Expression of FGFs and FGFRs in Human Hepatocellular Carcinoma Cell Lines

Jae Woo Kim

The Graduate School

Yonsei University

Department of Medicine

Expression of FGFs and FGFRs in Human Hepatocellular Carcinoma Cell Lines

A dissertation submitted to the Department of Medicine and the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Jae Woo Kim

July 2007

This certifies that the dissertation of Jae Woo Kim is approved.

Thesis Supervisor : [Byung-Il Yeh]

[Sang Ok Kwon: Thesis Committee Member]

[In Deok Kong: Thesis Committee Member]

[Mee Yon Cho: Thesis Committee Member]

[Soon Koo Baik: Thesis Committee Member]

The Graduate School

Yonsei University

July 2007

Acknowledgements

First, I thank my colleagues who help to write this thesis. Working hard and accomplish the aims in one kind of work is thought to be infinite happiness and pleasure of life.

Thank for the trust, guidance, and patience of professor Byung-Il Yeh at the department of biochemistry. Also, thanks Dr. Jun Namkung for contribution of this research.

Thanks deeply professor, Sang Ok Kwon and Soon Koo Baik at the department of internal medicine, In Deok Kong at the department of physiology, and Mee Yon Cho at the department of pathology, Wonju Christian hospital, for helpful suggestions on the thesis.

Finally, give thanks to my companion and dear wife Yoon Kyung Youm.

July 2007 Jae Woo Kim

CONTENTS

List of figures

List of tables

ABSTRACT

Expression of FGFs and FGFRs in Human Hepatocellular Carcinoma Cell Lines

Fibroblast growth factors (FGFs) play important roles in angiogenesis, wound repair, and development of cancer cell growth. That is, FGFs will activate signal transduction pathways to stimulate cell growth by promoting cell cycle progression and inhibiting pathways of cell death. This study aimed to seek for FGFs and FGFRs which can be used as new therapeutic targets by search of those related with the pathogenesis of hepatocellular carcinoma (HCC). To establish the quantitative expression profiles in HCCs comparing with normal hepatocyte, I performed real-time RT-PCR targeting all FGFs and FGFRs.

Six HCC cell lines (SNU-398, SNU-449, SNU-761, SNU-739, SNU-886 and Hep3B) and 2 hepatoblastoma cell lines (HepG2 and PLC/PRF/5) were cultured and obtained their cDNAs by reverse transcription. As a control, commercially available normal hepatocyte cDNA was purchased. On the screening of expressions including all 22 FGFs (FGF-1~FGF-23 except FGF-15) and 8 FGFRs

(FGFR1IIIb~FGFR5) by RT-PCR, FGF-16 and 22 were not expressed in all cancer cell lines and normal hepatocyte didn't express FGF-3, 4, 5, 8 and 20. FGFR-1 and 3, which have isotype IIIb and IIIc via alternative splicing, were excluded in quantitative analysis because exon-specific primers could not distinguish the primary transcripts before alternative splicing and mature transcripts.

Compared with that in normal hepatocyte, expression of most FGFs and FGFRs in cancer cells are increased. On the basis of FGFs-FGFRs specific activity, most FGF subfamilies showed increased expressions in both ligands and their receptors. However, FGF-6, 7, 21, and 23 were increased in normal hepatocyte or not expressed in cancer cells.

This is the first study of expression profiles for targeting all known FGFs and FGFRs in HCC and hepatoblastoma cell lines compared with those in normal hepatocyte.

Key words: fibroblast growth factor, fibroblast growth factor receptor, hepatocellular carcinoma cell lines, gene expression

Expression of FGFs and FGFRs in Human Hepatocellular Carcinoma Cell Lines

<Directed by Professor Byung-Il Yeh>

Jae Woo Kim

Department of Medicine

The Graduate School of Yonsei University

I. INTRODUCTION

The growth of cancer cells is characterized as sustained and dysregulated features, and it is the main problem in clinical aspects. Several growth factors like IGF, HGF, and TGF are identified as a cause of cancer cell growth and a target of cancer treatment. Fibroblast growth factors (FGFs) which are 22 membered polypeptide growth factors and their receptors (FGFRs) belonging to receptor

tyrosine kinase (RTK) are one of growth factors related with hepatocarcinogenesis. 23 FGFs have been identified in mice, but human beings have 22 FGFs: FGF-19 is the human ortholog of mouse FGF-15. Five FGFRs have been identified in human, FGFR-1 to 5[1-4] with alternative splicing in FGFR-1, 2 and 3 in the Ig-like domain III which divide them to isoform IIIb and IIIc.[1, 2] FGFs are grouped as seven subfamilies on the basis of their sequence similarity, and biochemical and developmental properties.[5] FGFs bind to their specific FGFRs with different activities[6] except FGF-11 to 14 which of their receptors still not identified yet.

FGFs play important roles in angiogenesis,[7] wound repair,[8] and development of cancer cell growth.[9] That is, FGFs will activate signal transduction pathways to stimulate cell growth by promoting cell cycle progression and inhibiting pathways of cell death. These are two mechanisms of FGFs signaling in cancers. One is due to altered expression level: increased expression of FGFs and(or) FGFRs make more functional units of FGFs-FGFRs signaling. The other is genetic alteration: the function of the mutants of FGFs may be altered by the changes of affinity to receptors and mutant FGFRs may function independently of

presence of their ligands.

FGF-23 is elevated in ovarian cancers.[10] In acute leukemia, overexpression of FGF-8 and FGF-17 was detected.[11] FGF-17 is increased in prostate cancer.[12] Over-expression of FGFR-1 or FGFR-3 has been shown in breast cancer[13] or thyroid carcinoma,[14] respectively. Several mutations in FGFR3 is identified in transitional cell carcinoma of bladder,[15-18] cervical carcinoma,[15, 17, 19] colorectal cancer,[20, 21] peripheral T cell lymphoma[22] and multiple myeloma.[23] In head and neck squamous cell carcinoma, FGFR-4 polymorphism has been studied.[24] Class switch of IIIb isoform to IIIc isoform of FGFR-2 in prostate cancer[25] or FGFR-2 splice site mutation in gastric cancer[20] are also known mutations of FGFRs in human cancers.

Hepatocellular carcinoma (HCC) accounts for between 85% and 90% of primary liver cancers[26] which are one of the fifth most common cancer worldwide, the third most common cause of mortality.[27] In Korea, epidemic area of HBV and HCV infection which are well-established risk factors of HCC, liver cancers are the third and the sixth most common cancer in male and female, respectively.[28] The

expression of insulin-like growth factor (IGF), hepatocyte growth factor (HGF), transforming growth factor (TGF) and wingless (Wnt) signaling have been considered as causative pathways in human HCCs.[29] For FGFs, several ligands and receptors have been studied.

FGF-2 was related to the capsular invasion into surrounding tissues[30] and FGF-3 over-expression was suggested to be associated with metastasis and recurrence.[31] Opposite to concept of FGFs as tumor promoting growth factors, FGF-21, preferentially expressed in the liver,[32] could delay initiation of chemically induced hepatocarcinogenesis.[33] It is also reported FGF-1 was not expressed only in normal hepatocyte, and FGF-7 was not both in normal hepatocyte and hepatoma-derived cell lines. For FGFRs, normal hepatocytes did not express FGFR-2 IIIb, 2 IIIc and 3 IIIb but in HCC cell lines.[34] Finally, over-expression of FGFR-3 in HCCs[35] was associated with poor tumor differentiation and high nuclear grade and acyclic retinoid acid, which down-regulate expression of FGFR-3, resulted in growth inhibition of HCCs.[36]

To sum up previous studies mentioned above, expression profiling of FGFs

and FGFRs in HCCs are not fully completed. A result of RT-PCR targeting all FGFs and FGFRs in HCCs is not published yet. Even some papers showing quantitative analysis using RT-PCR data are published, the discrepancy between intensity of the PCR product under the UV transilluminator and initial concentration of target mRNA have not been considered. Furthermore, the other previous papers which performed real-time PCR data of FGFR-1, 2, and 3, didn't distinguish isotype IIIb and IIIc which result in pointless data.

To establish the first quantitative expression profile in HCCs comparing with normal hepatocyte as control, I intended to perform reat-time RT-PCR targeting all FGFs and FGFRs.

