Over-expression of fibroblast growth factor receptor 3 in human hepatocellular carcinoma

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers in many parts of the world such as the Far East, Southern Sahara and Southern Europe, accounting for nearly half a million deaths worldwide[1]. Recent epidemiological data suggest that the incidence of HCC is increasing in USA, and the annual rate of new cases is 17 300[2]. Many risk factors, such as viral hepatitis, alcohol abuse, biliary disease, metabolic disorders, drugs, and toxins are related to carcinogenesis in HCC. These risk factors result in hepatocellular injury and replacement of normal acini by nodules. Regenerative hyperplasia of fibrous tissue and acini nodules profoundly disturbs the hepatic parenchymal structure and vascular network, which can directly or indirectly contribute to carcinogenesis, as a final stage of liver disease. Therefore, neoplastic transformation of HCC constitutes a long and complex course related to hepatocyte damage and the repair process, which involves multiple oncogenes and tumor suppressor genes.

With the development of the new molecular technology, DNA microarrays make it possible to study gene expression profiles in a global setting and screen thousands of genes rapidly and efficiently. Based on the panel of genes provided in the DNA microarray, further studies can be performed to determine the function of genes which have been identified to be significantly enriched or decreased, and these results can be used to discover new therapeutic target genes for the improvement of HCC prognosis[3]. This technology has been applied successfully in the identification of key genes in neoplastic transformation and as a diagnosis tool in molecular pathology[4].

In a previous study, a DNA microarray is used to compare the gene expression profile of HCC tissues with that of matched non-neoplastic tissue[5]. In this study, we focused on the expression change of transcription and translation-related genes in microarray gene profile. Among the 17 genes whose expression levels changed most significantly in this profile, 10 over-expressed genes and 7...
under-expressed genes were identified in HCC. FGFR3 is a member of the FGFR family, which is involved in cell growth control, cell differentiation and migration. Based on the unclear role of amplification of these receptors in carcinogenesis in digestive tract carcinoma, we studied the role of FGFR3 in carcinogenesis in HCC. The expression of FGFR3 significantly increased in HCC tissue, which was confirmed by Northern blot. Quantitative real-time PCR study further demonstrated that the mean ratio of FGFR3 mRNA to GAPDH mRNA in HCC tissue was 0.250, whereas the ratio in non-neoplastic liver tissue was 0.014 ($P<0.01$). Immunohistochemical (IHC) studies of 43 cases of HCC proved the over-expression of FGFR3 in HCC and the extent of over-expression was associated with tumor differentiation and nuclear grade of HCC. Thus, over-expression of FGFR3 may play an important role in carcinogenesis in HCC.

**MATERIALS AND METHODS**

**Patients and specimens**

Four specimens of fresh HCC tissue, coupled with non-neoplastic liver tissue from each individual, were obtained from City of Hope National Medical Center and St. Vincent Hospital. All tissue specimens were submitted for routine pathological evaluation and diagnosis confirmation. Forty-three HCC tissue paraffin blocks were collected from surgical pathology files at the City of Hope National Medical Center with a highly controlled manner to ensure patient confidentiality. A section was stained with hematoxylin and eosin (H&E) and examined to confirm the diagnosis and tumor type.

**DNA microarray and Northern blot analysis**

All fresh tissue samples were cut into small pieces and snap-frozen in liquid nitrogen immediately. The tissues were confirmed by histopathologic examination to validate the adequate HCC cells in the tumor samples. No HCC cells were contaminated in non-neoplastic liver tissues which were reassured before further approach. RNA isolation, cRNA preparation and microarray hybridization were performed as described previously. The microarray format, scanning and data analysis were performed at the Microarray Core Facility, Children’s Hospital, Los Angeles, USA. For each sample, at least two hybridizations were carried out. The average of median ratios from replicates was calculated for each spot. We then used 2.0 SD as our cut-off for the determination of expression outlines.

Normal human embryonic liver cell line CL-48, and human HCC cell lines, HepG2 and Hep3B, were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in high glucose Dulbecco’s modified Eagle’s medium (DMEM), 10% fetal bovine serum (FBS) and 1% P/S (100 U/mL penicillin and 100 μg/mL streptomycin) at 37 °C in 5% CO$_2$. Total RNA was isolated from the HCC and non-neoplastic liver tissues used for microarray analysis and from logarithmically growing CL-48, HepG2 and Hep3B cells using RNaseasy Mini kit (Qiagen, Valencia, CA, USA). The RNA was electrophoresed in 1.2% formaldehyde-agarose gel, blotted to a Hybond-N membrane (Amersham, Arlington, IL) and UV-cross-linked. 

