ELSEVIER

Journal of Hepatology 50 (2009) 118–127

Journal of Hepatology

www.elsevier.com/locate/jhep

Fibroblast growth factor receptor 4 regulates proliferation, anti-apoptosis and alpha-fetoprotein secretion during hepatocellular carcinoma progression and represents a potential target for therapeutic intervention☆

Han Kiat Ho[1,*], Sharon Pok[1], Sylvia Streit[1], Jens E. Ruhe[1], Stefan Hart[1], Kah Suan Lim[1], Hooi Linn Loo[1], Myat Oo Aung[2], Seng Gee Lim[2], Axel Ullrich[1,3]

[1]*Singapore OncoGenome Laboratory, Institute of Medical Biology, A*STAR, 8A Biomedical Grove, #06-06 Immunos, Singapore 138648, Singapore*
[2]*Division of Gastroenterology, National University Hospital, Singapore*
[3]*Department of Molecular Biology, Max-Planck-Institute of Biochemistry, Martinsried, Germany*

***Background/Aims*: FGFR4, a member of the fibroblast growth factor receptor family, has been recently associated with progression of melanoma, breast and head and neck carcinoma. Given its uniquely high expression in the liver, we investigated its contributory role to hepatocellular carcinoma (HCC).**

***Methods*: We performed a comprehensive sequencing of full-length FGFR4 transcript in 57 tumor/normal HCC tissue pairs, and quantified their mRNA expressions. Notable mutations and expression patterns were correlated with patient data. Clinically significant trends were examined in *in vitro* models.**

***Results*: We found eight genetic alterations including two highly frequent polymorphisms (V10I and G338R). Secretion of alpha-fetoprotein (AFP), a HCC biomarker, was increased among patients bearing homozygous Arg388 alleles. One-third of these patients exhibited increased FGFR4 mRNA expression in the matched tumor/normal tissue. Subsequent *in vitro* perturbation of FGFR4 signaling through both FGF19-stimulation and FGFR4 silencing confirmed a mechanistic link between FGFR4 activities and tumor aggressiveness. More importantly, inhibition of FGFR activity with PD173074 exquisitely blocked HuH7 (high FGFR4 expression) proliferation as compared to control cell lines.**

***Conclusions*: FGFR4 contributes significantly to HCC progression by modulating AFP secretion, proliferation and anti-apoptosis. Its frequent overexpression in patients renders its inhibition a novel and much needed pharmacological approach against HCC.**

***Keywords*: FGFR4; Tyrosine kinase; HCC; Alpha-fetoprotein; Proliferation; Apoptosis**

Received 22 April 2008; received in revised form 22 August 2008; accepted 29 August 2008; available online 12 October 2008
Associate Editor: J.M. Llovet

☆ The authors declare that they do not have anything to disclose regarding funding from industries or conflict of interest with respect to this manuscript.

* Corresponding author. Tel.: +65 64070271; fax: +65 64642049.
E-mail address: hankiat.ho@imb.a-star.edu.sg (H.K. Ho).

Abbreviations: AFP, alpha-fetoprotein; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; FGF19, fibroblast growth factor 19; FGFR, fibroblast growth factor receptor; FRS2α, FGFR substrate protein-2α; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide-formazan; siRNA, small interfering ribonucleic acid; SNP, single nucleotide polymorphism; TKIs, tyrosine kinase inhibitors.

0168-8278/$34.00 © 2008 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.jhep.2008.08.015

1. Introduction

Hepatocellular carcinoma (HCC) is one of the deadliest cancers, causing about half a million deaths each year [1]. While its etiology is well-established (predominantly linked to viral hepatitis), effective treatment has not been forthcoming. Curative approaches like surgical resection and transplantation are only available for a limited number of patients and chemotherapeutic options are at best, palliative [2,3]. Consequently, prognosis of advanced HCC is poor.