II. MATERIALS & METHODS

Cell culture

Six human HCC cell lines (SNU-398, SNU-449, SNU-739, SNU-761, SNU-886 and Hep3B) and 2 human hepatoblastoma cell lines (HepG2, PLC/PRF/5) were purchased from Korean Cell Line Bank (Seoul, Korea). All cell lines except HepG2 were cultured in RPMI-1640 with L-glutamine (300 ml/L), 25 mM HEPES and 25 mM $NaHCO_3$ supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin and streptomycin. HepG2 cell lines were cultured in Dulbecco's Modified Eagle Media (DMEM) with other supplements same as the other cell lines (all from Invitrogen, Carlsbad, CA). All cells were maintained on T-25 cell culture flask (from Becton Dickinson Labware, Franklin Lakes, NJ) at 37'C in a 5% CO_2 – 95% air atmosphere. All cells were observed grossly and microscopically twice a day. Subcultures were considered when cell populations were roughly 80% of confluency.

Cell harvest

After microscopic confirmation of confluency, cells were washed with phosphate-buffered saline then detached using 0.25% trypsin-EDTA solution (from Invitrogen, Carlsbad, CA), followed by pipetting with growth media including FBS. After centrifugation at 2000 rpm for 3 minutes, supernatants were discarded. Remaining cell pellets were washed using phosphate-buffered saline again and centrifuged once more. Final cell pellets were used for RNA isolation.

RNA isolation & Reverse transcription

Purified total RNA were obtained from harvested cell lines using RNeasy method according to manufacturer's protocol (from Qiagen, Hilden, Germany). The concentration and purity of purified RNA were measured using UV spectrophotometer (from Biochrom, Cambridge, UK) and checked 260/280 ratio higher than 1.80. All RNA samples were diluted with RNase-free water to final concentration of 100 ng/ul. cDNAs from purified RNAs were obtained using reverse transcription according to manufacturer's protocol (from Qiagen, Hilden,

Germany). cDNAs were diluted by adding same volume of distilled water.

Normal hepatocyte cDNA

As a control to human HCC cell lines, normal hepatocyte cDNA library and normal hepatocyte cDNA were purchased from Takara Bio Inc (Tokyo, Japan) and Ambion Inc (Austin, TX), respectively.

Primers for RT-PCR and real-time RT-PCR

To analyze the expression of human FGF 1 to 23 and FGFR-1 IIIb to 5 in HCC cell lines and normal hepatocyte, the primers for PCR were designed using web resource of Primer3 (http://frodo.wi.mit.edu/primer3/input.htm) and asked Genotech (Daejon, Korea) to synthesize them. Primer picking conditions were specified as product size ranging from 80 bp to 150 bp and the others as default settings. For some genes which have several transcript variants, primer sets existing in all transcript variants were selected. For FGFR-1, 2 and 3, which have isotype IIIb and IIIc, primers are designed located in exon 8 for isotype IIIb in

FGFR-1 and 3 and exon 9 in FGFR-2 and exon 9 for isotype IIIc in FGFR-1 and 3 and exon 10 in FGFR-2. And additional primers located in exon 6 and 10 are designed to investigate the presence of other isotypes (sequences are not shown). Sequences of primers are listed in (table 1).

Table 1. Primer lists used for RT-PCR and real-time PCR

Gene	Genebank ID	Forward primer (5'-3')	Reverse primer (5'-3')	Target
FGF1	NM_000800	GGGCTTTTATACGGCTCACA	TTTGCAGCTCCCATTCTTCT	397-540
FGF2	NM_002006	CAATTCCCATGTGCTGTGAC	GGCAGACGAATGCCTTATGT	2607-2753
FGF3	NM_005247	ACCGGACGGTGTCTAGTACG	CAGGGAGGACTTCTGTGTGC	892-1022
FGF4	NM_002007	GATGAGTGCACGTTCAAGGA	GAGGAAGTGGGTGACCTTCA	776-928
FGF5	NM_004464	TTGTCCTTCCTCCTCCTCCT	GGCAGAAGAGGAAGACATAG	244-414
FGF6	NM_020996	CCAGCTTCCAAGAAGAATGC	TTACCCGTCCGTATTTGCTC	496-612
FGF7	NM_002009	GACATGGATCCTGCCAACTT	GGGCTGGAACAGTTCACATT	463-591
FGF8	NM_033165	TCATCCGGACCTACCAACTC	ACTCGGACTCTGCTTCCAAA	286-433
FGF9	NM_002010	GGGGAGCTGTATGGATCAGA	CTCGGGGTCCCATCTTTATT	542-696
FGF10	NM_004465	CCGTACAGCATCCTGGAGAT	CCCCTTCTTGTTCATGGCTA	319-414
FGF11	NM_004112	ACTAGGGCTCTGTGCTGGAA	GCCAAAGCCAACAGCTAGAC	1439-1580
FGF12	NM_004113	TTCTGGAACACCAACCATGA	TCCTTGGGTGGATTTACTGG	722-866
FGF13	NM_004114	TGTGCATAAGAATGCCAAGC	TTCTTGGTGGGAGAGCAAAT	1591-1740
FGF14	NM_004115	AATGAATGGAGGCAAACCAG	ACTTCCTGCTGCTCTTCAGC	702-845
FGF16	NM_003868	GGAGTGGACTCTGGCCTGTA	CTGTTCCCGGAAAACACATT	400-501
FGF17	NM_003867	ACCAGTACGTGAGGGACCAG	TGAGCTTGGCAAACTTGTTG	113-271
FGF18	NM_003862	TGTGCTTCCAGGTACAGGTG	GGATGTGTTTCCCACTGGTC	590-736
FGF19	NM_005117	GGAGGAAGACTGTGCTTTCG	GGCAGGAAATGAGAGAGTGG	811-963
FGF20	NM_019851	CCACAGCCTCTTCGGTATCT	GCTCCCTAAAGATGCATTCG	406-557
FGF21	NM_019113	ACTCCAGTCCTCTCCTGCAA	ATCCTCCCTGATCTCCAGGT	248-348
FGF22	NM_020637	CAGGACAGCATCCTGGAGAT	CTGCAGTCCACGGTGTAGAG	208-341
FGF23	NM_020638	CAAGCCTTCGTTCTTCCTTG	CCTCATTTCAGCAAGCATCA	1452-1555
FGFR1IIIb	NM_023110	TACCAGCTGGATGTCGTGGA	CTGCGGGTCACTGTACACC	1669-1794
FGFR1IIIc	NM_023110	ACCACCGACAAAGAGATGGA	GCAGAGTGATGGGAGAGTCC	1894-2003
FGFR2IIIb	NM_000141	GCAGAAGTGCTGGCTCTGTT	TGTTTTGGCAGGACAGTGAG	1601-1689
FGFR2IIIc	NM_022970	CACCACGGACAAAGAGATTG	TAGAATTACCCGCCAAGCAC	1611-1729
FGFR3IIIb	NM_000004.10	CCTGGATCAGTGAGAGTGTGG	AAATTGGTGGCTCGACAGAG	9020-9116
FGFR3IIIc	NM_000004.10	CACCGACAAGGAGCTAGAGG	CCACGCAGAGTGATGAGAAA	9814-9925
FGFR4	NM_002011	CAAAGACAACGCCTCTGACA	CACCAAGCAGGTTGATGATG	1682-1782
FGFR5	AF279689	AGACAAGGACCTTCCCTCGT	GGTACAACTTAGGGCCAGCA	1315-1446
GAPDH	NM_002046	GGCCTCCAAGGAGTAAGACC	AGGGGTCTACATGGCAACTG	1095-1241

RT-PCR

Conventional RT-PCR was performed for preliminary scanning before real-time RT-PCR. cDNAs were used as templates for PCR. The reaction volumes were 20 ul containing 0.5 ul of template cDNA, 2 ul of primer set of each 10 pmol, 2 ul of 10X PCR buffer, 2 ul of dNTP mixture, 0.25 ul Taq DNA polymerase (from Takara Bio Inc, Shiga, Japan) and 14.25 ul of distilled water. PCR was performed as follows: 95'C at 3 minutes, followed by 35 cycles of denaturation at 95'C for 30 seconds, annealing at 60'C for 30 seconds, extension at 72'C for 30 seconds then final extension at 72'C for 2 minutes using programmable thermal cycler from Corbett Research (Mortlake, Austria) and MJ Research (Watertown, MA). GAPDH was used as positive control. 4 ul of DNA loading buffers (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in H_2O) were loaded into PCR products and electrophoresed as follows : 2% agarose (from Cambrex Bio Science Rockland, Rockland, ME) in 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA pH 8.0), horizontal gel unit from SCIE-PLAS (Warwickshire, UK). Gels were visualized at UV-transilluminator from Vilber Lourmat (Marne-la-Vallee, France)

and gel-documation system (from Eastman Kodak Company, Rochester, NY). Targets which expressed in normal hepatocyte were selected for real-time RT-PCR.

