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

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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.

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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 specific ligand for FGFR4 [20], demonstrated AFP elevation and subsequently developed HCC [21]. These findings suggest that aberration of FGFR4 signaling may have a strong pathophysiological impact in the liver. In 2007, FGFR1–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, t-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
sequencing apparatus (ABI 3730XL, Foster City, CA). Sequence traces were analyzed to identify genomic alterations using Mutation Surveyor (SoftGenetics, State College, PA). Coding sequence of FGFR4 was aligned to NCBI reference sequence (NM_002101.2). Alterations were compared to published mutations from PubMed or public databases (Supplementary Table S2).

2.4. Quantitative real-time PCR

Quantitative real-time PCR was performed using Applied Biosystems 7300 (ABI) with TaqMan Gene Expression Assays for FGFR4 using GAPDH as control. The samples were triplicated with 4 μL of prediluted cDNA each. Real-time PCR for Twist was performed with SYBR Green PCR mix using primers described previously [25]. Data were obtained as average CT values, and normalized against control as ΔCT. Expression changes in FGFR4 transcripts between normal vs. tumor tissue were expressed as fold change using 2ΔΔCT (difference between the ΔCT of the matched pairs).

2.5. Ligand stimulation assay

HepG2 or HuH7 cells were serum-starved for 24 h prior to FGF19 (R&D Systems, Minneapolis, MN, 50–100 ng/ml final concentration) stimulation. Heparin was added 2 h before stimulation. At 8 h, aliquots of the supernatant were harvested for AFP ELISA assay as described below.

2.6. Immunoblot assay

Samples were resolved using 7.5% SDS–PAGE and transferred onto nitrocellulose membranes. Immunodetection was by chemiluminescence (SuperSignal West Dura Extended, Pierce) using specific antibodies diluted in PBS with 0.05% (v/v) Tween 20 and 5% (v/v) powdered milk. Anti-phospho-FRS2α and anti-PARP were from Cell Signaling Technology (Beverly, MA), anti-FGFR4, anti-PCNA and anti-HSP60 from Santa Cruz Biotechnology (Santa Cruz, CA), anti-E- and -N-cadherin from Abcam (Cambridge, UK). Secondary antibodies were anti-mouse and anti-rabbit horseradish peroxidase conjugated antibodies (Pierce).

2.7. AFP ELISA assay

Supernatants from FGF19 stimulation and siRNA experiments were assayed with DELFIA hAFP ELISA kit (Perkin-Elmer, Boston, MA). Fluorescence was detected on a SpectraMax M5 (Molecular Devices, Sunnyvale, CA). The calculated AFP values were normalized to the cell count as represented by ATP content using Cell-Titer-Glo assay (Promega, Madison, WI).

2.8. Gene silencing by siRNA

Custom-made ON-TARGETplus siRNA designed for silencing FGFR4 was purchased from Dharmacon (Chicago, IL). Transfection was performed according to manufacturer’s recommendations. Immunoblot and AFP ELISA assays were subsequently performed after 72 h incubation with FGFR4 siRNA.

2.9. Cell viability assay

Various concentrations (0–10 μM) of PD173074 (1-(t-butyl-3-(6-(3,5-dimethoxyphenyl)-2-(4-diethylaminobutylamino)-pyrido[2,3- d]pyrimidin-7-yl)urea), EMD Chemicals, San Diego, CA) and gefitinib (LC Laboratories, Woburn, MA) were added to cells for 72 h. Cell-Titer-Glo (Promega) assay was performed according to manufacturer’s instruction. Data were expressed as percentage of viability vs. vehicle-treated controls. All chemicals were obtained from Sigma–Aldrich (St. Louis, MO) unless otherwise stated.

2.10. Caspase-3 activity assay

Cells were treated with PD173074 for 24 h prior to harvesting. Caspase-3 activities were determined by incubating cell lysates at 37 °C in caspase assay buffer as described previously [26]. Fluorescence was by the release of hydrolyzed AMC substrates detected with excitation/emission wavelengths = 380/450 nm. Data were expressed as RFU/μg lysate/h incubation.