$^{32}$P-labeled FGFR3 probe was prepared according to the protocol of the random priming probe kit from Roche (Indianapolis, IN, USA). The blots were hybridized for 1 h at 68 °C, washed twice with 2×SSC/0.1% SDS at room temperature, and washed twice with 0.1×SSC/0.1% SDS at 60 °C. After hybridization, membranes were exposed for 18-48 h to Kodak XAR-5 film (Eastman Kodak, Rochester, NY, USA) with intensifying screens and exposed to a phosphorimager screen for 18 h, then read by phosphorimager scanner. All experiments were repeated thrice for confirmation. The DNA probe for FGFR3 was generated by RT-PCR from CL-48 total RNA with the following primers: 5’-ATG GCC GCC CCT GCC TGC-3’ (sense primer starting with FGFR3 cDNA nucleotide 40) and 5’-GTG GTG TGT TGG AGC TCA TG-3’ (anti-sense primer starting with FGFR3 nucleotide 1 337). The PCR product included FGFR3 axons 2 and 3 sequence (according to the FGFR3 sequence NM_000142 in GenBank). The PCR conditions were as follows: 1 cycle at 95 °C for 15 min; 35 cycles at 94 °C for 40 s, at 60 °C for 40 s, at 72 °C for 2 min; and 1 cycle at 72 °C for 10 min. The products were purified and cleaned up using a gel extract and purification kit (Qiagen).

**Quantitative real-time PCR**

To further confirm and validate the microarray data, quantitative real-time PCR was employed to measure the FGFR3 messenger RNA expression in HCC tissue and matched non-neoplastic liver tissue. RNAs isolated from the fresh-frozen tissues for microarray were treated using RNA-free DNase I (Promega, Madison, WI, USA) according to the protocol to remove contaminated DNA. cDNA was reverse transcribed from total RNA using AMVRT and Oligo (dT) 12-18 primer. The forward primer 5’-TGC TGA ATG CCT CCC AGC -3’, reverse primer 5’-CGT CTT CGT CAT CTC CCG AG-3’, and probe 5’-FAM- TGT GCG GTT GAC AGA CGA GCC TCC AT-TAMRA-3’ were designed to span axons 2 and 3. Pre-developed TaqMan assay reagent control kit (Perkin-Elmer Applied Biosystems, Foster City, CA, USA), including forward, reverse primer and VIC-probe of GAPDH, was used as internal control. FGFR-3-pCDNA3.1 plasmid and GAPDH-pT7T3D-PAC plasmid, containing the full-length FGFR3 and GAPDH structure genes respectively, were used to generate the standard curve. The PCR conditions were as follows: 1 cycle at 50 °C for 2 min, at 95 °C for 10 min; 40 cycles at 95 °C for 15 s, at 60 °C for 60 s. Each point of data was performed in duplicate. Quantification of gene expression was performed using the ABI PRISM 7700 sequence detection system (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) according to the Pre-developed TaqMan assay reagent gene expression quantification protocol. The normalized reporter signal was defined as the intensity of fluorescent reporter dye (FAM or VIC) relative to the passive reference dye TAMRA. The threshold line was determined as high as 10 times of the standard deviation of the baseline fluorescence signal. After reaching the threshold, the sample was designated positive and the threshold cycle (Ct) was
defined as the cycle number at this point. Standard curves for FGFR3 and GAPDH were generated by serial dilution of control plasmids and the amount of mRNA of FGFR3 and GAPDH in each test sample was calculated based on the standard curve. Values were expressed as the ratio of FGFR3 to GAPDH in each sample.

Immunohistochemical study
Paraffin sections were deparaffinized in xylene and rehydrated in a graded alcohol series. After retrieval in pH 8.0 EDTA for 20 min, the slides were blocked with DAKO protein block serum for 5 min (DAKO, Carpinteria, CA, USA). The primary rabbit anti-human polyclonal FGFR3 antibody (200 µg/mL, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at an 1:100 dilution for 25 min and washed thrice (5 min each) with DAKO wash buffer. After 7 min of hydrogen peroxide treatment, the slides were incubated with labeled polymer HRP anti-rabbit antibody (DAKO, Carpinteria, CA, USA) for 30 min. Then, DAB (0.05 g DAB and 100 µL 30% H2O2 in 100 mL PBS) was applied for 5 and 10 min respectively. Each slide was counterstained with DAKO’s hematoxylin. For all IHC studies, PBS was used as a negative control. Granular cytoplasmic staining was assessed as positive. The evaluation of staining intensity was performed by three independent observers.