Real-time RT-PCR

Selected targets were performed for quantitative expression profiling using real-time RT-PCR. Reactions were as follows: total reaction volume was 12.5 ul containing 6.25 ul of SYBR Master Mix (from Qiagen, Hilden, Germany), 0.5 ul of template cDNA, 0.75 ul of primer set mix of each 10 pmol and 5 ul of distilled water and programmed at 95'C for 15 minutes for heat activation, followed by 40 cycles of denaturation at 94'C for 20 seconds, annealing at 60'C for 30 seconds, and extension at 72'C for 30 seconds with fluorescence detection at the end of each cycles. Melt analysis was performed after all reactions to identify homogeneity of PCR products by waiting at 70'C for 30 seconds, then ramp to 99'C rising by 1'C with fluorescence detection. Real-time RT-PCR was performed using Rotor Gene 3000 (from Corbett, Austria). All reactions were performed in triplicate. GAPDH was used as control to calculate ΔCt values. Thresholds to determine Ct values

were manually selected at the starting point of exponential phase.

As there exist so many kinds of FGFs and FGFRs, real-time RT-PCR was performed and grouped on the basis of the 7 subfamilies.

DNA sequencing

To confirm whether the PCR products are the same FGFs and FGFRs as I expected to amplify DNA sequencing was tried as followings.

Amplified cDNAs of FGFs and FGFRs were isolated from agarose gel and purified with DNA PrepMate^{TM} (Bioneer, Chungwon, Korea). They were then subcloned by inserting the cDNA into a pMOSBlue T-vector (Amersham Pharmacia Biotech., Uppsala, Sweden). Plasmid containing amplified products of cDNAs of FGFs and FGFRs were selected by α-complementation. The clones from each FGFs and FGFRs were randomly selected and plasmid DNAs prepared from each clone were used as templates for DNA sequencing. It was progressed by Genotech (Daejon, Korea).

Statistics

ΔCt values of real-time RT-PCR were expressed as mean ± standard deviation (S.D.) from triplicated reactions. For determining statistical significances, ΔCt values of each target were tested to confirm a normal distribution using one-sample Kolmogorov-Smirnov test. All values were fitted to a normal distribution (data not shown). To comparing with the values got from normal hepatocyte and other cell lines, one-way ANOVA test was performed. All tests were performed using SPSS version 12.0 (from SPSS Inc, Chicago, IL). Statistical significances were considered in case P-value of < 0.05.

III. RESULTS

Preliminary scanning of expressions of FGFs and FGFRs in cancer cell line

RT-PCR was performed to find expression profiles of 22 FGFs and 8 FGFRs by use of normal hepatocyte cDNA (data not shown) and purified RNA from SNU-739 cell line (figure 1).

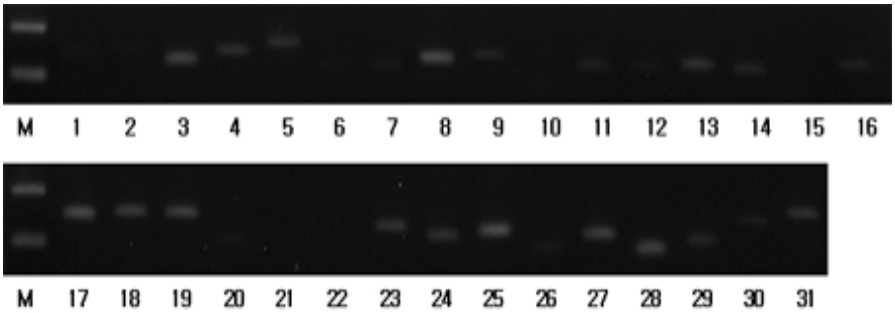

Figure 1. Preliminary scanning of 22 FGFs and 8 FGFRs in SNU-739 cell line
FGF 16 and 22 were not expressed in SNU-739 cell line. The numbers of the lanes indicate as followings: 1, FGF-1; 2, FGF-2; 3, FGF-3; 4, FGF-4; 5, FGF-5; 6, FGF-6; 7, FGF-7; 8, FGF-8; 9, FGF-9; 10, FGF-10; 11, FGF-11; 12, FGF-12; 13, FGF-13; 14, FGF-14; 15, FGF-16; 16, FGF-17; 17, FGF-18; 18, FGF-19; 19, FGF-20; 20, FGF-21; 21, FGF-22; 22, FGF-23; 23, FGFR-1 IIIb; 24, FGFR-1 IIIc; 25, FGFR-2 IIIb; 26, FGFR-2 IIIc; 27, FGFR-3 IIIb; 28, FGFR-3 IIIc; 29, FGFR-4; 30, FGFR-5; 31, GAPDH and M, molecular weight size marker (100 bp DNA ladder). Two bands shown in lane M indicate the DNA size of 100 and 200bp.

All of the FGFs were expressed in cancer celll lines used in this study, except FGF-16 and 22 (figure 2). These two FGFs are excluded in quantitative expression profiling.

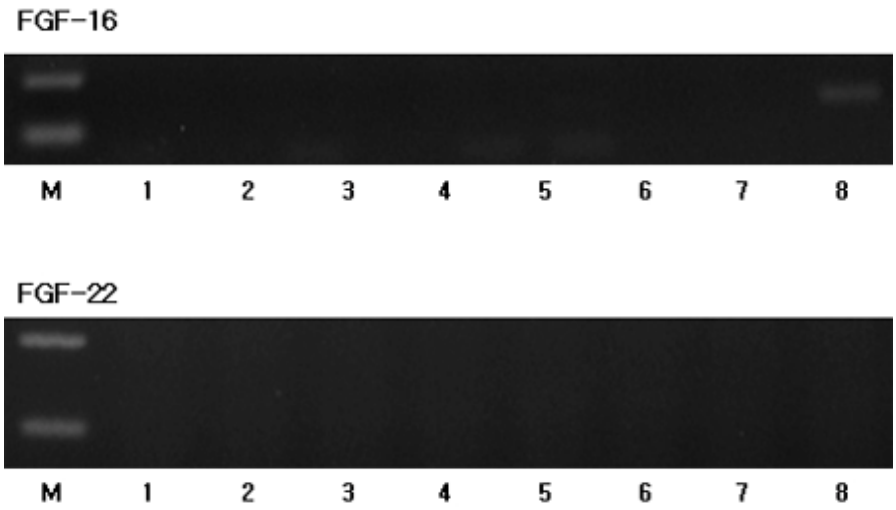

Figure 2. mRNA expressions of FGF-16 and 22 on 6 hepatocellular cell lines and 2 hepatoblastoma cell lines

All tumor cell lines did not express FGF-16 and 22. The numbers of the lanes indicate as followings: 1, SNU-761; 2, SNU-886; 3, SNU-449; 4, SNU-739; 5, SNU-398; 6, Hep3B; 7, HepG2; 8, PLC/PRF/5 and M, molecular weight size marker (100 bp DNA ladder). Two bands shown in lane M indicate the DNA size of 100 and 200 bp.

FGFs expression profiles in normal hepatocyte

RT-PCR product from normal hepatocyte cDNA showed that FGF-3, 4, 5, 8, and 20 were not expressed (figure 3). However, these five FGFs were amplified from SNU-739 cell line. To confirm the differences of expressions of these FGFs between normal hepatocyte and cancer cell lines, RT-PCRs were repeated using the RNAs from all of cell lines used in this study (figure 4). In all of the HCC cell lines FGF-3, 4, 5, 8, and 20 are expressed well, but not in normal hepatocyte .

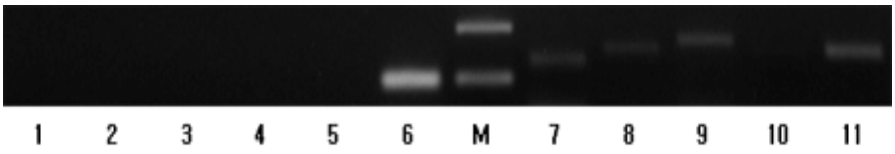

Figure 3. mRNA expressions of FGF-3, 4, 5, 8 and 20 in normal hepatocyte compared with in HCC cell line (SNU-739)

In normal hepatocyte, FGF-3, 4, 5, 8 and 20 were not expressed. PCR products from normal hepatocyte are loaded in lane 1 to 6, and SNU-739 in lane 7 to 11. The numbers of the lanes indicate as followings: 1 and 7, FGF-3; 2 and 8, FGF-4; 3 and 9, FGF-5; 4 and 10, FGF-8; 5 and 11, FGF-20; 6, GAPDH and M, molecular weight size marker (100 bp DNA ladder). Two bands shown in lane M indicate the DNA size of 100 and 200 bp.