3. Results

3.1. Sequencing and mutational analysis of HCC samples

A comprehensive sequencing of FGFR4 was performed on 57 pairs of matched HCC samples using cDNAs as a template. Overlapping PCR amplification reactions provided complete transcript coverage (Supplementary Table S1). From the analyses, we found eight mis-sense mutations, including three known SNPs (i.e. V10I, L136P and G388R), and five previously unreported alterations (i.e. D126N, T179A, G426D, D709G and a deletion of aa 450–802). These alterations were found in both the tumor and corresponding normal tissues of the respective samples (Supplementary Table S2). Two SNPs occurred at relatively higher frequencies: V10I is distributed at 36.5%/40.4%/23.1% for the VV/VI/II genotype respectively; G388R is distributed at 46.6%/29.3%/24.1% for GG/GR/RR, respectively. To determine if these frequencies were consistent with general population of the same ethnicity, sample Asian population genomic DNA (n = 91) was used for establishing the genotype distribution of G388R. This subsequent sequencing revealed that distribution of G388R in our patient group is not significantly different from control population (Table 1A). Likewise, V10I was distributed similarly to a control Asian population as published previously (Table 1B).

3.2. Correlation of genotypes to clinical parameters

Patient profiles obtained at surgical resection were collated for analysis (Supplementary Table S3). Statistical correlations between each parameter with the geno-

<p>| Table 1A | FGFR4–G388R genotype distribution in HCC patients and control Asian population. Control Asian genomic DNA taken from the same general population as the patient samples were genotyped for the G388R polymorphism. Similar distribution is observed for both samples. χ² test was 5.280 with 2 degrees of freedom (p &gt; 0.05, two-tailed). |</p>
<table>
<thead>
<tr>
<th>Genotype</th>
<th>HCC samples</th>
<th>Control Asian population</th>
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<tbody>
<tr>
<td>GG</td>
<td>46.6% (27/58)</td>
<td>34.1% (30/88)</td>
</tr>
<tr>
<td>GR</td>
<td>29.3% (17/58)</td>
<td>43.2% (38/88)</td>
</tr>
<tr>
<td>RR</td>
<td>24.1% (14/58)</td>
<td>22.7% (20/88)</td>
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type of the major polymorphisms were performed using ANOVA and $\chi^2$ analysis. Homozygous Arg388 displayed an increased secretion of HCC tumor marker, AFP, as compared to wild-type FGFR4. Heterozygous genotype showed an intermediate level of AFP secretion (Fig. 1).

3.3. Elevation of FGFR4 transcript expression

To determine the functional relevance of FGFR4 polymorphisms, as well as to detect expression changes during disease progression, FGFR4 transcript levels were quantified by real-time PCR. We found that 31.6% of the patients (18 of 57) displayed at least a 2-fold increase in FGFR4 expression from the normal to the corresponding tumor (Table 2). Four patients have more than 10-fold increase in expression, with one showing an approximate 170-fold increase (Fig. 2). While elevated FGFR4 expression did not correlate with gross patient information, we performed subsequent in vitro studies to better characterize the effects of FGFR4 on HCC.

3.4. Elevation of AFP production with FGF19 stimulation

Increased FGFR4 expression in tumors vs. normal tissue, as well as the differential effects of genotypes on tumor parameters (G388R with AFP production), indicates a possible relationship between FGFR4 and HCC progression. We further investigated this by stimulating HCC cell lines with FGFR4-specific ligand, FGF19, and measured tumor response like AFP production. To select a suitable cell-based model, five liver cell lines (four liver cancer lines: HuH7, HepG2, SK-Hep1, Hs817.T; and one normal control line: Hs1.Li) were assayed for FGFR4 transcript expression. All five lines possess good mRNA expression of FGFR4 with the highest and lowest expressing being HuH7 and HepG2 respectively (Supplementary Table S4). HuH7 and HepG2 were also sequenced to be homozygous for the

![Image](http://www.example.com/image.png)
388Arg allele (Supplementary Fig. S5). Thus, both cell lines were suitable as model cell systems to address the effects of FGFR4 and the 388Arg SNP on HCC progression.

Using FGF19 (50 and 100 ng/ml) stimulation, AFP release in HuH7 and HepG2 was determined by ELISA after 8 h of cumulative production. From our results, HepG2 displayed dramatic increase in AFP after both 50 and 100 ng/ml of FGF19 (Fig. 3A). On the other hand, HuH7 production of AFP was moderately stimulated by both concentrations (Fig. 3B). AFP productions were normalized by their respective cell counts using ATP content to exclude any AFP increase due to the stimulation of cell proliferation through FGF19-FGFR4 signaling. To ensure activation of FGFR4, phosphorylation of adaptor protein, FRS2α, was monitored by immunoblot assay. Results confirmed a strong FGFR4 activation in HepG2 under the conditions used for ligand stimulation assay (Fig. 3C). Likewise, the modest AFP stimulation in HuH7 after FGF19 treatment was accompanied by an equivalent FRS2α phosphorylation (Fig. 3D).

