferation of HCC cells (Hep3B: 82.0%±16.3%; Huh7: 72.6%±9.1%). We also studied 194 HCC patients who underwent primary liver resection or primary radiofrequency ablation from 2008 to 2017. Serum REIC/Dkk-3 protein levels were measured by an enzyme-linked immunosorbent assay and compared to the prognostic data. The 3-year disease-free survival of the REIC/Dkk-3 high group was significantly higher than that in the REIC/Dkk-3 low group. In conclusion, this is the first study investigating the relationship between HCC patient survival and serum REIC/Dkk-3 protein levels in a large population. Based on the results, the serum REIC/Dkk-3 protein level should be considered a new prognostic marker for patients with HCC.

Key words: enzyme-linked immunosorbent assay, liver resection, primary radiofrequency ablation, Huh7, Hep3B

Although treatment strategies for hepatocellular carcinoma (HCC) have greatly improved, the prognosis remains poor. In most cases, HCC develops in patients with chronic hepatitis or liver cirrhosis (LC) — conditions that are characterized by persistent hepatic injury and concurrent liver regeneration. Chronic inflammation causes the accumulation of genetic and epigenetic changes in hepatocytes, resulting in the development of hepatocellular carcinoma. Despite advances in this area, the molecular pathogenesis of HCC is poorly understood. Different studies have reported different sets of genes that are frequently altered in patients with HCC. Nonetheless, changes in Wnt pathway-associated genes in HCC are clearly among the major gene alterations, and a molecule related to Wnt signaling has been shown to exhibit anti-cancer activity against HCC [1].

The reduced expression in immortalized cells (REIC)/Dickkopf-3 (Dkk-3) gene was identified as a tumor suppressor gene, and is downregulated in various cancers [2]. Overexpression of the *REIC/Dkk-3* gene induces endoplasmic reticulum stress, which is responsible for apoptosis through the c-Jun-NH2-kinase pathway in cancer cells [2]. The *REIC/Dkk-3* gene expression is decreased or lost in various cancers, including hepatocellular carcinoma [3-7], and the adenovirus-medi-

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ated overexpression of *REIC/Dkk-3* induces cancer cell death. The *REIC/Dkk-3* gene was found to be consistent with the human *Dkk-3* gene and belongs to the Dkk protein family. Dkk-3 is a 350 amino acid secreted glycoprotein, which is comprised of an N-terminal signal peptide and 2 conserved cysteine-rich domains that are divided by 12 amino acid linker regions [8]. The function of *REIC/Dkk-3* under normal physiological conditions is still unknown. A Dkk-3 knockout mouse study revealed no major alterations in their phenotype [9].

Cancer cells are thought to evade immune surveillance by disabling the immune system function of CD8+ cytotoxic T lymphocytes (CTL) and natural killer (NK) cells [10]. Although some studies have reported that REIC/Dkk-3 functions in the regulation of anti-tumor immune responses, we and others reported that REIC/Dkk-3 actually activated an anti-tumor immune response [11-13]. In addition, in peripheral blood mononuclear cells (PBMCs), it was reported that the REIC/Dkk-3 protein upregulated the differentiation of monocytes to a dendritic cell (DC)-like phenotype and subsequently activated the CTL function [14-16]. It is possible that this immune-stimulatory mechanism is related to the recognition of major histocompatibility complex class I-presented tumor antigens between DCs and CTLs [14]. In our previous study, we evaluated the REIC/Dkk-3 protein-induced immunological antitumor effects on pancreatic cancer cells *in vivo* and *in vitro* when pancreatic cells were co-cultured with PBMCs, in order to elucidate the synergistic immunological effects of REIC/Dkk-3 protein and PBMCs [17]. We thus considered it would be useful to examine whether the REIC protein induces an antitumor effect in HCC cell lines. In the present study, therefore, we evaluated the REIC/Dkk-3 protein-induced immunological antitumor effects on HCC cells and retrospectively examined the serum REIC/Dkk-3 levels in HCC patients.

Materials and Methods

Assessment of immunological effects on HCC cells in vitro

1. Cell lines and cell culture

The Huh7 HCC cell line was obtained from the Japanese Cancer Resources Bank (Tokyo), and the Hep3B cell line was obtained from DS Pharma Biomedical (Osaka, Japan). Each of the cell lines were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA), which was supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich Japan, Tokyo), 1% non-essential amino acids (Sigma), 1% penicillin/streptomycin solution (Sigma), and amphotericin B (0.5 μg/mL). The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air and were made quiescent at subconfluence under restricted serum conditions with 0.1% dialyzed FBS for 24 h before the experiment. PBMCs were obtained from Lonza (Tokyo).