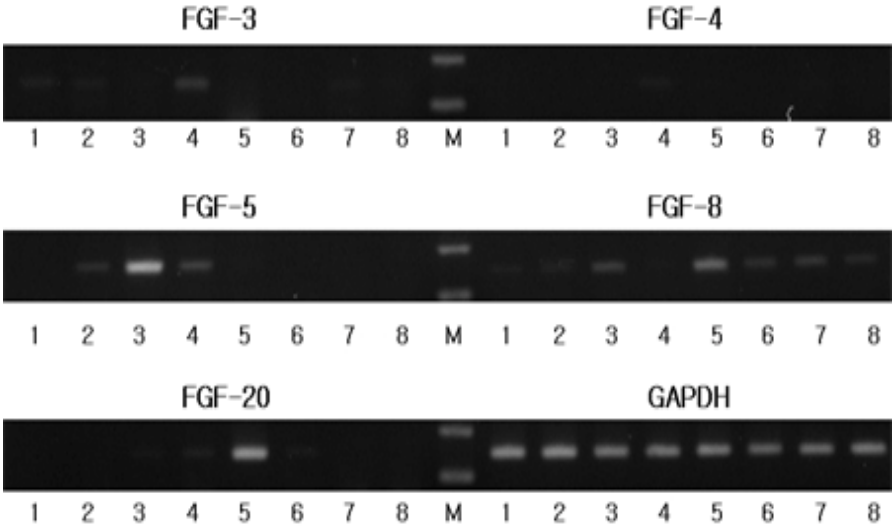

Figure 4. mRNA expressions of FGF-3, 4, 5, 8 and 20 in cancer cell lines

In all cancer cell lines, FGF-3, 4, 5, 8 and 20 were expressed. The numbers of the lanes indicate as followings: 1, SNU-761; 2, SNU-886; 3, SNU-449; 4, SNU-739; 5, SNU-398; 6, Hep3B; 7, PLC/PRF/5; 8, HepG2 and M, molecular weight size marker (100 bp DNA ladder). Two bands shown in lane M indicate the DNA size of 100 and 200 bp.

Isotype specific RT-PCR

Exon-specific primer sets were used in RT-PCR of FGFR-1, 2 and 3 to check the presence of linked type of isotype b and c in the Ig-like domain III. Using forward primer located in exon 8 (for FGFR-1 and 3) or exon 9 (for FGFR-2) and reverse primer located in exon 9 (for FGFR-1 and 3) or exon 10 (for FGFR-2), I got the unexpected PCR product which containing both exon 8 (or 9) and exon 9 (or 10) in FGFR-1 and 3 (figure 5), not in FGFR-2 (data not shown).

Quantitative expression profiles of FGFs and FGFRs

Expressions of 15 FGFs (FGF-1 to 23 except 3, 4, 5, 8, 16, 20 and 22) and 4 FGFRs (FGFR-2 IIIb, 2 IIIc, 4 and 5) were evaluated by real-time PCR according to grouping by subfamily and specific activities on receptors.

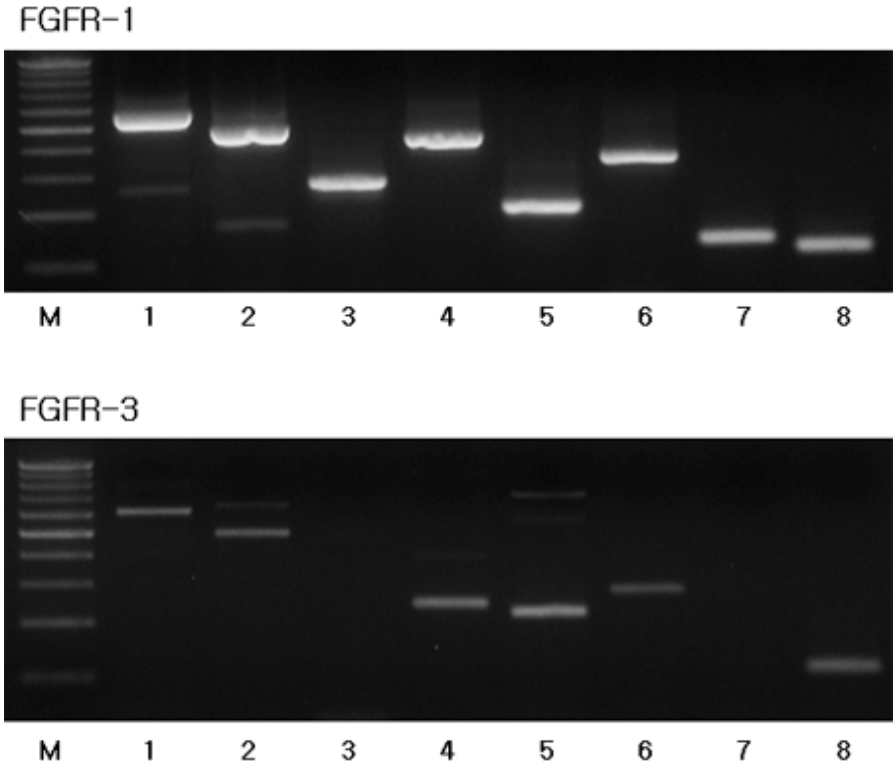

Figure 5. mRNA expressions of FGFR-1 and 3 using exon-specific primers FGFR-1 and 3 contained both exon 8 (or 9) and 9 (or 10). SNU-449 was used as template. The numbers of the lanes indicate as followings: 1, exon 6 - exon 10; 2, exon 6 - exon 9; 3, exon 6 - exon 8; 4, exon 8 - exon 10; 5, exon 9 - exon 10; 6, exon 8 - exon 9; 7, exon 8 - exon 8; 8, exon 9 - exon 9 and M, molecular weight size marker (100 bp DNA ladder).

DNA Sequencing

I found a few PCR products which were not amplified in previous reports[34]. That is, I could see the DNA bands for FGF-1 and FGF-7 in normal hepatocyte and FGF-7 was also amplified from HCC cell lines. To confirm the PCR products I sequenced all of the PCR products. All of the sequences were right ones, and the PCR products for FGF-1 and FGF-7 were proved to be originated from FGF-1(figure 6) and FGF-7 (data not shown).

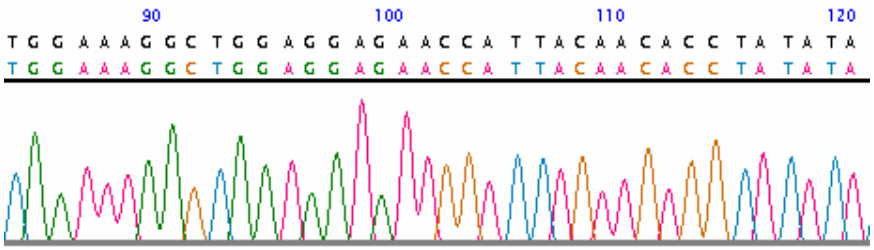

Figure 6. Nucleotide sequences of FGF-1 from normal hepatocyte

These sequences corresponds to the sequences from 443rd to 480th human FGF-1.

Expression of FGF subfamily 1

Table 2 shows expression profile of FGF subfamily 1. Significant differences including over-expressions of FGF-1 of SNU-449, FGF-2 of SNU-398, FGFR-4 of Hep3B, HepG2, PLC/PRF/5 and FGFR-5 of SNU-739, SNU-761 and HepG2 and down-expressions of FGF-2 of HepG2, FGFR-2 IIIb of SNU-886, SNU-449 and PLC/PRF/5 (last two cell lines showed no expression) and FGFR-2 IIIc of SNU-449, SNU-886 and PLC/PRF/5 were observed.

Expression of FGF subfamily 4

FGF-6 (table 3) was the only target of subfamily 4 available in this study. 4 cell lines (SNU-398, SNU-449, SNU-761 and SNU-886) showed no expression and Hep3B showed significant down-regulation.