Scoring and data analysis
The diagnosis of HCC, tumor cell differentiation, nuclear grade and growth pattern were assessed based on the examination of H&E-stained sections, according to the Edmondson’s grade and nuclear grade criteria[7,8]. Briefly, well-differentiated HCC was composed of cells with minimal atypia and an increased nuclear/cytoplasmic ratio. In moderately-differentiated HCC, tumor cells were arranged in trabeculae of three or more cells in thickness and had abundant eosinophilic cytoplasm and round nuclei with distinct nucleoli. In poorly-differentiated HCC, tumor cells had an increased nuclear/cytoplasmic ratio, frequent pleomorphism and bizarre giant cells. Nuclear grade was classified in a four-tiered system. Nuclear grade I HCC comprised of adenoma-like cells that had abundant cytoplasm showing little variation in size and shape and was closely similar to benign hepatocyte nuclei, with an even distribution of chromatin. Nuclear grade II HCC contained cells with large and prominent nucleoli, some degree of nuclear membrane irregularity, and chromatin clumping, and were characterized by greater nuclear pleomorphism, and anaplastic giant cells. Nuclear grade III HCC was noted by its distinct pleomorphism, hyperchromatism, and anaplastic giant cells. Tumor growth pattern was also classified according to its trabecular, compact, pseudoglandular, cirrhotic and solid architecture. In cases that had several architectural patterns, the predominant one was captured for the statistics. The staining pattern of FGFR3 was classified in a subjective spectrum of 0 to ++: 0 as negative expression in tissue, + as weak staining, and ++ as strong staining. For each level of staining, the percentage of cells with the corresponding score was estimated visually. A combined weighted score, consisting of the sum of the percentage of cells at each staining intensity level, was calculated for each sample. In each case, staining of FGFR3 in matched surrounding non-neoplastic hepatocytes was evaluated at the same time. Statistical analysis of FGFR3 expression differences in HCC and surrounding non-neoplastic liver tissue was conducted using the Wilcoxon signed rank test. The association between the expression of FGFR3 and clinicopathologic parameters was examined by the non-parametric X2 tests. The relationship of the patients’ demographic features, such as age, gender and hepatitis B status, to FGFR3 expression was examined with the F-test statistic for linear effects and non-parametric Kruskal-Wallis test for singly ordered tables. SPSS 10.0 statistical analysis software was used to calculate the statistics. P<0.05 was considered statistically significant.

RESULTS
Microarray and Northern blot analysis
The partial result of microarray has been published in our previous study[6]. Among the 59 genes whose expression levels were most significantly changed, 30 over-expressed genes and 29 under-expressed genes were identified in HCC. In this study, we focused on the expression change of transcription and translation-related genes in microarray gene profile. In this profile, a total of 10 genes including translational initiation factor EIF-2, GTP binding protein, CDC2-related protein kinase, FGFR3, elongation factor-1β, were over-expressed in HCC. Whereas, insulin-like growth factor, MAP2K5, cyclin D1 and other 4 genes were under-expressed (Table 1). Our results revealed that transcription and translation-related genes also played a very important role in hepatocarcinogenesis besides oncogenes and tumor suppressor genes. Based on the increasing importance of FGFR3 in carcinogenesis in digestive tract carcinoma, we studied the role of FGFR3 in carcinogenesis in HCC. Northern blot showed that the FGFR3 gene was strongly expressed in HCC tissue, HepG2 and Hep3B HCC cell

Table 1 Different expressions of transcription and translation-related gene in HCC