2. Human REIC/Dkk-3 protein

Human REIC/Dkk-3 protein [18] was obtained from the Innovation Center Okayama for Nano-bio-targeted Therapy, Okayama University. Stock solutions of REIC/Dkk-3 protein were maintained at −80°C until use.

3. Assessment of lymphocyte cytotoxicity

Huh7 and Hep3B cells (5.0×10^4 cells/well) were seeded in flat-bottomed 6-well plates, and co-cultured with or without PBMCs (5.0×10^5 cells/well) in the absence or presence of REIC/Dkk3 protein (10 μg/ml). After 5 days of incubation, cell viability was evaluated using a methyl thiazole tetrazolium (MTT) assay. PBMCs were removed by washing twice with cold phosphate-buffered saline before the MTT assay.

Examination of the impact of serum REIC/Dkk-3 protein in HCC patients

1. *Patients*

A total of 194 HCC patients who were treated at Okayama University Hospital were recruited. We retrospectively studied HCC patients who underwent primary liver resection or primary radiofrequency ablation for HCC admitted to our unit from February 2008 to January 2017. The average observation time was 1441 days. All patient serums were gathered before therapy. The subjects included HBV-positive (n = 70), HCV-positive (n = 70) and HBV/HCV-negative (n = 54) patients. The diagnosis of HCC was made based on vascularization, which was detected on dynamic computed tomography (CT) or magnetic resonance imaging (MRI) after contrast enhancement. The diagnosis of HCC was confirmed by CT, MRI, or biopsy results. The study protocol was reviewed and approved by the Ethics Committee of the Okayama University (approval number: ken1703-048). Written informed consent was obtained from the subjects. The major characteristics of the study subjects are summarized in Table 1.

2. Enzyme-linked immunosorbent assay (ELISA)

Peripheral blood samples were collected before sur-

Table 1 The major characteristics of the patients

Parameters	†: median (range)	n = 194
Age†		70.5 (38–87)
Sex (male: female)		138 (71%): 56 (29%)
HBs Ag (+)		40 (21%)
HCV Ab (+)		122 (63%)
Alcohol consumption†(g)		10 (0–310)
Stage (I/II/III/IV)		94 (48%)/78 (40%)/18 (9%)/4 (2%)
Tumor size, mm†		15.5 (6–105)
Multiple tumors		53 (27%)
Child Pugh grade (A/B/C)		180/13/1
Serum REIC/Dkk-3 protein, pg/mL†		16741 (2642–33281)

gery or radio frequency ablation (RFA). Samples were centrifuged for 5 min at 540 × *g* and separated into plasma and serum. The serums were stored at −20°C until use. The serum REIC/Dkk-3 protein levels were measured using a commercially available enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. The optical density was measured at 450 nm and referenced to 570 nm on a Multiskan™ GO microplate spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The REIC/Dkk-3 concentrations were obtained with a 2-parameter logistic curve fitted against a standard curve and multiplied by the dilution factor.

Statistical analyses

The results were analyzed using the Mann-Whitney *U*-test and Student's *t*-test. The JMP12 software program (SAS Institute Japan, Tokyo) was used to perform the statistical analyses. A Kaplan-Meier survival analysis was performed to obtain the survival curves. The log-rank test was used to compare survival between the high and low REIC/Dkk-3 protein level groups. The duration of disease-free survival was determined from the day following surgery or RFA to the day that initial recurrence or metastasis was detected by a clinical examination. Overall survival was calculated from the day following surgery or RFA to the last follow-up appointment. The data are presented as the mean ± standard deviation (SD). *p*-values of < 0.05 were considered to indicate statistical significance.

Results

Combined therapy with REIC/Dkk-3 protein and PBMCs induces antitumor effects. REIC/Dkk-3 protein slightly reduced the proliferation of HCC cells (Hep3B: 96.9% ± 12.9%; Huh7: 94.2% ± 2. 8%); this effect was greater when the cells were co-cultured with PBMCs (Hep3B: 82.0% ± 16.3%; Huh7: 72.6% ± 9.1%) (Fig. 1).

