Research Article

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# Diacylglycerol kinase alpha enhances hepatocellular carcinoma progression by activation of Ras–Raf–MEK–ERK pathway

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**Background & Aims**: Diacylglycerol kinases (DGKs) were recently recognized as key regulators in cell signaling pathways. We investigated whether DGKα is involved in human hepatocellular carcinoma (HCC) progression.

**Methods**: We silenced or overexpressed DGKα in HCC cells and assessed its effect on tumor progression. DGKα expression in 95 surgical samples was analyzed by immunohistochemistry, and the expression status of each sample was correlated with clinicopathological features.

**Results**: DGKα was detected in various HCC cell lines but at very low levels in the normal liver. Knockdown of DGKα significantly suppressed cell proliferation and invasion. Overexpression of wild type (WT) DGKα, but not its kinase-dead (KD) mutant, significantly enhanced cell proliferation. DGKα knockdown impaired MEK and ERK phosphorylation, but did not inhibit Ras activation in HCC cells. In a xenograft model, WT DGKα overexpression significantly enhanced tumor growth compared to the control, but KD DGKα mutant had no effect. Immunohistochemical studies showed that DGKα was expressed in cancerous tissue, but not in adjacent non-cancerous hepatocytes. High DGKα expression ($\geqslant$20%) was associated with high Ki67 expression ($p$ <0.05) and a high rate of HCC recurrence ($p$ = 0.033) following surgery. In multivariate analyses, high DGKα expression was an independent factor for determining HCC recurrence after surgery.

**Conclusions**: DGKα is involved in HCC progression by activation of the MAPK pathway. DGKα could be a novel target for HCC therapeutics as well as a prognostic marker.



Keywords: Liver cancer; Diacylglycerol; Phosphatidic acid; Diacylglycerol kinase; MAP kinase.

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*Abbreviations:* HCC, hepatocellular carcinoma; MAPK, mitogen-activated protein kinase; DGK, diacylglycerol kinase; DG, diacylglycerol; PA, phosphatidic acid; siRNA, small interfering RNA; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; HGF, hepatocyte growth factor; WT, wild type; KD, kinase dead; FACS, fluorescence activated cell sorting.

## Introduction

Hepatocellular carcinoma (HCC) is one of the most common solid tumors worldwide and its incidence is continuing to increase [1,2]. The main therapies for HCC are curative strategies such as liver resection or liver transplantation [3,4]. As these treatments are only viable for patients with preserved liver function, or for those with access to a donor organ, there are many patients with incurable HCC [5,6]. In addition, the long-term outcome after these therapies remains unsatisfactory because of high recurrence rates [3,5]. Therefore, new novel therapeutic strategies for HCC are required.

HCC is associated with increased expression and activity of mitogen-activated protein kinase (MAPK) signaling intermediates [7,8]. Activated Ras induces the Raf–MAPK/ERK kinase (MEK)-extracellular signal-regulated kinase (ERK) cascade, which regulates various cellular responses, including proliferation, survival, and migration [7–9].

Diacylglycerol kinase (DGK) catalyzes the phosphorylation of diacylglycerol (DG) to generate phosphatidic acid (PA) [10–14]. DG and PA are recognized as important second messengers, and play key roles in signal transduction and cellular function [11–14]. DGKs have critical tasks in signal transmission from many receptors, and modulate diverse cellular processes, regulating both DG and PA levels. To date, 10 mammalian DGK isozymes (α–θ) have been identified, and all have the catalytic region in common [10–14]. DGKα is subdivided into the type I group due to its calcium-binding EF-hand motifs and recoverin homology domain [15–17]. This enzyme was first identified in T lymphocytes/thymus and enhances interleukin 2-induced T cell proliferation [15,18,19]. Another report demonstrated that DGKα overexpression resulted in a defect in T cell receptor signaling characteristic of anergy [20,21]. These reports collectively suggest that DGKα has various biological roles.

Here, we found that DGKα was expressed in several human liver cancer cell lines, but only at very low levels in the normal liver. In order to identify HCC-specific functions of DGKα, this isoform was downregulated and then conversely overexpressed in two types of HCC cell lines by transfecting small interfering RNA (siRNA) and DGKα expression plasmids, respectively. Interestingly, this study clarified that DGKα positively regulated proliferation and invasion of human HCC cells through activation of MAPK signaling. Furthermore, immunohistochemical studies

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of surgical samples suggested that high DGKα expression was associated with HCC recurrence after surgery.

## Materials and methods

*Cell culture*

Human HCC cell lines, HuH7, PLC/PRF/5, HLE, and Hep3B, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 mg/ml streptomycin sulfate (Life Technologies, Inc., Carlsbad, CA). All cells were maintained at 37 °C in 5% $CO_2$.

*Antibodies and reagents*

Anti-pig DGKα polyclonal antibodies (cross-reactive with human DGKα) were prepared as described previously [22]. Other antibodies were obtained from commercial sources as follows: anti-Ras antibody (Upstate Biotechnology, Inc., Waltham, MA), anti-ERK1/2, anti-phosphorylated-ERK1/2 (Thr-202/Tyr-204), anti-MEK1/2, and anti-phosphorylated-MEK1/2 (Ser-217/221) antibodies (Cell Signaling Technology Inc., Beverly, MA), anti-actin, anti-GAPDH and anti-cyclin D1 monoclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-Ki67 monoclonal antibodies (Dako, Tokyo, Japan) and anti-GFP monoclonal antibodies (Nacalai Tesque, Kyoto, Japan). Recombinant human hepatocyte growth factor (HGF) was purchased from Peprotech (Rocky Hill, NJ).