| Genename                                      | Coding number |
|-----------------------------------------------|---------------|
| Most over-expressed genes in HCC              |               |
| Translational initiation factor EIF-2, alpha subunit | 1 154         |
| Homo sapiens GTP binding protein mRNA complete code | 38 708        |
| Human mRNA for elongation factor-1-β          | 35 748        |
| Human CDC2-related protein kinase mRNA        | 1 793         |
| Fibroblast growth factor receptor 3           | 36 165        |
| Elongation factor-1-γ                         | 1 676         |
| mRNA for erythropoietin receptor              | 396           |
| Human G protein-coupled receptor              | 1 097         |
| Glutathione S-transferase                     | 31 919        |
| Homo sapiens NADH-ubiquinone oxidoreductase subunit CI-SGDH | 32 232        |
| Least expressed genes in HCC                 |               |
| Human insulin-like growth factor binding protein gene | 1 232         |
| Homo sapiens mRNA for TGF-βIIIR α             | 1 815         |
| Heterogeneous nuclear ribonucleoprotein H     | 41 292        |
| Polyubiquitin Ubc                             | 32 334        |
| Nucleic acid binding protein gene             | 32 841        |
| MAP2K5                                        | 33 071        |
| Cyclin D1                                     | 40 436        |
lines were opposed to match non-neoplastic liver tissue and CL-48 human normal embryonic liver cell lines (Figure 1). Under the image quantity measurement relative to the internal control by GAPDH, the expression of FGFR3 in HCC and HCC cell lines was as high as 4- or 2.5-folds in non-neoplastic liver tissue and CL-48, respectively. Therefore, our Northern blot analysis was consistent with the expression patterns generated by microarray data.

**Quantitative analysis of mRNA expression in FGFR3 by real-time PCR**

The standard curve formulas $Y = 45.01 - 3.86X (r^2 = 0.994)$ for FGFR3 and $Y = 38.38 - 3.31X (r^2 = 0.991)$ for GAPDH were derived from the lines of the calibration curves. The results from four liver cancer tissue and matched non-neoplastic liver tissue specimens were plotted on the standard curve. The mRNA expression in FGFR3 was calculated as mentioned above. The mean ratio of FGFR3 mRNA to GAPDH mRNA in HCC tissue was 0.250, and the ratio in non-neoplastic liver tissue was 0.014. The average mRNA expression in HCC tissue was significantly less than that in non-neoplastic liver tissue ($P<0.01$), compared to GAPDH. The typical analysis result is shown in Figure 2.

**IHC study**

To validate the specificity of DNA microarray and real-time PCR, IHC analysis was performed in paraffin sections of HCC tissue with surrounding non-neoplastic liver tissues (Figure 3). Immunostaining was performed under 10 high power fields ($\times 400$) in the most densely cellular areas. Areas having necrosis or fibrosis were excluded. Among the 43 HCC cases, 2 were cirrhotic, 24 trabecular, 6 psuedoglandular, 3 solid, 7 compact, and 1 sarcomatoid. H&E staining was examined first and used as a control. The positive staining of FGFR3 was diffuse brownish cytoplasmic staining. FGFR3 was predominantly localized in the tumor cells, with lower levels in the non-neoplastic hepatic cells. Among the 43 cases of HCC, only 2 cases of non-neoplastic tissues demonstrated very weak staining, and the difference in the distribution of FGFR3 staining in the HCC area and non-neoplastic liver tissue was noticeable ($P<0.01$) (Figure 3). Stromal cells, bile ductal epithelial cells, vascular epithelial cells and fibrotic tissue were negative for FGFR3. Less intense staining was observed in infiltrated lymphocytes, Kupffer's cells and smooth muscle cells.

Figure 1 Northern blot validation of FGFR3 expression in matched HCC/non-neoplastic liver tissue and cell lines.

Figure 2 Standard curve and amplification plots of FGFR3 and GAPDH by quantitative real-time PCR. Starting quantity FGFR3 (A) and GAPDH (B); cycle
Clinicopathological features correlated with FGFR3 expression

We further examined the expression of FGFR3 in 43 cases of HCC tissue samples to confirm our above findings. As shown in Table 2, the age of the patients ranging from 26 to 82 years with a mean age of 59.79 years, 79% were men (34/43) and 21% were women (9/43). HBV positive patients accounted for 65.12% (28/43). Compared to the surrounding non-neoplastic liver tissue, 23.26% (10/43) of patients showed moderate staining for FGFR3 and 48.84% (21/43) of patients showed strong staining for FGFR3 in HCC tissue, indicating significant over-expression of FGFR3 in malignant tissues vs non-malignant tissues ($P < 0.01$).