In the last decade, tyrosine kinase inhibitors (TKIs) have emerged as an exciting class of anti-cancer agents. Among them, imatinib has been used successfully against chronic myelogenic leukemia bearing Bcr-Abl mutation. EGFR inhibitors: gefitinib and erlotinib, have also shown therapeutic benefits against non-small cell lung carcinoma [4]. Despite these developments, limited efforts explore TKIs against HCC. Sporadic reports associating various tyrosine kinases to HCC have been published, but none has delivered promising results. For example, while overexpression of PYK2, FAK, PDGFRα, IGF-1R and FGFR3 were described in HCC and may correlate with disease severity [5–9], attempts to identify potential pro-oncogenic activating mutation of tyrosine kinases (such as EGFR) proved negative [10,11]. FDA-approved TKIs like gefitinib, erlotinib and imatinib, as well as monoclonal antibodies like cetuximab, are being explored in HCC clinical trials to expand their indications for usage. However, such approaches are highly empirical in the absence of prevailing evidence for tyrosine kinase signaling aberration(s) in HCC patients. Not surprisingly, many TKI trials yielded marginal responses [12–15]. It is therefore necessary to systematically examine the contribution of specific tyrosine kinases to HCC, in order to establish a sound basis for future use of such inhibitors, consequently improving treatment outcome. Herein, we perform a comprehensive mutation and expression analysis of FGFR4 in HCC as a test case for this strategy.

The rationale for investigating FGFR4 in HCC stems from multiple recent findings: FGFR4 is a member of the FGFR family which plays a pivotal role in embryonic development, CNS control, tissue repair and even tumor invasiveness and angiogenesis [16,17]. Disruption of FGFR signaling has resulted in numerous developmental defects like dwarfism (FGFR2), bone overgrowth (FGFR3) and Pfeiffer syndrome (FGFR1) [16]. Importantly, FGFR4 is an attractive target for HCC progression because the hepatocyte is the only human cell type where FGFR4 is the predominant isoform of the FGFRs [18]. It was also reported that the liver has the highest transcript expression of FGFR4 as compared to other major organs [19]. Furthermore, mice ectopically expressing FGF19 (in skeletal muscle), a specific ligand for FGFR4 [20], demonstrated AFP elevation and subsequently developed HCC [21]. These findings suggest that aberration of FGF19–FGFR4 signaling may have a strong pathophysiological impact in the liver. In 2007, FGF19–FGFR4 signaling mechanism was re-defined when the recruitment of a co-receptor, klotho or βklotho, was shown to amplify downstream FGFR4 signaling [19,22]. Interestingly, βklotho expression was limited to a few tissue types, with the highest levels found in the liver [19]. Altogether, the liver uniquely possesses the complete FGFR4 activating machinery and thus represents a good system to better understand FGFR4 signaling, and reciprocally, to explore the impact of its perturbation on hepatic disorders including HCC.

Hence, we performed a comprehensive mutation analysis of FGFR4 in primary HCC samples. Genetic alterations, as well as FGFR4 transcript expressions were correlated with various clinical parameters of respective patients. We found an SNP that correlated with AFP secretion. FGFR4 expression was elevated in normal-to-tumor transition in one-third of the patients. Further *in vitro* studies provided strong evidence that FGFR4 activity directly affects AFP production and plays a major role in tumor proliferation and anti-apoptosis. These findings entail preliminary, yet convincing evidence for a modulatory role of FGFR4 on HCC growth and progression, pointing to a novel drug target against this disease.

2. Experimental procedures

2.1. Cell culture

HuH7 was obtained from Dr. P. Hofschneider (Max Planck Institute, Martinsried, Germany). HepG2, Hs1.Li, SK-Hep1 and Hs817.T cells were from ATCC (Manassas, VA). HepG2 and Hs1.Li were maintained in MEM supplemented with non-essential amino acids, L-glutamine, sodium pyruvate and 10% FCS. Other cells were maintained in DMEM supplemented with sodium pyruvate and 10% FCS. All cell culture reagents were obtained from Invitrogen (Carlsbad, CA).

2.2. Sample preparation

HCC samples ($n = 57$) with matched normal tissue were obtained from resected livers of patients from the National University Hospital (Singapore). The tumor and adjacent normal tissues were visually separated. RNA extraction from tissue was performed by TriZol method as previously described [23]. mRNA was purified from total RNA using Oligotex mRNA kit (Qiagen, Valencia, CA). cDNAs were synthesized using method as described previously [24].

2.3. Sequencing and mutational analysis

Primers were designed using Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), and synthesized by Proligo (SigmaAldrich, Singapore). PCR reactions were performed as previously described [24]. Direct sequencing was done with automated