Cancer Letters 309 (2011) 95–103

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Hepatocellular carcinoma with progenitor cell features distinguishable by the hepatic stem/progenitor cell marker NCAM

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ARTICLE INFO

Article history:
Received 27 March 2011
Received in revised form 14 May 2011
Accepted 18 May 2011

Keywords:
NCAM
HCC with progenitor features
Soluble NCAM
FGFR
Intrahepatic metastasis

ABSTRACT

We analyzed hepatocellular carcinoma (HCC) with progenitor cell features using hepatic stem/progenitor cell marker neural cell adhesion molecule (NCAM). Approximately 8.3% of the operated HCC cases expressed NCAM, and 22.3% of the HCC patients had soluble NCAM levels >1000 ng/ml (the "highly soluble" NCAM group). Soluble NCAM status was a significant independent factor predictive of long-term survival in patients with HCC, and high levels of soluble NCAM were significantly related to intrahepatic metastasis. The 140-kDa NCAM isoform was specifically detected in the "highly soluble" NCAM group of HCC patients and its related signals are potential drug targets for NCAM+ HCC.

1. Introduction

The two major primary liver cancers in adults are hepatocellular carcinoma (HCC) and cholangiocellular carcinoma (CC). In addition, mixed/combined forms of HCC and CC have been reported and their relationship with hepatic stem/progenitor cells is being actively analyzed [1,2]. Recently, further detailed immunohistochemical analysis of HCC using stem/progenitor cell markers revealed the existence of HCC in which a fraction of the tumor cells express stem/progenitor cell markers. These HCC are not otherwise recognizable by routine diagnosis by hematoxylin–eosin staining. These HCC are termed HCC with progenitor cell features and analysis of their prognosis revealed a high frequency of recurrence after hepatic resection and transplantation and a poor prognosis compared with conventional HCC [3–5]. Nevertheless, these tumors are clinically diagnosed and treated without distinguishing them from conventional HCC. Thus, the diagnosis of HCC with progenitor cell features without tumor biopsy and appropriate treatment are crucial for improving the prognosis of HCC patients. In the present study, we analyzed HCC with progenitor cell features using the previously reported stem/progenitor cell marker neural cell adhesion molecule (NCAM).

Abbreviations: HCC, hepatocellular carcinoma; NCAM, neural cell adhesion molecule; CK19, cytokeratin 19; IM, intrahepatic metastasis; CC, cholangiocellular carcinoma; FGFR, fibroblast growth factor receptor; NK, natural killer; GPI, glycosyl-phosphatidylinositol; DAPI, 4′ 6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle medium, SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; FACS, fluorescence-activated cell sorter; PE, phycoerythrin; PCR, polymerase chain reaction; Fyn, Src-related tyrosine kinase $p59^{fyn}$; GAP43, growth-associated protein 43; FRS2, fibroblast growth factor receptor substrate 2; Rac-1, ras-related C3 botulinum toxin substrate 1; Cdc42, cell division cycle 42; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence II, PSA, polysialic acid; EMT, epithelial mesenchymal transition; TGF, transforming growth factor; PLC, phospholipase C.

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doi:10.1016/j.canlet.2011.05.021

NCAM is a cell adhesion molecule that was originally detected in the study of neurons and that belongs to the immunoglobulin superfamily. NCAM can homophilically bind to another NCAM, resulting in a zipper-like formation, and can bind heterophilically to L1-CAM, fibroblast growth factor receptor (FGFR) and extracellular components such as chondroitin, sulfate proteoglycan, heparin sulfate proteoglycan, collagen and laminin [6]. The large number of NCAM-interacting partners suggests that NCAM possesses important functions. In the normal human body, a variety of cells or tissues express NCAM, including NK cells, neuroendocrine gland, the central and peripheral nervous system and cardiomyocytes [6]. In addition, NCAM is also expressed by a variety of malignant cells such as neuroblastoma [7], rhabdomyosarcoma [8,9], small-cell lung cancer [10] and brain tumors [7], as well as by multiple myeloma [11] and acute myeloid leukemia [12]. The extracellular part of NCAM consists of five immunoglobulin-like domains and two fibronectin type III-like domains. Alternative mRNA splicing results in three major isoforms: a 120-kDa NCAM isoform is connected by a glycosyl-phosphatidylinositol (GPI) anchor to the cell membrane and is predominantly expressed in normal and well-differentiated tissues. Isoforms of 140- and 180-kDa, which contain a transmembrane domain, are found predominantly in less differentiated and malignant cell types [6]. Intracellular signaling of 140-kDa and 180-kDa NCAM is well understood from the study of neurons. It is also known that these isoforms and their downstream signals are involved in FGFR and N-cadherin signaling, as well as neurite motility and outgrowth [13]. Furthermore, NCAM can be released to the serum as a soluble form of NCAM, which includes NCAM forms with or without transmembrane domains. These soluble NCAM forms arise by processes such as alternative splicing of NCAM1 transcripts and by enzymatic processing of extracellular domains at the cell membrane. Thus, soluble NCAM has the potential to identify NCAM-expressing cells [14].

