Biochemical and Biophysical Research Communications 465 (2015) 167–173

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Targeted disruption of fibrinogen like protein-1 accelerates hepatocellular carcinoma development

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ARTICLE INFO

Article history:
Received 30 June 2015
Accepted 15 July 2015
Available online 28 July 2015

Keywords:
Fibrinogen like protein-1
Hepatocellular carcinoma
Diethyl nitrosamine
Hepatoprotecatant
Mitogen

ABSTRACT

Fibrinogen like protein-1 (Fgl1) is a predominantly liver expressed protein that has been implicated as both a hepatoprotectant and a hepatocyte mitogen. Fgl1 expression is decreased in hepatocellular carcinoma (HCC) and its loss correlates with a poorly differentiated phenotype. To better elucidate the role of Fgl1 in hepatocarcinogenesis, we treated mice wild type or null for Fgl1 with diethyl nitrosamine and monitored for incidence of hepatocellular cancer. We find that mice lacking Fgl1 develop HCC at more than twice the rate of wild type mice. We show that hepatocellular cancers from Fgl1 null mice are molecularly distinct from those of the wild type mice. In tumors from Fgl1 null mice there is enhanced activation of Akt and downstream targets of the mammalian target of rapamycin (mTOR). In addition, there is paradoxical up regulation of putative hepatocellular cancer tumor suppressors; tripartite motif-containing protein 35 (Trim35) and tumor necrosis factor super family 10b (Tnfrsf10b). Taken together, these findings suggest that Fgl1 acts as a tumor suppressor in hepatocellular cancer through an Akt dependent mechanism and supports its role as a potential therapeutic target in HCC.

1. Introduction

Fgl1 is a 68kd protein that comprises two disulfide linked 34kd homodimers [1]. Although predominantly expressed in the liver, we recently demonstrated low-level transcription of Fgl1 in brown and white adipose in the setting of liver injury [2], and others have documented low-level expression in the pancreas [3]. Existing evidence supports a role for Fgl1 in liver regeneration and hepatoprotection: Fgl1 enhances tritiated thymidine uptake by primary hepatocytes and human hepatocellular carcinoma (HCC) cell lines [4], is upregulated following 70% partial hepatectomy in rodents [5], and the administration of recombinant Fgl1 protects hepatocytes from acute liver injury [6].

The pathogenesis of HCC involves a multistep process with inactivation of tumor suppressor genes (TSGs) and upregulation of proto-oncogenes [7]. A role for Fgl1 in HCC development has been suggested i) Fgl1 is frequently reduced or absent in HCC tissues [8,9], ii) restoration of Fgl1 expression in cultured HCC cell lines inhibits cell growth [8], iii) human FGL1 is located on the short arm of chromosome 8, a region rich in putative TSGs that are frequently deleted in HCC, and iv) additive loss of putative TSGs on human chromosome 8p, including Fgl1, is associated with increased growth of HCC, suggesting a synergistic effect of allelic loss of individual TSGs in promoting tumor growth [10]. These paradoxical effects of Fgl1 as both a mitogen in non-tumor hepatocytes and a growth inhibitor in HCC has been suggested to result from differing modes of Fgl1 dependent signaling: autocrine in hepatocyte proliferation versus intracrine in hepatocarcinogenesis [11].

Because previous studies on the role of Fgl1 in HCC has been limited to analysis of archived patient tumor samples [8,9] or cultured cells [8] or through shRNA driven reduction in cellular Fgl1 content in a murine model of HCC [10], we set out to determine whether Fgl1 directly influenced the development of HCC. Using

Abbreviations: HCC, hepatocellular cancer; DEN, diethyl nitrosamine; Pb, phenobarbital; Fgl1, fibrinogen like protein-1; UHRF1, ubiquitin-like with PHD and ring finger domains-1; Trim35, tripartite motif containing 35; Tnfrsf10b, tumor necrosis factor receptor superfamily, member 10b; AFP, alfa fetoprotein.

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http://dx.doi.org/10.1016/j.bbrc.2015.07.078
0006-291X/

our previously generated Fgl1 knockout mouse (Fgl1KO), we show that Fgl1KO mice develop HCC at a faster rate than Fgl1 wild type mates (Fgl1WT), following tumor induction with DEN and tumor promotion by low dose phenobarbital (Pb). We find that livers of Fgl1KO have more proliferative foci at 8 months and more than a two fold increase in prevalence of HCC at 12 months of DEN treatment. Although tumors from Fgl1WT and Fgl1KO were grossly and histologically similar, tumors from Fgl1KO were molecularly distinct as they exhibited 1) enhanced activation of Akt dependent signaling and 2) increased expression of putative HCC tumor suppressors *Trim35* and *Tnfrsf10b*.

2. Materials and methods

2.1. Animals

Fgl1KO mice were as previously described [2] and available from the Jackson labs (B6; 129-Fgl1tm1Cuko/J, JAX Stock number: 0024004). Ethics Statement: All animal experiments were performed according to institutional guidelines of Harvard Medical School. All protocols with animal experimentation conformed to criteria outlined in the National Institutes of Health "Guide for the Care and Use of Laboratory Animals." Protocols were reviewed and approved by the animal use committee of Harvard Medical School (protocol number 04193).