The presence of REIC/Dkk-3 proteins in serum samples of HCC patients. The clinical background of the study patients is shown in Table 1. The rates of 3-year disease-free and overall survival were 42.7% and 82.4%, respectively (Fig. 2a, b). The 194 HCC patients enrolled in the present study were divided into two groups according to the mean serum REIC/Dkk-3 protein level (16741 pg/mL; determined by ELISA): the REIC/Dkk-3 high group (> 16741 pg/mL; n = 97) and the REIC/Dkk-3 low group (≤ 16741 pg/mL; n = 97). There were no significant differences between the REIC/Dkk-3 high and REIC/Dkk-3 low groups with regard to age, sex, HBV infection, HCV infection, alcohol consumption, TNM stage or Child-Pugh grade (Table 2). Figure 2 shows the differences in survival according to the serum REIC/Dkk-3 level. The 3-year disease-free survival rates of the REIC/Dkk-3 high and REIC/Dkk-3 low groups were 49.0% and 35.1%, respectively (Fig. 2a). The serum REIC/Dkk-3 level significantly affected disease-free survival ($p = 0.04$). The 3-year overall survival rates of the REIC/Dkk-3 high and REIC/Dkk-3 low groups were 84.1% and 80.1%, respectively (Fig. 2b). Although the difference was not statistically significant ($p = 0.08$), the REIC/Dkk-3 high group showed a more favorable prognosis.

Discussion

The *REIC/Dkk-3* gene was isolated and cloned as an immortalization-related gene at Okayama University in

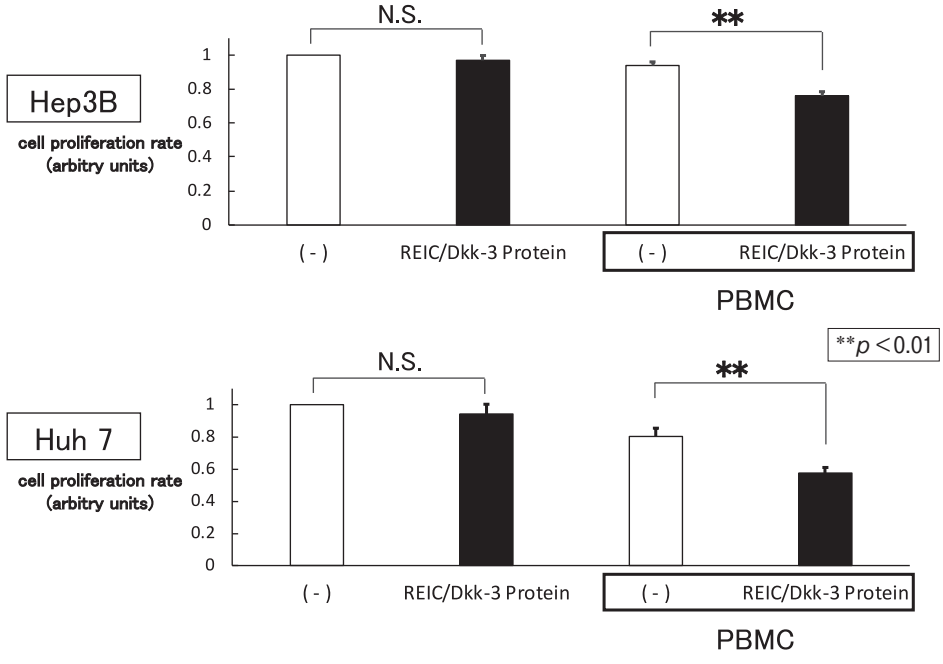

Fig. 1 Cell viability induced by REIC/Dkk-3 protein. Hep3B and Huh7 cells were co-cultured with or without PBMCs for 5 days in the absence or presence of REIC/Dkk-3 protein. Cell viability was evaluated using an MTT assay. The results are presented as the mean ± standard deviation (n = 21). $^{**}p < 0.01$.