*Human tissue samples*

Samples from 95 patients who had undergone liver resection for HCC without preoperative treatment at the Department of Surgery and Science, Kyushu University Hospital, between January 1998 and December 2002 were analyzed by immunohistochemistry. Patients' clinical features are shown in Table 1. Histological diagnoses of the tumors were based on the General Rules for the Clinical and Pathological Study of Primary Liver Cancer by the Liver Cancer Study Group of Japan [23]. Written, informed consent was obtained from each patient for the study of tissue excised from surgical specimens. The Kyushu University Medical human investigation committee gave approval for this study.

*Plasmids*

cDNAs encoding wild type (WT) DGKα and kinase-dead (KD) DGKα were generated as previously described [15,24] and were subcloned into pEGFP-C3 and pcDNA 1.1 vectors. Cells were transiently transfected using Lipofectamine LTX (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. To generate stable cell lines that permanently expressed exogenous GFP alone, GFP–DGKα WT or GFP–DGKα KD, 1 μg of linearized DNA was transfected, and cells were selected for neomycin resistance using 2 mg/ml of G418. Individual clones were isolated and tested for expression of GFP by Western blot analysis.

*Immunohistochemistry*

Paraffin sections of samples were deparaffinized. Heat-induced epitope retrieval was performed in 0.1 M NaOH citrate buffer (pH 7.0), and the samples were heated in an autoclave. Immunoreactivity was independently graded by two liver pathologists. At least 1000 cancer cells in five high-power fields were counted.

*RNA interference*

To silence the expression of human DGKα, the following oligonucleotides (Invitrogen, Carlsbad, CA) were used: *DGKα1* sense; 5′-CGAGGAUGGCGAGAUGGCUAAAUAU-3′, and *DGKα2* sense; 5′-GCGAGUCAAGCAUUGGUCUUGGCAA-3′. As a negative control, scrambled siRNA was used. The annealed oligonucleotide duplex siRNA (10 nM) was transfected into cells using Lipofectamine RNAi max (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

*Cell proliferation assays*

PLC/PRF/5 and HuH7 cells were seeded in 60 mm dishes at a density of $2 \times 10^5$. After days 0, 2, 4, 6, and 8 of transfection with plasmid or siRNA, cells were trypsinized. Cells excluding trypan blue were counted using a hemocytometer.

*Invasion assays*

Invasion analyses were performed as described previously [25]. Invasive indexes were calculated using the following formula: Invasion index (%) = (number of cells that invaded through Matrigel insert membrane)/(number of cells that migrated through control insert membrane). Each experiment was performed in triplicate wells and repeated three times.

*Protein extraction and Western blot analysis*

Protein extraction and Western blot analysis were performed as described [26]. To measure the relative density of immunoreactive bands, images were scanned and analyzed by Image J software (National Institute of Health., Bethesda, MD).

*Affinity precipitation of activated Ras*

Cells were lysed in lysis buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.5 M NaCl, and 2% Igepal). The supernatant was incubated with 10 μl of Raf–Ras–binding domain (RBD)–GST beads (Cytoskeleton Inc., Denver, CO), which selectively interacted with active GTP–bound Ras. The beads were washed three times with wash buffer (25 mM Tris pH 7.5, 30 mM $MgCl_2$, 40 mM NaCl) containing 5 mM $MgCl_2$, and then boiled in SDS sample buffer. Ras associated with Raf–RBD–GST and total Ras in cell lysates were detected with anti-Ras antibody using Western blot analysis.

*Fluorescence-activated cell sorting (FACS)*

HCC cells were transfected with siRNA. After 48 h, cells were incubated with 40 ng/ml of HGF for 48 h. Adherent and floating cells were then pooled and washed with ice-cold PBS. Cells were fixed with ice-cold 70% ethanol and labeled with PI, followed by FACS. G1, S and G2/M populations were quantified using FACS Scan Cell Sorter (BD Biosciences, Tokyo, Japan) using FlowJo software (Tree Star, Ashland, OR).

*Xenograft model*

BALB/c male nude mice (Charles River, Yokohama, Japan) were maintained according to the Institutional Animal Care and Use Committee of the Kyushu University Graduate School of Medical Sciences. Tumors were generated by subcutaneously injecting $5 \times 10^6$ PLC/PRF/5 cells stably expressing endogenous GFP alone, GFP–DGKα WT, or GFP–DGKα KD. Tumor dimensions were measured once a week, and tumor volume was calculated using the following formula: tumor volume ($mm^3$) = (the largest diameters)/2 × (the smallest diameters)/2 [27]. Mice were euthanized when tumors reached 10% of their body weight or when the skin overlying tumors became ulcerated.

*Statistical analysis*

JMP 8J Version (SAS Institute, Cary, NC) was used for all analyses. All experiments were independently performed three times in triplicate. Comparisons between groups were made using Wilcoxon test with continuous variables and Fisher's exact test for comparisons of proportions. Survival curves were estimated using the Kaplan–Meier method, and the differences in survival rates between groups were compared by the log-rank test. Multivariate analysis was performed using Cox's proportional hazard regression model to evaluate the independent factors predictive of patients' survival. By multivariate analysis, we examined the following six clinicopathological factors, which were significant factors in the univariate analysis: (1) positive for hepatitis C virus antibody; (2) indocyanine green 15-min retention test (>15% *vs.* ⩽15%); (3) positive for intrahepatic metastasis; (4) DGKα (high *vs.* low expression); (5) liver cirrhosis, (6) AFP (>40 *vs.* ⩽40). Data are expressed as mean ± standard deviation. $p$ Values of <0.05 were considered to be significant.