Table 2. mRNA expressions in FGF subfamily 1

	FGF-1	FGF-2	FGFR-2 IIIb	FGFR-2 IIIc	FGFR-4	FGFR-5
Normal	16.32±0.36	14.97±1.63	14.75±0.81	14.54±2.72	9.99±0.85	10.51±0.20
SNU-398	18.38±1.47	7.49±1.37*	18.56±2.10	14.17±1.99	8.11±0.82	8.01±2.68
SNU-449	10.02±0.58*	15.23±1.21	No expression	23.79±1.15*	8.02±0.94	9.21±0.87
SNU-739	13.68±0.68	17.73±3.28	12.46±0.66	7.86±1.06*	7.99±1.80	6.50±0.36*
SNU-761	12.79±1.74	13.15±0.77	16.97±1.27	13.91±1.10	5.99±0.63	5.97±0.18*
SNU-886	13.29±0.59	12.93±0.61	25.81±2.18*	21.97±2.50*	9.13±0.29	8.85±0.32
Hep3B	20.27±1.95	20.24±0.66	10.2±0.99	13.36±0.25	6.27±3.00*	7.92±1.13
HepG2	17.45±1.17	24.20±2.60*	19.16±2.52	18.69±1.51	4.91±0.25*	5.93±0.82*
PLC/PRF/5	17.93±1.41	17.07±0.54	No expression	22.41±0.59*	4.36±0.63*	9.29±0.67
HCCs	14.74±3.82	14.46±4.41	14.00±8.74	15.84±5.95	7.59±1.21	7.74±1.28*
HBs	17.69±0.34	20.64±5.04	19.16±2.52	20.55±2.63	4.64±0.39*	7.61±2.38
All cancers	15.47±3.52	16.00±5.04	17.19±5.37	17.02±5.46	6.85±1.99*	7.71±1.65*

ΔCt values are expressed in mean ± standard deviation of triplicate reactions. Activities to FGFRs for FGF subfamily 1 are equal to all FGFRs, but FGFR-5 is known as receptor for FGF-2 only. FGFR-1 IIIb, IIIc, 3 IIIb and 3IIIc, receptors for this subfamily, are excluded. * indicates p-value < 0.05 in one-way ANOVA; HCCs, average of ΔCt values of HCC cell lines except those of non-expressing ones; HBs, average of ΔCt values of hepatoblastoma cell lines except those of non-expressing ones; All cancers, average of ΔCt values of all cell lines except those of non-expressing ones.

Table 3. mRNA expressions in FGF subfamily 4

	FGF-6	FGFR-2 IIIc	FGFR-4
Normal	14±0.48	14.54±2.72	9.99±0.85
SNU-398	No expression	14.17±1.99	8.11±0.82
SNU-449	No expression	23.79±1.15*	8.02±0.94
SNU-739	15.12±2.95	7.86±1.06*	7.99±1.80
SNU-761	No expression	13.91±1.10	5.99±0.63
SNU-886	No expression	21.97±2.50*	9.13±0.29
Hep3B	20.27±1.78*	13.36±0.25	6.27±3.00*
HepG2	16.41±1.02	18.69±1.51	4.91±0.25*
PLC/PRF/5	18.18±2.12	22.41±0.59*	4.36±0.63*
HCCs	ND	15.84±5.95	7.59±1.21
HBs	17.30±1.25	20.55±2.63	4.64±0.39*
All cancers	ND	17.02±5.46	6.85±1.99*

ΔCt values are expressed in mean ± standard deviation of triplicate reactions. FGF-4 and FGF-5, members of subfamily 4, and FGFR-1 IIIc and 3 IIIc, receptors for this subfamily, are excluded. Order of activities to FGFRs in FGF subfamily 4 is FGFR-1 IIIc and FGFR-2 IIIc > FGFR-3 IIIc and FGFR-4. *indicates p-value < 0.05 in one-way ANOVA; HCCs, average of ΔCt values of HCC cell lines except those of non-expressing ones; HBs, average of ΔCt values of hepatoblastoma cell lines except those of non-expressing ones; All cancers, average of ΔCt values of all cell lines except non-expressing ones; ND, average value is not determined because there were no PCR products in four HCC cell lines.

Expression of FGF subfamily 7

FGF-3, 22 and FGFR-1 IIIb were excluded in quantitative profiling. As shown in (table 4), expression of FGF-7 is significantly decreased in SNU-449, SNU-761, SNU-886, HepG2 and PLC/PRF/5 or even similar to non-template control (NTC) in Hep3B. SNU-449, SNU-739, SNU-761, HepG2 and PLC/PRF/5 showed no expression of FGF-10 and SNU-886 was significantly less expressed. But SNU-398 showed up-regulated FGF-10 expression with statistical significance.

Expression of FGF subfamily 8

Although SNU-398 showed up-regulated expression of FGF-17, SNU-449 was down-regulated and PLC/PRF/5 showed no expression. Over-expression of FGF-18 was observed in SNU-398 and SNU-739 (table 5).

Expression of FGF subfamily 9

SNU-398 showed significant over-expression of FGF-9, but SNU-449 did not express at all (table 6).

Table 4. mRNA expressions in FGF subfamily 7

	FGF-7	FGF-10	FGFR-2 IIIb
Normal	11.18±0.56	18.18±0.37	14.75±0.81
SNU-398	11.81±0.80	13.44±0.42*	18.56±2.10
SNU-449	16.33±1.06*	No expression	No expression
SNU-739	8.77±0.29	No expression	12.46±0.66
SNU-761	16.97±0.72*	No expression	16.97±1.27
SNU-886	16.16±0.92*	20.32±0.84*	25.81±2.18*
Hep3B	No expression	19.08±0.48	10.20±0.99
HepG2	17.56±1.37*	No expression	19.16±2.52
PLC/PRF/5	17.07±0.91*	No expression	No expression
HCCs	14.01±3.38	ND	14.00±8.74
HBs	17.32±0.35*	No expression	19.16±2.52
All cancers	14.95±3.26	ND	17.19±5.37

ΔCt values are expressed in mean ± standard deviation of triplicate reactions. FGF-3 and FGF-22, members of subfamily 7, and FGFR-1 IIIb, receptor for this subfamily, are excluded. Order of activities to FGFRs in FGF subfamily 4 is FGFR-2 IIIb > FGFR-1 IIIb. * indicates p-value < 0.05 in one-way ANOVA; HCCs, average of ΔCt values of HCC cell lines except those of non-expressing ones; HBs, average of ΔCt values of hepatoblastoma cell lines except those of non-expressing ones; All cancers, average of ΔCt values of all cell lines except those of non-expressing ones; ND, average value is not determined because there were no PCR products in four HCC cell lines.

Table 5. mRNA expressions in FGF subfamily 8

	FGF-17	FGF-18	FGFR-4	FGFR-2 IIIc
Normal	17.01±1.24	16.77±0.18	9.99±0.85	14.54±2.72
SNU-398	11.08±0.86*	8.31±0.48*	8.11±0.82	14.17±1.99
SNU-449	19.95±1.19*	14.85±0.19	8.02±0.94	23.79±1.15*
SNU-739	14.62±0.18	9.90±0.79*	7.99±1.80	7.86±1.06*
SNU-761	14.66±0.35	16.21±1.34	5.99±0.63	13.91±1.10
SNU-886	14.91±0.45	18.28±0.78	9.13±0.29	21.97±2.50*
Hep3B	15.63±0.23	15.57±0.66	6.27±3.00*	13.36±0.25
HepG2	15.37±0.92	14.20±0.63	4.91±0.25*	18.69±1.51
PLC/PRF/5	No expression	15.48±1.14	4.36±0.63*	22.41±0.59*
HCCs	15.14±2.84	13.85±3.88	7.59±1.21	15.84±5.95
HBs	15.37±0.92	14.84±0.91	4.64±0.39*	20.55±2.63
All cancers	15.17±2.53	14.10±3.26	6.85±1.99*	17.02±5.46

ΔCt values are expressed in mean ± standard deviation of triplicate reactions. FGF-8, members of subfamily 8, and FGFR-3 IIIc, 1 IIIc and 3 IIIb, recpetors for this subfamily, are excluded. Order of activities to FGFRs in FGF subfamily 8 is FGFR-3 IIIc > FGFR-4 > FGFR-2 IIIc > FGFR-1 IIIc >> FGFR-3 IIIb. * indicates p-value < 0.05 in one-way ANOVA; HCCs, average of ΔCt values of HCC cell lines except those of non-expressing ones; HBs, average of ΔCt values of hepatoblastoma cell lines except those of non-expressing ones; All cancers, average of ΔCt values of all cell lines except those of non-expressing ones.

Table 6. mRNA expressions in FGF subfamily 9

	FGF-9	FGFR-2 IIIc	FGFR-4
Normal	17.03±3.54	14.54±2.72	9.99±0.85
SNU-398	7.37±0.23*	14.17±1.99	8.11±0.82
SNU-449	No expression	23.79±1.15*	8.02±0.94
SNU-739	14.93±0.59	7.86±1.06*	7.99±1.80
SNU-761	16.16±0.38	13.91±1.10	5.99±0.63
SNU-886	20.56±2.47	21.97±2.50*	9.13±0.29
Hep3B	21.49±0.63	13.36±0.25	6.27±3.00*
HepG2	18.02±1.24	18.69±1.51	4.91±0.25*
PLC/PRF/5	20.04±0.48	22.41±0.59*	4.36±0.63*
HCCs	16.10±5.30	15.84±5.95	7.59±1.21
HBs	19.03±1.43	20.55±2.63	4.64±0.39*
All cancers	16.94±4.69	17.02±5.46	6.85±1.99*

ΔCt values are expressed in mean ± standard deviation of triplicate reactions. FGF-16 and FGF-20, members of subfamily 9, and FGFR-3 IIIc, 1 IIIc and 3 IIIb, receptors for this subfamily, are excluded. Order of activities to FGFRs in FGF subfamily 9 is FGFR-3 IIIc > FGFR-2 IIIc > FGFR-1 IIIc and FGFR-3 IIIb >> FGFR-4. * indicates p-value < 0.05 in one-way ANOVA; HCCs, average of ΔCt values of HCC cell lines except those of non-expressing ones; HBs, average of ΔCt values of hepatoblastoma cell lines except those of non-expressing ones; All cancers, average of ΔCt values of all cell lines except those of non-expressing ones.