2.2. Hepatocarcinogenesis

Mice were injected with DEN (Sigma, St Louis, MO) 5 mg/kg at 15 days of age. Mice were fed a standard chow diet (LabDiet, Brentwood, MO) and were given free access to water supplemented with 0.07% phenobarbital as previously described [12]. Mice were euthanized at 6, 8, 10–12 months of age. Mouse liver and tumor tissue were collected and placed in 10% formalin or snap frozen at −80 °C for later analysis.

2.3. Western immunoblot assays

Liver and tumor tissue was homogenized in RIPA buffer (25 mM Hepes pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, and 2 mM EDTA). The resulting homogenate was centrifuged at 13,000 RPM and the supernatant was transferred to clean tubes. Protein concentrations were determined and lysates denatured with Laemmli sample buffer as previously described [13]. The following antibodies were used: eukaryotic transcription factor 4E binding protein 1(4EBP1), p4EBP1 (Thr37/46), protein kinase B (Akt), pAkt (Ser473), p38, p-p38 (Thr180/Tyr182), p-p70S6K (S371), p70S6K (Cell Signaling, Beverly, MA), UHRF1 (Santa Cruz Biotechnology, Dallas, TX), and β-actin (Sigma, St Louis, MO). Western immunoblot assays were performed as previously described [13]. Band intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

3. Nucleic acid assays

Quantitative PCR assays were performed as previously described [14]. mRNA levels were determined relative to cyclophilin A. Table S1 shows the primer sequences that were used for these assays.

3.1. Immunohistochemistry

Liver tissue fixed in 10% formalin was paraffin embedded and sectioned onto glass slides. Sections were deparaffinized in xylene and pretreated with epitope retrieval solution (IHC World, Elliot City, MD). Slides were blocked with 10% normal goat serum (Invitrogen, Carlsbad, CA). The following primary antibodies were used proliferating cell nuclear antigen (PCNA), ki-67, and β-catenin (Cell Signaling, Beverly, MA). The SuperPicture™ 3rd Generation IHC Detection Kit (Life Technologies, Carlsbad, CA) was used per manufacturer protocol.

4. Results

4.1. Disruption of Fgl1 enhances DEN induced hepatocarcinogenesis

Fgl1KO mice did not develop spontaneous HCC even at 15 months of age (not shown). To determine whether there were differences in hepatocarcinogenesis between Fgl1WT and Fgl1KO we treated mice with DEN + Pb and evaluated for incidence of HCC at 6, 8 and 12 months. At 6 months gross and histologic analysis showed no evidence of any liver lesions (not shown). At 8 months we noted the appearance of numerous discrete foci in histologic sections of Fgl1KO livers (n = 4) with only one such lesion in Fgl1WT (n = 4). These foci contained proliferating cells as shown by the presence of ki-67 positive cells (Fig. 1A and B). At 10 months of the expected 12 months for the last cohort, we noted that a subset of Fgl1KO (3 of 12) displayed marked evidence of generalized deterioration characterized by hair loss, inability to groom, and weight loss. The 3 Fgl1KO mice and 3 random age matched littermates from the Fgl1WT cohort were euthanized. All 3 of the Fgl1KO mice harbored large and multiple liver lesions compared to 0 of the Fgl1WT mice (Fig. 1C and D). Analysis of the remaining cohort at 12 months post DEN, showed that 6 of the 9 Fgl1KO mice exhibited gross evidence of liver cancer compared to 1 of 6 of the Fgl1WT. In total, 10 months–12 months after tumor induction with DEN, 9 of 12 Fgl1 WT and 1 of 9 Fgl1WT developed HCC. At baseline, Fgl1KO mice were larger than their WT mates[2], however at 12 months, we noted that Fgl1KO mice were smaller despite the larger tumor burden when compared to Fgl1WT mice (22.1 gm ± 2.1 vs. 27.6 ± 0.9) (Fig. 1E).

Histologic analysis confirmed that the liver lesions (including the solitary tumor from Fgl1WT) were HCCs, with numerous histopathologic features typical of human HCCs including nuclear atypia, mitotic figures, thickened cords and multinucleated cells (Fig. 2B, D and E). Not surprisingly these tumors showed positive staining for ki-67 and PCNA (Fig. 2G and F). Consistent with the diagnosis of HCC, the tumors had elevated expression of transcripts for alpha feto-protein (AFP) and the hepatocellular oncogene, ubiquitin-like with PHD and Ring fingers domain 1 (UHRF1) [15] when compared to non-tumor liver (Fig. 2H and I). UHRF1 protein usually undetectable in normal livers [16] was markedly elevated in the tumors (Fig. 2J) consistent with its known enhancement in rapidly dividing cells. To ensure that the findings were not spurious, we repeated the experiment to confirm increased incidence of HCC at 12 months after induction. Again we found that 13 out of 13 Fgl1KO and 6 of 11 Fgl1WT developed HCC. Over the course of these experiments 22 of 25 Fgl1KO developed tumors while only 7 of 20 Fgl1WT (Fig. 2K) had HCC. These data reveal that Fgl1KO are more prone to DEN induced carcinogenesis.

4.2. Akt signaling is enhanced in Fgl1KO liver tumors

HCC is characterized by aberrant activation of a number of signaling pathways. Principal among these pathways are those regulated by protein kinase b (Akt), Yap/Hippo, p38 MAP kinase and β-catenin [17]. To examine differences in signaling pathways between the Fgl1KO and Fgl1WT tumors, we used lysates from Fgl1WT and Fgl1KO tumors from the second experimental cohort where we have more than 1 tumor from the Fgl1WT cohort for western immunoblot analysis. We first examined whether p38