Table 3 demonstrates the correlation between FGFR3 expression and clinicopathological features. Higher expression of FGFR3 was observed in most moderately- and poorly-differentiated HCC tissue samples. Nine of 20 cases with strong FGFR3 expression were poorly-differentiated. In 4 cases of well-differentiated HCC tissue samples, only 1 case showed very weak staining, whereas no FGFR3 staining was detectable in the other 3 cases. In the same manner, 11 of 20 cases with strong FGFR3 expression were nuclear grade 3, and 9 were nuclear grade 2. Only 1 of 3 HCC tissue samples with nuclear grade 1 showed very weak staining, whereas no FGFR3 staining was detectable in the other 3 cases. A significant correlation was observed between cancer differentiation ($P < 0.05$) and nuclear grade ($P < 0.05$) based on $\chi^2$ test. These findings suggested that HCC could be distinguished by a significant increase of FGFR3, being compatible with the current clinical parameters. The association between FGFR3 staining and HCC differentiation and nuclear grade is illustrated in Figure 4. HCC with poor differentiation and nuclear grade 3 demonstrated very strong staining of FGFR3.

Table 2 Patients demographic features and FGFR3 expression ($n = 43$)

| Demographic features | n = 43 |
|----------------------|--------|
| Age (yr)             | 59.79  |
| Gender               |        |
| Male                 | 34     |
| Female               | 9      |
| Hepatitis B          |        |
| Positive             | 28     |
| Negative             | 15     |
| FGFR3 expression (%) |        |
| 0                    | 3 (6.98)|
| +                    | 20 (46.51)|
| ++                   | 20 (46.51)|

DISCUSSION

To understand the characteristics of gene expression in HCC and the molecular processes involved in the cellular transformation from normal cells to neoplastic cells, global gene expression profiling with microarray was used to analyze the expression level change of 12 588 genes and oligos in HCC tissue and matched non-neoplastic liver tissue. Among the 17 transcription and translation-related genes whose expression levels changed most significantly, 10 over-
expressed genes and 7 under-expressed genes were identified in HCC tissue. Due to the high false positive and false negative rates in microarray study, significant over-expression of FGFR3 in HCC tissue was validated by Northern blot and quantitative real-time PCR.

FGFR family has been reported to encode a set of transmembrane tyrosine kinase receptors, which are composed of an extracellular ligand-binding domain, a transmembrane domain, and a split intracellular kinase domain\[9\]. FGFR family can bind to a number of related mitogenic fibroblast growth factors, resulting in receptor dimerization, autophosphorylation, and downstream signal transduction\[10\]. Up to now, four members of the family have been identified with domains of similar constitution, namely extracellular immunoglobulin-like domain, transmembrane domain, and intracellular kinase domain\[11\]. Since the alternative mRNA splicing of FGFR1, FGFR2 and FGFR3, seven prototype receptors have been reported\[12\]. Each prototype receptor has a different ligand binding capacity and tissue distribution\[13\]. Given the multiple forms of the receptors, the enormous potential for diversity of FGFR family is involved in cell growth control, cell differentiation and migration. Recently a number of studies have provided increasing evidence that FGFR family may be involved in carcinogenesis\[14,15\]. However, the detailed mechanism still needs to be further elucidated.

Activation and dimerization of FGFR3 result in cell proliferation or differentiation via signal transduction pathways\[16\]. Point mutations in the transmembrane domain of FGFR3 have been identified as a causative factor for skeletal development disorders such as achondroplasia and hypochondroplasia\[16,17\]. Moreover, activating mutations of FGFR3 have been reported to be related with superficial bladder and the presence of wild-type FGFR3 often precipitates multiple lesions in different regions of the bladder\[23\]. Several novel mutant transcripts caused by aberrant splicing and activation of cryptic splice sequences have been reported recently in digestive tract tumors, and high frequency of dysregulation of mRNA splicing is proposed to confer a selectable advantage on clones of cells in colorectal tumorigenesis\[23\]. More recently, two novel mutations of FGFR3 in colorectal carcinomas have also been identified\[24\]. All identified mutations occur at highly conserved sequences, not only in the FGFR family of molecules but also throughout evolution and are clustered in the immunoglobulin-like loop-III domain, highlighting the binding capacity and tissue distribution.

As malignant cells are characterized by uncontrolled growth genes that are over-expressed in malignant cells and not detectable in normal tissues, studies are conducted to elucidate the complicated process of carcinogenesis. In our study, IHC study of 43 clinical cases of HCC revealed that expression of FGFR3 increased significantly in HCC. More importantly, over-expression of FGFR3 was correlated with pathological factors including HCC differentiation and nuclear grade, suggesting that clinicopathologic index, morphologic grade, and molecular grade are consistent. Therefore, activation of FGFR3 may play an important role in hepatocarcinogenesis. The identification of FGFR3 somatic mutation in HCC is currently under investigation. However, the detailed mechanism needs to be further studied.

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