Expression of FGF subfamily 19

Even in same subfamily, members of this subfamily showed obvious contrast of expressions (table 7). FGF-19 was up-regulated in SNU-398, SNU-739, SNU-886 and Hep3B. But FGF-21 were down-regulated in the same cell lines except Hep3B, and HepG2 also showed decreased expression. 6 of 8 cell lines used in this study showed no expression of FGF-23 and HepG2, one of expressing ones, was significantly decreased.

Table 7. mRNA expressions in FGF subfamily 19

	FGF-19	FGF-21	FGF-23	FGFR-2 IIIc	FGFR-4
Normal	16.1±0.84	6.35±0.42	13.33±0.9	14.54±2.72	9.99±0.85
SNU-398	10.21±0.78*	13.8±1.32*	No expression	14.17±1.99	8.11±0.82
SNU-449	16.83±0.82	19.03±0.49*	No expression	23.79±1.15*	8.02±0.94
SNU-739	11.14±0.16*	15.17±0.53*	13.36±0.83	7.86±1.06*	7.99±1.80
SNU-761	7.81±0.13	14.95±0.51*	No expression	13.91±1.10	5.99±0.63
SNU-886	12.36±1.00*	18.3±0.35*	No expression	21.97±2.50*	9.13±0.29
Hep3B	9.23±0.20*	9.04±0.79	No expression	13.36±0.25	6.27±3.00*
HepG2	14.1±0.92	14.42±4.29*	18.36±2.23*	18.69±1.51	4.91±0.25*
PLC/PRF/5	14.99±0.67	15.12±0.43*	No expression	22.41±0.59*	4.36±0.63*
HCCs	11.26±3.14*	15.05±3.58*	ND	15.84±5.95	7.59±1.21
HBs	14.55±0.63	14.77±0.49*	ND	20.55±2.63	4.64±0.39*
All cancers	12.08±2.98*	14.98±3.21*	ND	17.02±5.46	6.85±1.99*

ΔCt values are shown with mean ± standard deviation of triplicate reactions. FGFR-1 IIIc and 3 IIIc, receptors for subfamily 19 are excluded. Activities of FGFRs for FGF subfamily 19 are equally weak in FGFR-1 IIIc, 2 IIIc, 3 IIIc and 4. * indicates p-value < 0.05 in one-way ANOVA; HCCs, average of ΔCt values of HCC cell lines except non-expressing; HBs, average of ΔCt values of hepatoblastoma cell lines except non-expressing; All cancers, average of ΔCt values of all cell lines except non-expressing; ND, average value is not determined because there were no PCR products in five HCC cell lines and one hepatoblastoma cell line.

Expression of FGF subfamily 11

FGF subfamily 11, which known as fibroblast growth factor homologous factors (FHFs),[37] have 4 members but no receptors identified yet. Significant over-expressions of FGF-11 (FHF-3) of SNU-398, SNU-739, HepG2 and PLC/PRF/5, FGF-12 (FHF-1) of SNU-449, Hep3B, PLC/PRF5, FGF-13 (FHF-2) of HepG2 and FGF-14 (FHF-4) of SNU-398, SNU-739, SNU-761, SNU-886 and Hep3B were observed. But down-regulation of FGF-14 of SNU-449 was also existed and many cells showed no expression of members in this family. FGF-12 expression was not detected in SNU-398 and HepG2. 6 cell lines including SNU-398, SNU-449, SNU-739, SNU-886, Hep3B and PLC/PRF/5 didn't express FGF-13. 2 hepatoblastoma cell lines, HepG2 and PLC/PRF/5, didn't show a sign of amplification of FGF-14 (table 8).

Table 8. mRNA expressions in FGF subfamily 11

	FGF-11	FGF-12	FGF-13	FGF-14
Normal	13.96±0.50	25.06±1.60	21.55±1.97	17.86±0.32
SNU-398	5.65±0.37*	No expression	No expression	13.41±0.89*
SNU-449	11.84±0.58	18.59±0.67*	No expression	22.10±0.95*
SNU-739	9.93±0.64*	23.06±0.22	No expression	14.47±1.32*
SNU-761	11.54±0.57*	23.06±2.25	17.10±1.17	12.76±0.5*
SNU-886	15.37±0.54	26.2±1.58	No expression	15.07±0.42*
Hep3B	12.08±0.95	20.26±0.48*	No expression	13.17±0.58*
HepG2	9.38±0.72*	No expression	12.80±1.96*	No expression
PLC/PRF/5	10.31±0.29*	15.67±1.77*	No expression	No expression
HCCs	11.07±3.19*	22.24±2.92	17.10±1.66	15.16±3.37
HBs	9.69±0.44*	15.67±1.77*	12.80±1.96*	No expression
All cancers	10.72±2.70*	21.14±3.70	14.95±2.76	15.1±3.37

ΔCt values are shown with mean ± standard deviation of triplicate reactions. There's no known about activities of FGF subfamily 11. * indicates p-value < 0.05 in one-way ANOVA; HCCs, average of ΔCt values of HCC cell lines except those of non-expressing ones; HBs, average of ΔCt values of hepatoblastoma cell lines except those of non-expressing ones; All cancers, average of ΔCt values of all cell lines except those of non-expressing ones.

Summary of expression profiles of FGFs and FGFRs

Compared with that in normal hepatocyte, expression of most FGFs and FGFRs in cancer cells are increased. However, FGF-6, 7, 21 and 23 were increased in normal hepatocyte or not expressed in hepatocellular carcinoma cell lines (table 9).

Table 9. Summary of Expression Profiles of FGFs and FGFRs

	Normal	HCCs (6)	HBs (2)	All cancers (8)
FGF-1	16.32±0.36	14.74±3.82	17.69±0.34	15.47±3.52
FGF-2	14.97±1.63	14.46±4.41	20.64±5.04	16.00±5.04
FGF-6	14.00±0.48	ND	17.30±1.25	ND
FGF-7	11.18±0.56	14.01±3.38	17.32±0.35*	14.95±3.26
FGF-9	17.03±3.54	16.10±5.30	19.03±1.43	16.94±4.69
FGF-10	18.18±0.37	ND	No expression	17.61±3.22
FGF-11	13.96±0.50	11.07±3.19*	9.69±0.44*	10.72±2.70*
FGF-12	25.06±1.60	22.24±2.92	15.67±1.77*	21.14±3.70
FGF-13	21.55±1.97	17.10±1.66	12.80±1.96*	14.95±2.76
FGF-14	17.86±0.32	15.16±3.37	No expression	15.16±3.37
FGF-16	No expression	No expression	No expression	No expression
FGF-17	17.01±1.24	15.14±2.84	15.63±0.23	15.17±2.53
FGF-18	16.77±0.18	13.85±3.88	14.84±0.91	14.10±3.26
FGF-19	16.10±0.84	11.26±3.14*	14.55±0.63	12.08±2.98*
FGF-21	6.35±0.42	15.05±3.58*	14.77±0.49*	14.98±3.21*
FGF-22	No expression	No expression	No expression	No expression
FGF-23	13.33±0.90	ND	ND	ND
FGFR-2 IIIb	14.75±0.81	14.00±8.74	19.16±2.52	17.19±5.37
FGFR-2 IIIc	14.54±2.72	15.84±5.95	20.55±2.63	17.02±5.46
FGFR-4	9.99±0.85	7.59±1.21	4.64±0.39*	6.85±1.99*
FGFR-5	10.51±0.20	7.74±1.28*	7.61±2.38	7.71±1.65*

ΔCt values are shown with mean ± standard deviation of triplicate reactions. The real-time RT-PCR for FGF-3, 4, 5, 8, and 20 was not performed because RT-PCR showed no products with normal hepatocyte. Among the 5 SNU cell lines FGF6 and FGF23 were not amplified by RT-PCR in 4 cell lines. * indicates p-value < 0.05 in one-way ANOVA; HCCs, average of ΔCt values of HCC cell lines except those of non-expressing ones; HBs, average of ΔCt values of hepatoblastoma cell lines except those of non-expressing ones; All cancers, average of ΔCt values of all cell lines except those of non-expressing ones. ND, average value is not determined because there were no PCR products in more than half of the HCC cell lines and hepatoblastoma cell line. ND also indicates mRNA expression is very low compared with that in normal hepatocyte.