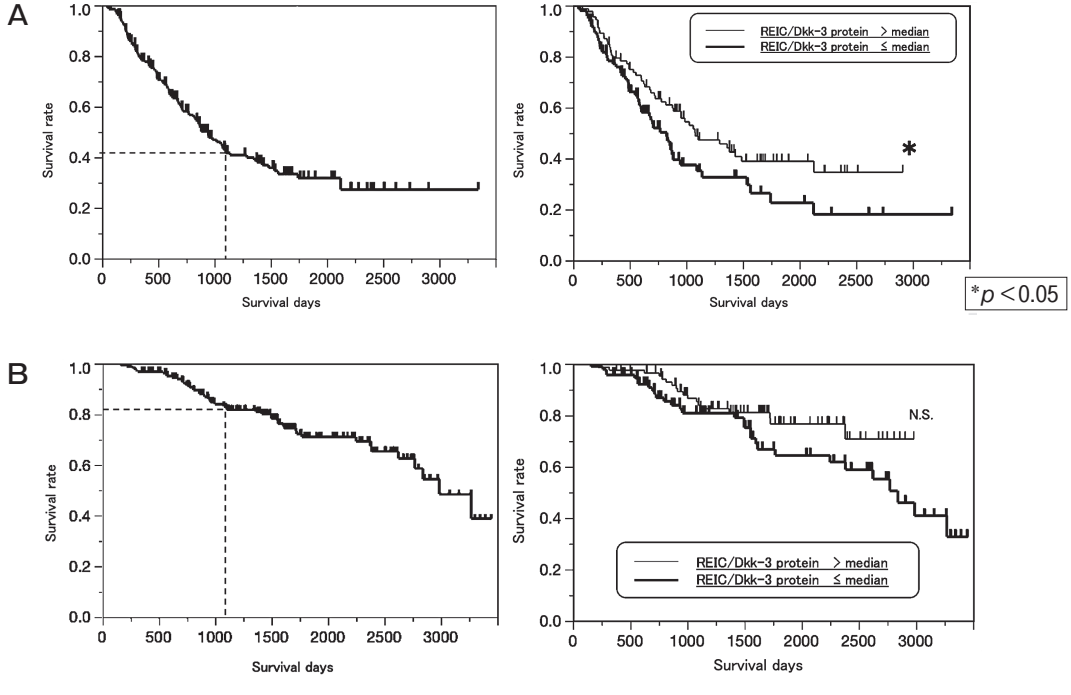

Fig. 2 Disease-free survival and overall survival in the study population. A Kaplan-Meier analysis was performed to investigate the relationships between the serum REIC/Dkk-3 protein levels and disease-free survival (**A**) and overall survival (**B**). The dotted line indicates the 3-year survival rate. In both figures, the Kaplan-Meier curve of all patients (left) and those of the REIC/Dkk-3 high and low groups (right) are shown separately. $^{*}p < 0.05$.

Table 2 The clinicopathological characteristics according to the REIC/Dkk-3 protein level

Parameters (†average)	REIC/Dkk-3 protein High (n=97)	Low (n=97)	*p*-value
Age†(year)	73.76	74.04	0.85
Sex			
Male	74 (54%)	64 (46%)	0.11
Female	23 (41%)	33 (59%)	
HBs Ag			
(+)	21 (53%)	19 (47%)	0.69
(−)	76 (49%)	78 (51%)	
HCV Ab			
(+)	59 (49%)	63 (51%)	0.61
(−)	38 (53%)	34 (47%)	
Alcohol consumption†(g)	※missing value: 82		
	41.9	34.7	0.52
TNM stage			
I, II	86 (50%)	86 (50%)	0.15
III, IV	11 (50%)	11 (50%)	
Child-Pugh grade			
A	91 (51%)	89 (49%)	0.34
B or C	6 (43%)	8 (57%)	
Initial treatment			
Liver resecetion	39 (67%)	19 (33%)	0.001
RFA therapy	58 (43%)	78 (57%)	

2000 and identified as a new tumor suppressor gene [2]. *REIC/Dkk-3* gene therapy was demonstrated to induce immunological effects in several studies as well as cancer cell apoptosis in a prostate cancer study [19-21]. The aberrant expression of Wnt or the downregulation of Wnt antagonists has repeatedly been reported in several types of malignancy [1]. The degree of *REIC/Dkk-3* methylation in the tissues of HCC patients is higher than that in the tissues of cirrhosis patients without HCC [22]. Moreover, high levels of *REIC/Dkk-3* gene methylation are associated with a poor prognosis in HCC [22]. Thus, it is possible that *REIC/Dkk-3* may function as a tumor suppressor gene in HCC. *Dkk-3* has been found to be overexpressed in hepatoblastoma and HCC, suggesting that the function of *REIC/Dkk-3* may differ according to the tissue of origin [23].

We previously demonstrated that the REIC/Dkk-3 protein reduced pancreatic cancer cell proliferation by inducing cytotoxic effects in the presence of PBMCs [17]. Moreover, it was demonstrated that recombinant REIC/Dkk-3 protein induced the differentiation of monocytes into a DC-like phenotype, and that immunological activation appears to play an important role in tumor antigen presentation to CTL cells. The REIC/Dkk-3 protein may be the key mediator of antitumor immunity via the induction of monocyte differentiation. Therefore, in the present study we conducted an experiment to evaluate the effect of purified REIC/Dkk-3 protein on HCC cell proliferation in the presence of PBMCs. We used purified REIC/Dkk-3 protein to avoid the side-effects of adenovirus vector infection and focused on the antitumor effects of the protein itself.

