Research Article

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Dysadherin can enhance tumorigenesis by conferring properties of stem-like cells to hepatocellular carcinoma cells

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Background & Aims: Hepatocellular carcinoma (HCC) is associated with a high potential for metastasis and disease recurrence, even after surgical resection. The cancer stem cell (CSC) hypothesis proposes that CSCs are responsible for chemo-resistance, recurrence, and metastasis. Dysadherin is a prognostic indicator of metastasis and poor survival in many different cancer types. In this study, we investigated the possible link between dysadherin and CSC in HCC.

Methods: We analyzed the functional implications of dysadherin on cancer stemness by modification of the *dysadherin* gene in HCC cell lines.

Results: The transfection of dysadherin cDNA into the liver cancer cell line PLC/PRF/5 enhanced the properties of CSCs, including anti-apoptosis, their sphere-forming ability, side population phenotype, and tumor initiation ability *in vivo*. Furthermore, knockdown of dysadherin in the liver cancer cell line SK-Hep1 suppressed its stem cell-like properties.

Conclusions: These results show that dysadherin give rise to properties of CSC in HCC. Therefore, these findings suggest that dysadherin may be a potential molecular prognostic marker of HCC and may aid in the development of more effective therapies.

Cancer

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer [1]. Unfortunately, the fact that HCC is resistant to conventional chemotherapy and radiotherapy leaves patients suffering from this disease with no effective therapeutic options and a poor prognosis if HCC is metastatic [2]. HCC also commonly metastasizes to lungs, lymph nodes, adrenal gland, and bones, including the skull [2]. Therefore, it is very important to understand the molecular changes that correlate with recurrence or metastasis of HCC.

The stem cell model for carcinogenesis suggests that cancers originate from, and are maintained by, a small fraction of cells called cancer stem cells (CSCs). Like normal stem cells, CSCs possess the ability to self-renew, differentiate, and proliferate following a prolonged period of quiescence [3]. CSCs may also be resistant to chemotherapy and radiation, causing recurrences and metastasis of cancer following standard therapy [3]. CSCs may generate solid tumors in various forms of cancer [4–6]. Different markers characterize CSCs in various cancer types; for example, $CD44^{+}CD24^{-/low}$ in breast cancer [4], $CD44^{+}\alpha2\beta1^{hi}$ $CD133^{+}$ in prostate cancer [5], and $CD133^{+}CD166^{+}$ in colon cancer [6].

Recently, several reports identified and characterized CSCs in HCC. Chiba and colleagues reported the presence of a side population (SP) of cells with stem cell features in Huh7 and PLC/PRF/5 liver cancer cell lines [7]. These SP cells were highly proliferative and formed tumors in NOD/SCID mice, whereas the non-SP cells were not tumorigenic. Suetsugu and colleagues demonstrated that CD133 could be a marker for the putative HCC stem cell, as only $CD133^{+}$-expressing Huh7 liver cancer cell lines were able to form tumors in NOD/SCID mice [8]. CD90 is another putative CSC marker that has been investigated in HCC [2]. However, individual markers such as SP, CD133 and CD90 may not be sufficient to represent all of the characteristics of CSCs, because cells lacking such markers have been shown to exhibit CSC properties, depending on the cancer cell type or the patient [9,10]. Therefore, an important area of CSC research is to find representative markers for characterizing CSCs. Moreover, the development of a representative marker of CSCs in HCC could provide a new insight into the HCC tumorigenic process and have great therapeutic implications.

Dysadherin is a membrane glycoprotein that plays important roles in tumor development and promotes cancer metastasis [11]. Clinical data suggest that overexpression of dysadherin is a prognostic indicator of metastasis and/or poor survival in many different cancer types [12]. Experimental data also suggest that expression of dysadherin leads to cancer metastasis in a number

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Abbreviations: HCC, hepatocellular carcinoma; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PPIA, cyclophilin A; MTT, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; PBS, phosphate-buffered saline; SD, standard deviation.

of ways, including down-regulation of E-cadherin-mediated cell adhesion and up-regulation of chemokine production [11,12]. Furthermore, a cDNA sequence almost identical to dysadherin was isolated from a library of human $CD34^+$ hematopoietic stem/progenitor cells, suggesting that it may be a regulator of stem cell properties [11]. Together, these studies suggest a possible link between CSCs and dysadherin. In this study, we introduced dysadherin into liver cancer cell lines to clarify the role of dysadherin in cancer stemness, in terms of tumorigenic potential and defined stem cell-like properties. The purpose of this work was to investigate whether selective inhibition of dysadherin can be a feasible strategy for stem-specific cancer therapy

Materials and methods

Cell lines and culture conditions

The human liver cancer cell lines Hep3B, Huh7, HepG2 and SK-Hep1 were obtained from the Korean Cell Line Bank (Seoul, South Korea). PLC/PRF/5, AV1 and AL3-1 cells were provided by Dr. Setsuo Hirohashi (National Cancer Center, Toyko, Japan) and were established as reported previously [11]. The characteristics of these liver cancer cell lines were described in Supplemental Table 1. Cells were cultured as previously described [13].