IV. DISCUSSION

As a kind of medical therapy for cancer the inhibition of growth by blocking the signal transduction pathway is being tried. One group of the FDA approved widely prescribed drugs is composed of monoclonal antibodies for growth factor receptors or receptor tyrosine kinase (RTK) inhibitors. For colorectal cancers, bevacizumab (Avastin®) or cetuximab (Erbitux®) and panitumumab (Vectibix®) are designed to inhibit the growth signaling by blocking VEGF or EGFR, respectively. Trastuzumab, famous for their brand name Herceptin®, blocks growth of breast cancer by blocking Her2. Inhibitors of receptor tyrosine kinase like ZD6474 (Zactima®), GSK572016 (Lapatinib®) and Erlotinib (Tarceva®) are indicated to treat non-small cell lung cancer or breast cancer by inhibiting RTK of VEGF, EGFR and Erb2.

The choice of treatment for hepatocellular carcinoma (HCC), which can be expected complete cure, is surgical operation for primary cancer with no metastasis. However, the operability is dependent upon the size of tumor and general conditions of patients. As many cases are diagnosed after the cancer is progressed

to the later there used to be a big problem to try surgery for HCC. Less invasive treatments including percutaneous ethanol injection, arterial chemoembolization and radio frequency ablation are other strategies to treat HCC, but they are not definitive therapies so far. To secure survival benefit and enhanced quality of life in even advanced stages, medical treatment targeting cancer growth should be accentuated.

As the drugs mentioned above being progressed, drug development targeting HCC begins by profiling of growth factors and their receptors. This is the first study of expression profile targeting all known FGFs and FGFRs in HCC and hepatoblastoma cell lines compared with normal hepatocyte which enables establish the targets in further investigation like cell proliferation assay.

Among 22 FGFs, FGF-16 and 22 were not expressed in all target cDNAs including normal hepatocyte. FGF-3, 4, 5, 8, and 20 were not performed real-time PCR because of no expression in normal hepatocyte. But these 5 FGFs were expressed in several cell lines, showing definitive different of ‘all-or-none’, not ‘much more’ compared with normal hepatocyte. These ligands will be the first

targets of cell proliferation study using blocking their signaling by neutralizing antibodies and/or siRNAs.

Asada et al.,[34] reported FGF-1 was only expressed in hepatoma-derived cell lines and FGF-7 was not both in normal hepatocyte and hepatomoma-derived cell lines. However, I could get the PCR product, and tried DNA sequencing to confirm the product. DNA sequencing showed the PCR product of FGF-1 and FGF-7 are true ones. I tried RT-PCR once again with the primers he used, but I couldn't get any PCR products. Therefore, I determined Asada's primers were not good and FGF-1 and FGF-7 are expressed both in normal hepatocyte and HCC cell lines.

FGF subfamily 11, including FGF-11, 12, 13 and 14, is no known about activities to FGFRs. But, expression level of FGF subfamily 11 was quite different from normal hepatocyte. Further studies about identifying specific receptors for FGF subfamily 11 should be needed.

This study initially aimed to complete the quantitative expression profiling of all FGFs and FGFRs, but isotype IIIb and IIIc of FGFR-1 and 3 were impossible to perform quantitative comparison because of the low specificity of the primers

and existences of a variety of DNA fragments after splicing. Primers used in this study are designed using exon 8 and exon 9 sequences for isotype IIIb and IIIc, respectively. RT-PCR using other primers located in exon 6 and exon 10 showed unexpected product which has a product of exon 8 and 9 simultaneously. This can be explained in two ways. One is that there's another isotype having both exon 8 and exon 9 in Ig-like domain III. For FGFR-1, I could find this isotype cloned, but not for FGFR-3. The other is that primary mRNA transcript before alternative splicing was detected by RT-PCR. Primers used in reverse transcription were a mixture of oligo-dTs and random hexamers, thus it was possible for cDNA synthesis to be performed using primary mRNA transcript. To distinguish these, as primers located in only exon 8 or exon 9 can be imperfect, specific primers including boundary between exon 8 and exon 10 for isotype IIIb, exon 7 and exon 9 for isotype IIIc, which don't exist in primary transcripts has to be used for RT-PCR. I tried to design these isotype-specific primers, but I couldn't make specific primers because there are high G/C content at boundary regions. Besides conventional RT-PCR of isotypes and real-time PCR with no distinction of isotypes,

there's no previous reports performed real-time PCR targeting isotype IIIb and IIIc. The authors might have experienced the same situation with me.

Although many FGFs and their receptors are increased in cancer cells, I could find some exceptions. FGF-6, 21 and 23 were increased in normal hepatocyte or not expressed in cancer cells and these are consistent with previous study[33]. Further investigations will have to be proceeded to confirm what this phenomenon means. If we can interpret the effect and function of the FGF-6, 7, 21, and 23 in HCC pathogenesis, it'll be very helpful to set up strategies for solution of HCC.

Taken together, this study could establish expression profiles of FGFs and FGFRs in HCCs. In most FGFs and FGFRs, cancer cells showed up-regulated expressions, but FGF-6, 7, 21 and 23 might have anti-cancer properties in normal hepatocyte. FGFs or FGFRs which show significant differences will be used as targets for novel pathway of cancer growth inhibition.

In summary, I completed the of expression profiles of all known FGFs and FGFRs in HCC cell lines compared with those in normal hepatocyte. To discover

the new therapeutic target for HCC future study is needed to develop the method for blocking the expression of FGF-3, 4, 5, 8, and 20 and to know the role of FGF-6, 7, 21, and 23 for HCC pathogenesis.

REFERENCES

1. Powers, C.J., S.W. McLeskey, and A, Wellstein. *Fibroblast growth factors, their receptors and signaling.* Endocr Relat Cancer, 2000. **7**(3): p. 165-97.
2. Ornitz, D.M., et al. *Receptor specificity of the fibroblast growth factor family.* J Biol Chem, 1996. **271**(25): p. 15292-7.
3. Givol, D. and A, Yayon. *Complexity of FGF receptors: genetic basis for structural diversity and functional specificity.* Faseb J, 1992. **6**(15): p. 3362-9.
4. Jaye, M., J. Schlessinger, and C.A., Dionne. *Fibroblast growth factor receptor tyrosine kinases: molecular analysis and signal transduction.* Biochim Biophys Acta, 1992. **1135**(2): p. 185-99.
5. Ornitz, D.M. and N, Itoh. *Fibroblast growth factors.* Genome Biol, 2001. **2**(3): p. REVIEWS3005.
6. Zhang, X., et al. *Receptor specificity of the fibroblast growth factor family. The complete mammalian FGF family.* J Biol Chem, 2006. **281**(23): p. 15694-700.
7. Folkman, J. and Y, Shing. *Angiogenesis.* J Biol Chem, 1992. **267**(16): p. 10931-4.
8. McNeil, P.L., et al. *Growth factors are released by mechanically wounded endothelial cells.* J Cell Biol, 1989. **109**(2): p. 811-22.
9. Chai, N., et al. *FGF is an essential regulator of the fifth cell division in preimplantation mouse embryos.* Dev Biol, 1998. **198**(1): p. 105-15.
10. Tebben, P.J., et al. *Elevated fibroblast growth factor 23 in women with malignant ovarian tumors.* Mayo Clin Proc, 2005. **80**(6): p. 745-51.
11. Nezu, M., et al. *Expression of the fetal-oncogenic fibroblast growth factor-8/17/18 subfamily in human hematopoietic tumors.* Biochem Biophys Res Commun, 2005. **335**(3): p. 843-9.
12. Heer, R., et al. *Fibroblast growth factor 17 is over-expressed in human prostate cancer.* J Pathol, 2004. **204**(5): p. 578-86.
13. Yoshimura, N., et al. *The expression and localization of fibroblast growth factor-1 (FGF-1) and FGF receptor-1 (FGFR-1) in human breast cancer.*

Clin Immunol Immunopathol, 1998. **89**(1): p. 28-34.