3.5. Suppression of AFP production with FGFR4 silencing

To substantiate FGFR4’s role on AFP production in HCC, FGFR4 silencing was performed in HuH7, chosen for its high basal level of FGFR4 expression. Using siRNA against FGFR4, we depleted expression by more than 50% at 72 h incubation (Fig. 4A). Consequently, the supernatant of these cells secreted approximately 20% less AFP than non-silenced cells (Fig. 4B). Furthermore, shRNA-based silencing was performed to achieve a stable knock-down of FGFR4 and downstream FRS2α phosphorylation in HepG2 and HuH7. Here, we observed a consistent trend of reduced AFP production in FGFR4-decimated cells (Supplementary Fig. S6).

3.6. Inhibition of FGFR signaling with inhibitor, PD173074 represses AFP production and display superior anti-proliferative potential as compared to gefitinib

The clinical relevance FGFR4 inhibition in HCC was investigated using a non-selective FGFR inhibitor, PD173074, administered to HuH7 cells. While 1 μM has negligible effect on AFP levels, both 5 and 10 μM PD173074 completely repressed AFP secretion to a serum-starved basal level (Fig. 5A). More distinctively, similar treatments abrogated FGF19-stimulated AFP production and 10 μM of PD173074 further repressed AFP production to below serum-starved level (Fig. 5B).

Subsequently, we determined PD173074’s anti-proliferative potential using viability assay. Submicromolar range strongly inhibited the survival of HuH7 (Fig. 6A). Notably, HuH7 exhibited much higher susceptibility towards PD173074 vs. other HCCs and a normal liver cell line Hepa1.6. We also compared the efficacy of PD173074 in HuH7 with EGFR inhibitor, gefitinib. Even 10 μM of gefitinib failed to invoke 50% reduction in viability (Fig. 6B).
3.7. Inhibition of FGFR signaling with inhibitor, PD173074 promotes apoptosis and blocks cell invasiveness in metastatic HCC

To further determine if PD173074 has pro-apoptotic effect beyond anti-proliferation, we monitored caspase-3 activity after treatment. We observed that PD173074 elicited strong caspase-3 activation at 24 h that preceded visible morphological changes. Caspase-3 activity increased dose-dependently where 10 μM evoked approximately 10-fold elevation of baseline (Fig. 7A). Additionally, this activation was accompanied by PARP cleavage and a less pronounced suppression of PCNA expression (Fig. 7B).

Finally, we considered the effect of PD173074 treatment on cell invasiveness. In HuH7, we did not observe any change in the protein level of both E- and N-cadherin (Fig. 8A). On the other hand, similar treatment in a metastatic liver cancer model, SK-Hep1, led to a suppression of Twist mRNA transcripts even at 1 μM treatment (Fig. 8B).

4. Discussion

Recent work on FGFR4 describes its high expression in the liver, as well as roles in regulating hepatobiliary functions and even HCC development [19,21,27–29]. However, these efforts remain rudimentary in comparison to studies on tyrosine kinases like EGFR, VEGFR and MET. Hence, we performed a comprehensive mutation and expression analysis of FGFR4 in primary HCC tumors and their adjacent histologically normal tissues. Our efforts generated several findings that confirm the contributory role of FGFR4 signaling in HCC.

Firstly, we uncovered a number of mis-sense genetic alterations in our HCC samples. Three of these mutations were previously reported as SNPs (i.e. V10I,
L136P and G388R). In particular, V10I and G388R are highly represented in our samples. These polymorphisms prevail more in Asian populations as compared to other ethnicities. While their occurrence does not deviate significantly from control Asian populations, their predominance among Asians could translate into critical consequences for HCC if these mutations modify FGFR4 function. This can be especially detrimental for Asia, being the epicenter of hepatitis B carriers, the leading etiology for HCC. One polymorphism, G388R, has attracted recent interest due to prior reports suggesting that Arg388 correlated to a reduced survival for head and neck carcinoma, as well as a more aggressive phenotype for colon, soft tissue, prostate and breast carcinomas [17,30–33]. Here, we found that homozygous Arg388 concur with patients secreting more AFP. AFP expression peaks during fetal liver development and decreases rapidly upon hepatocyte maturation. The reactivation of AFP in HCC characterizes the state of tumor dedifferentiation and the development of stem cell-like behavior [34]. Although AFP has not been mechanistically linked to HCC initiation, its level corresponds to disease progression and aggressiveness in about 70% of HCC and is a widely used biomarker [35]. Thus, our finding corroborated earlier reports demonstrating Arg388’s correlation with a poor prognosis in various malignancies. However, we did not observe a link of this mutation to reduced patient survival. Nonetheless, a strong correlation with important biomarker like AFP is highly suggestive that the endogenous FGFR4 itself could play a major role in HCC progression.