In this study, purified REIC/Dkk-3 protein slightly reduced the proliferation of HCC tumor cells (Fig. 1). This effect was significantly enhanced in the presence of PBMCs; hence, the REIC/Dkk-3 protein induced therapeutic effects by activating PBMCs and thereby inducing their anti-tumor activities. Because the HCC tumor cells and PBMCs were derived from different donors, they expressed different types of major histocompatibility complex (MHC). The REIC/Dkk-3 protein could induce an anti-tumor effect with PBMCs through an MHC-independent pathway. A possible mechanism is cd1d-dependent natural killer T cell activation resulting in cytokine production [24]. Further study is necessary to elucidate this mechanism.

We previously reported the efficacy of adenovirus-mediated *REIC/Dkk-3* gene therapy in a mouse model of HCC [6]. The overexpression of the *REIC/Dkk-3* gene induced apoptosis in HCC cells. The current results revealed the anticancer effects of REIC/Dkk-3 protein on HCC and the utility of combination therapy with the REIC/Dkk-3 protein and PBMCs in the treatment of HCC.

Based on the above, we focused on whether the serum REIC/Dkk-3 protein level could be used as a new prognostic biomarker in HCC patients. The significance of serum REIC/Dkk-3 protein levels was evaluated in a previous study. Kim *et al.* reported on the combination of insulin-like growth factor binding protein 2, pyruvate kinase M2, and Dkk-3, as a diagnostic panel for colorectal cancer. Even in HCC, Erdal *et al.* reported that the combination of Dkk-1 and AFP achieved better diagnostic results than AFP alone, and that AFP was superior to Dkk-1 and Dkk-3 when used individually [25,26]. They evaluated the utility of the serum REIC/Dkk-3 protein level as a diagnostic marker for HCC and concluded that it was not suitable. Thus,

we retrospectively investigated the serum REIC/Dkk-3 protein levels of 194 HCC patients. As shown in Fig. 2a, the 3-year disease-free survival rate of the REIC/Dkk-3 high group was significantly higher than that of the REIC/Dkk-3 low group. The 3-year overall survival rate of the REIC/Dkk-3 high group was also higher than that of the REIC/Dkk-3 low group; however, the result was not statistically significant. These data suggest that REIC/Dkk-3 protein acts as a tumor suppressor in human HCC.

Our results suggested a possible beneficial anti-tumor effect of *REIC/Dkk-3*. We therefore evaluated the utility of serum REIC/Dkk-3 protein levels as a prognostic marker for HCC patients, and found that high-REIC/Dkk-3 protein levels were associated with a fair prognosis. Our study also indicates that the REIC/Dkk-3 protein represents a useful mediator of antitumor immunity for HCC. Although further analysis is necessary to elucidate the exact mechanisms of the interaction between the REIC/Dkk-3 protein and immune cells, the immunomodulatory effects of combined treatment tend to be observed in locally treated tumor lesions following adenovirus-mediated *REIC/Dkk-3* gene therapy.

Finally, we should note that this study has some limitations due to its retrospective design. First, our population had an uneven background. As many as 40% of patients in the REIC/Dkk-3 high group underwent curative surgical treatment, compared to 20% of patients in the REIC/Dkk-3 low group. In contrast, 80% of patients in the REIC/Dkk-3 low group underwent RFA therapy. Many meta-analyses and RCTs have compared hepatectomy with RFA, but whether RFA or hepatectomy is better remains controversial [27-29]. Thus, the prognostic difference caused by the therapeutic modalities might have affected the survival analysis. Second, hepatectomy was performed in 4 patients with portal vein tumor thrombosis (HCC Stage IV). Those patients were all assigned to the REIC/Dkk-3 low group, which might have affected the disease-free and overall survival rates. Finally, this study did not document the treatments performed after primary therapy. Thus, further prospective studies with well-defined and stratified patient populations are needed.

In conclusion, this was the first study on the relationship between the HCC patient survival and serum REIC/Dkk-3 protein level in a large population. Based on the results, the serum REIC/Dkk-3 protein level should be considered a new prognostic marker for patients with HCC.

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