14. Onose, H., et al. *Overexpression of fibroblast growth factor receptor 3 in a human thyroid carcinoma cell line results in overgrowth of the confluent cultures.* Eur J Endocrinol, 1999. **140**(2): p. 169-73.
15. Cappellen, D., et al. *Frequent activating mutations of FGFR3 in human bladder and cervix carcinomas.* Nat Genet, 1999. **23**(1): p. 18-20.
16. Kimura, T., et al. *The incidence of thanatophoric dysplasia mutations in FGFR3 gene is higher in low-grade or superficial bladder carcinomas.* Cancer, 2001. **92**(10): p. 2555-61.
17. Sibley, K., P. Stern, and M.A., Knowles. *Frequency of fibroblast growth factor receptor 3 mutations in sporadic tumours.* Oncogene, 2001. **20**(32): p. 4416-8.
18. van Rhijn, B.W., et al. *The fibroblast growth factor receptor 3 (FGFR3) mutation is a strong indicator of superficial bladder cancer with low recurrence rate.* Cancer Res, 2001. **61**(4): p. 1265-8.
19. Wu, R., et al. *Somatic mutations of fibroblast growth factor receptor 3 (FGFR3) are uncommon in carcinomas of the uterine cervix.* Oncogene, 2000. **19**(48): p. 5543-6.
20. Jang, J.H., K.H. Shin, and J.G., Park. *Mutations in fibroblast growth factor receptor 2 and fibroblast growth factor receptor 3 genes associated with human gastric and colorectal cancers.* Cancer Res, 2001. **61**(9): p. 3541-3.
21. Jang, J.H., et al. *Novel transcripts of fibroblast growth factor receptor 3 reveal aberrant splicing and activation of cryptic splice sequences in colorectal cancer.* Cancer Res, 2000. **60**(15): p. 4049-52.
22. Yagasaki, F., et al. *Fusion of ETV6 to fibroblast growth factor receptor 3 in peripheral T-cell lymphoma with a t(4;12)(p16;p13) chromosomal translocation.* Cancer Res, 2001. **61**(23): p. 8371-4.
23. Chesi, M., et al. *Frequent translocation t(4;14)(p16.3;q32.3) in multiple myeloma is associated with increased expression and activating mutations of fibroblast growth factor receptor 3.* Nat Genet, 1997. **16**(3): p. 260-4.
24. Streit, S., et al., *Involvement of the FGFR4 Arg388 allele in head and neck*

squamous cell carcinoma. Int J Cancer, 2004. **111**(2): p. 213-7.

25. Kwabi-Addo, B., et al. *Alternative splicing of fibroblast growth factor receptors in human prostate cancer.* Prostate, 2001. **46**(2): p. 163-72.
26. El-Serag, H.B. and K.L., Rudolph. *Hepatocellular carcinoma: epidemiology and molecular carcinogenesis.* Gastroenterology, 2007. **132**(7): p. 2557-76.
27. Parkin, D.M. *Global cancer statistics in the year 2000.* Lancet Oncol, 2001. **2**(9): p. 533-43.
28. Shin HR, A.Y., Bae JM, Shin MH, Lee DH, Lee CW, Ohrr HC, Ahn DH, Ferlay J, Parkin DM, Oh DK, Park JG. *Cancer Incidence in Korea.* Cancer Res Treat, 2002. **34**(6): p. 405-408.
29. Breuhahn, K., T. Longerich, and P, Schirmacher. *Dysregulation of growth factor signaling in human hepatocellular carcinoma.* Oncogene, 2006. **25**(27): p. 3787-800.
30. Mise, M., et al. *Clinical significance of vascular endothelial growth factor and basic fibroblast growth factor gene expression in liver tumor.* Hepatology, 1996. **23**(3): p. 455-64.
31. Hu, L., et al. *Up-regulation of fibroblast growth factor 3 is associated with tumor metastasis and recurrence in human hepatocellular carcinoma.* Cancer Lett, 2007. **252**(1): p. 36-42.
32. Nishimura, T., et al. *Identification of a novel FGF, FGF-21, preferentially expressed in the liver.* Biochim Biophys Acta, 2000. **1492**(1): p. 203-6.
33. Huang, X., et al. *Forced expression of hepatocyte-specific fibroblast growth factor 21 delays initiation of chemically induced hepatocarcinogenesis.* Mol Carcinog, 2006. **45**(12): p. 934-42.
34. Asada, N., et al. *Expression of fibroblast growth factor receptor genes in human hepatoma-derived cell lines.* In Vitro Cell Dev Biol Anim, 2003. **39**(7): p. 321-8.
35. Qiu, W.H., et al. *Over-expression of fibroblast growth factor receptor 3 in human hepatocellular carcinoma.* World J Gastroenterol, 2005. **11**(34): p. 5266-72.
36. Shao, R.X., et al. *Acyclic retinoid inhibits human hepatoma cell growth by*

suppressing fibroblast growth factor-mediated signaling pathways. Gastroenterology, 2005. **128**(1): p. 86-95.

37. Goldfarb, M. *Fibroblast growth factor homologous factors: evolution, structure, and function.* Cytokine Growth Factor Rev, 2005. **16**(2): p. 215-20.

국문요약

인간 간세포암종 세포주에서 섬유모세포 성장인자와 섬유모세포 성장인자 수용체의 발현

<지도 예 병 일 교수>

연세대학교 대학원 의학과

김 재 우

섬유모세포 성장인자는 암세포 성장에 있어서 혈관 신생, 상처 치유 및 발달에 중요한 역할을 한다. 즉, 섬유모세포 성장인자가 세포 주기 진행을 촉진하고 세포 자멸사를 억제하는 신호체계를 활성화 시킬 것이다. 이번 연구는 인간의 간세포암종 세포주에서 섬유모세포 성장인자가 암세포 성장을 일으키는 주된 신호 중 하나라는 가설로 진행되었다. 정상 간세포와 간암세포를 비교하는 최초의 정량적인 발현 분석을 실시하기 위하여 본 연구자는 지금까지 알려진 모든 섬유모세포 성장인자와 그 수용체를 대상으로 한 실시간 역전사-중합효소 연쇄반응을 시도하였

다.

6개의 간세포암종 세포주와 (SNU-398, SNU-449, SNU-761, SNU-739, SNU-886, Hep3B)와 2개의 간모세포종 세포 (HepG2, PLC/PRF/5) 를 배양하여 역전사 효소를 이용하여 cDNA를 합성했다. 정상군으로는, 정상 간세포의 cDNA를 구입하여 사용하였다. 22개의 섬유모세포 성장인자 (1번부터 23번까지, 15번은 존재하지 않음)와 8개의 섬유모세포 성장인자 수용체 (1b 에서 5까지) 를 대상으로 한 선행 검색 결과에서, 섬유모세포 성장인자 16번과 22번은 모든 cDNA 에서 발현되지 않았으며, 정상 간세포는 성장인자 3번, 4번, 5번, 8번, 20번을 발현하지 않는 것으로 나타났다. 선택적인 접합을 통해 IIIb 와 IIIc 의 아형을 갖고 있는 수용체 1번과 3번은 exon 부위를 대상으로 하는 primer 를 이용으로 하는 정량 분석에서 제외되었는데, 이러한 primer 들은 일차로 만들어진 전사체와 최종적으로 성숙된 전사체를 구별할 수 없기 때문이었다. 성숙된 전사체에만 존재하는 부위를 대상으로 하는 아형 특이적인 primer 를 제작하려 했지만, 그 부위의 G/C 비율이 높아서 실패하였다.

정상 간세포의 경우와 비교해볼 때, 대부분의 섬유모세포 성장인자와

수용체가 암세포주들에서 증가되었다. 성장인자들이 갖는 수용체에 대한 특이적인 결합 능력에 근거한 비교에서도, 대부분의 subfamily 에서 성장인자와 수용체 모두가 증가했음을 확인했다. 그러나, 성장인자 6번, 7번, 21번, 23번 같은 경우는 정상에서 더 증가했거나 아예 암세포에서 발현되지 않았다.

본 연구는 지금까지 알려진 모든 섬유모세포 성장인자와 수용체를 대상으로 정상 간세포와 간세포암종 세포주에서의 발현 양상을 모두 확인한 첫번째 연구이며, 이상의 결과를 토대로 암세포주보다 정상 간세포에서 발현이 잘 되는 6번, 7번, 21번, 23번 섬유아세포 성장인자가 정상세포주에서 발현되도록 하는 방법과 정상 간세포에서는 발현되지 않으나 암세포주에서 발현이 잘 되는 3번, 4번, 5번, 8번, 20번 섬유아세포 성장인자의 발현을 막을 수 있는 방법을 찾는 것이 간세포암 해결을 위해 진행되어야 할 후속연구로 판단된다.

핵심되는 말: 섬유모세포 성장인자, 섬유모세포 성장인자 수용체, 간세포암종 세포주, 유전자 발현