In silico analysis of clinical microarray data

In silico analysis of published clinical microarray data was performed using the database and analysis tools at www.oncomine.org. Clinicopathological characteristics of liver tissue samples used in this analysis were described in Supplemental Table 2.

Quantitative reverse-trasncription-PCR (qRT-PCR)

qRT-PCR for quantification of cDNA was performed using SYBR green PCR master mix (Applied Biosystems) and the ABI 7300 Real-Time PCR system according to the manufacturer's instructions. Normal and tumor liver tissue cDNAs were purchased from Origene Technologies (Rockville, MD). Clinicopathological characteristics of tissues were described in Supplemental Table 3. qRT-PCR was done in triplicates. Cyclophilin A (PPIA) mRNA was used as a normalization control. The primer sequences are provided in Supplemental Table 4.

Cell proliferation, apoptosis, and cell cycle analysis

Cell proliferation was assessed via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma–Aldrich, St. Louis, MO) assay and apoptosis was quantified on pooled cells (floating and adherent) using the Cell Death Detection ELISA assay kit (Roche Diagnostics), respectively, according to the manufacturer's instructions. Cell cycle analysis was done as previously described [14].

Immunoblotting, immunohistochemisty, and immunofluorescent staining

Immunoblotting, immunohistochemistry, and immunoflurorescent staining were carried out as previously described [13]. The following antibodies were used: ABCB1 (Santa Cruz Biotechnology, CA), dysadherin (NCC-M53) (provided by Dr. Setsuo Hirohashi, National Cancer Center, Toyko, Japan) [11] and β-actin (Sigma–Aldrich) antibody.

Tumorsphere culture

Tumorsphere culture was performed as previously described [13]. After 10–14 days culture, wells were examined under an inverted microscope at 50× magnification and the number of spheres in 30–50 fields of view was counted according to cell density per well using the Image-Pro Plus program (MediaCybernetics, Silver Spring, MD).

RNA interference

The small interfering RNA (siRNA) specific to dysadherin (GeneBank accession No.AB072911)[15], ABCB1 (GeneBank Accession No.NM_000927, L-003868–00), and non-targeting siRNA #1 (D-001810-01) were purchased from Dharmacon (Lafayette, CO). Transfection was performed using DharmaFECT1 (Dharmacon), according to the manufacturer's instructions. Cells were treated for 48 h with siRNA at a final concentration of 100 nM.

In vivo limiting dilution assay

Female NOD/SCID mice were purchased from the Korea Research Institute of Bioscience and Biotechnology (KRIBB, South Korea). All mice were maintained according to IACUC approved protocols of the Lee Gil Ya Cancer and Diabetes Institute. For titration experiments, cells were harvested *in vitro* and varying cell densities from 100 to 5000 cells were suspended in a mixture of PBS and Matrigel (Growth Factor Reduced Matrigel, BD Biosciences) in a total volume of 50 μl (PBS/Matrigel, 1:1). Afterwards, 7-week-old female NOD/SCID mice were injected subcutaneously in the left and right hind flank regions under anesthesia ($n = 3$ for each group). Mice were euthanized at 7 weeks and primary tumors were excised for analysis. The volume of the primary tumor was measured as previously described [13].

Flow cytometry

FACS and SP analysis were carried out as previously described [7,13]. The following antibodies were used: allophycocyanin(APC)-conjugated dysadherin (NCC-M53) (PhycoLink APC Conjugation kit; Prozyme, CA), ABCB1(Santa Cruz Biotechnology), Oct4 (Abcam, Cambridge, MA), Nanog (Abcam), and Sox2 (Abcam) antibody. Alexa Fluor 488-conjugated mouse or rabbit IgG antibody (Molecular Probes, Eugene, OR) were used to visualize.

Statistical analysis

All experiments were conducted in triplicate ($n = 3$) or greater, and the results were expressed as the mean ± SD. Statistical analyses of these data were conducted via unpaired parametric Student's *t* test or nonparametric Mann–Whitney *U* test.

Results

Expression profiles of dysadherin in human liver cancer tissues and cell lines

To investigate the possible involvement of dysadherin in liver cancer progression, we performed an *in silico* analysis of dysadherin transcript levels in cancer tissues. Results from a large clinical microarray study showed that dysadherin mRNA levels increased in liver disease including cirrhotic, dysplastic liver and hepatocellular carcinoma [16] (Fig. 1A). We next examined the correlation between dysadherin expression and differentiation in HCC tissues. qRT-PCR analysis showed a significant trend toward increased mRNA expression of dysadherin with decreasing differentiation in HCC (Fig. 1B).

We also examined a possible association between stem cell markers and dysadherin expression in liver cancer cell lines including PLC/PRF/5, Hep3B, Huh7, HepG2, and SK-Hep1. qRT-PCR analysis showed that mRNA expression of dysadherin was partly associated with elevated mRNA expression of stem cell surface markers such as CK19, CD117, and CD90 (Supplementary Fig. 1) [2,17–18]. Furthermore, we examined the correlation between stem cell transcription factors and dysadherin expression in liver cancer cell lines by flow cytometry. FACS analysis showed that dysadherin-expressing cells account for less than 10% of total cells in Hep3B, Huh7, and HepG2 cells. In contrast, less than 1% of