Besides mutations, FGFR4 transcript expression was found to be significantly elevated in about one-third of the patients. This increase potentially connects FGFR4 activities with the commitment and progression of hepatocytes to HCC. However, this observation alone does not differentiate if FGFR4 upregulation is a cause or effect of HCC progression. Thus, we clarified this relationship with AFP-producing cell lines, either stimulating or inhibiting FGFR4, and monitoring AFP production as a marker for disease progression. HuH7 and HepG2 not only exemplify liver cancer cell lines
of high and low endogenous AFP production, respectively [36], they both contain the 388Arg homozygous genotype which renders them suitable for in vitro investigation. We activated FGFR4 signaling through stimulation with the specific ligand, FGF19, and detected significant increase in AFP production in both HuH7 and HepG2. AFP increase in HuH7 was modest as compared to HepG2, when subjected to identical stimulatory conditions. We speculate that the discrepancy arose from high basal AFP production in HuH7, which mitigated its sensitivity to further stimulation through the FGFR4-FGF19 axis. This likelihood was transcended downstream where the phosphorylation of FGFR4 adaptor protein, FRS2α, was found to be similarly more responsive to FGF19 in HepG2 than in HuH7. Subsequently, silencing of FGFR4 substantiated these findings whereby a decreased expression resulted in lower AFP in both cell lines. Overall, this is the first evidence, demonstrating that perturbation of FGFR4 activity can modify AFP production, a biomarker for HCC progression.

More significantly, we demonstrated as a consequence of this relationship, plausible application of FGFR4 as a therapeutic target against HCC. PD173074, which displays submicromolar inhibition of FGFR1-4 [37–41], was found to repress both serum- and FGF19-stimulated AFP production. While our results do not preclude possible contribution of FGFR1–3, specific and complete reversal of FGF19-stimulated AFP production by PD173074, justifies the predominance of FGFR4 for this effect. Moreover, PD173074 exerts a stronger anti-proliferative pressure on HuH7, the highest FGFR4 expressor among the NCI60 panel (http://symatlas.gnf.org/SymAtlas), as compared to other HCC cell lines of lower FGFR4 expression, and especially the non-cancerous liver cell line Hs1.Li. This observation ascertains the relative importance of FGFR4 among the other isoforms for PD173074’s efficacy. We also found this effect to be linked to an early activation of apoptosis which suggests its mechanism of action may be enhanced by HCC cytotoxicity besides cell-cycle arrest.

An additional therapeutic advantage of blocking FGFR4 activity is the suppression of cell invasiveness in metastatic HCC. While we did not observe any significant effect of FGFR4 inhibition on invasivity markers in HuH7, an epithelial cell line, inhibition of Twist expression in the more metastatic and mesenchymal-like SK-Hep1 was significant [25,42]. This finding suggests its relevance in curtailing secondary lesions, a common progression and cause of cancer mortality. Therefore, limiting cell invasiveness is both an independent and a complementary mechanism for the anti-cancer potential of FGFR4 inhibition.

Finally, our most remarkable finding is the superior inhibitory effect of PD173074 vs. classical EGFR inhibitor, gefitinib, currently used in HCC clinical trials [43]. Here, we show that HCC exhibits higher chemosensitivity to PD173074 than gefitinib, thus providing biochemical evidence that FGFR could be a better target than EGFR in HCC. Furthermore, FGFR4 knockout mice did not display much pathological phenotype [44], indicating that inhibition of FGFR4 may have a minimal adverse impact on liver physiology. Hence, we envisage this approach to be more effective against the subset of HCC patients with elevated FGFR4 during normal-to-tumor transition. The manifestation of raised FGFR4 levels found in many HCC patients makes this an attractive target for further development.

In conclusion, we provide an early report that FGFR4 plays a contributory role to HCC progression. An additional impact on the disease may also arise from the pre-eminence of functionally curious genetic polymorphisms like FGFR4-G388R among Asian populations. Most of all, the frequent occurrence of FGFR4 upregulation justifies future investigations and raises hope that many could gain from its therapeutic benefits. These could represent new opportunities in characterization and development of potentially new drug targets for an otherwise deadly and rampant malignancy.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2008.08.015.

References