NCAM is known as a stem/progenitor cell marker in liver and is expressed in ductular reactions of acute and chronic damaged livers [15]. We also reported that NCAM is expressed in a fraction of some HCC cell populations [16]. As a tumor marker, NCAM has a number of benefits compared with other markers: (1) NCAM is a cell surface marker; (2) NCAM has three isoforms, two of whose downstream signals have been elucidated in studies of other fields; and (3) NCAM can be detected as a soluble form of NCAM in serum. These properties suggested that NCAM may be a useful marker for the diagnosis and treatment of NCAM+ HCC. In this study we analyzed NCAM expression in cases of operated HCC as well as the frequency and clinical course of NCAM+ HCCs. The level of soluble NCAM was measured in the serum of HCC patients and its relationship to the prognosis and intrahepatic metastasis of HCC tumors was analyzed. We further identified the isoform of NCAM that can be specifically detected in patients with HCC and analyzed the growth and invasive ability of NCAM+ HCC cells and their related downstream signals in order to elucidate the reason for the poor prognosis of NCAM+ HCC.

2. Materials and methods

2.1. Specimen collection

We analyzed 60 cases of livers that were extirpated for resection of HCC. Paraffin-embedded liver tissues were analyzed after receiving IRB approval from Niigata University.

2.2. Staining

Liver tissue was fixed in 10% formalin and embedded in paraffin blocks. Four-micrometer sections were cut and mounted on silane-coated slides. For immunohistochemical analysis, after removing the paraffin, antigen retrieval was performed using antigen retrieval solution (BioGenex Laboratories, San Ramon, CA) for 15 min in a microwave oven. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol (Wako, Osaka, Japan) for 10 min at room temperature, and sections were incubated overnight with the primary antibody, mouse anti-NCAM (123C3; Santa Cruz Biotechnology, Santa Cruz, CA), diluted in PBS. Slides were then stained using the Vectastain® ABC kit and DAB TRIS tablets (Muto Pure Chemicals, Tokyo, Japan). For immunocytochemical analysis, cells were washed with PBS and then fixed in methanol at −20 °C for 10 min. Anti-NCAM antibody (Santa Cruz Biotechnology) and Cy3-conjugated anti-mouse IgG antibody were employed as first and second antibodies, respectively. Nuclei were labeled with DAPI VECTASHIELD mounting medium for fluorescence analysis (Vector Laboratories, Inc., Burlingame, CA).

2.3. ELISA

The sera from 92 patients with HCC and 32 patients with chronic hepatitis C were measured. The cohort of the patients for ELISA was mostly different cohort of those for immunohistochemical analysis. All patients were followed up in our hospital. The sera were measured using the DuoSet® ELISA Development System for human NCAM1/CD56 (R&D Systems, Inc., Minneapolis, MN), according to the manufacturer's protocol. Briefly, the sera were diluted 1000-fold and pipetted into wells in a 96-well polystyrene microplate (R&D Systems, Inc.), the surface of which was coated with mouse anti-human NCAM1 antibody. After incubation, biotinylated goat anti-human NCAM1 was added to the well. After incubation and washing, streptavidin conjugated to horseradish-peroxidase was added, and finally, a color development step was performed. Absorbance was measured at 450 nm using the Biotrak visible plate reader (GE Healthcare Life Science, Buckinghamshire, UK). All samples were measured in duplicate.

2.4. Western blot analysis

The sera of four patients with HCC from the highly soluble-NCAM group, one patient with HCC from the minimally soluble-NCAM group, one patient with chronic