Hepatocellular carcinoma cells cause different responses in expressions of cancer-promoting genes in different cancer-associated fibroblasts

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Abstract Cancer-associated fibroblast (CAF) is one of the most crucial components of the tumor microenvironment to promote the invasiveness of cancer cells. The interactions between cancer cells and CAFs are bidirectional. Our recent study showed that up-regulations of chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-C motif) ligand 26 (CCL26), interleukin 6 (IL6), and lysyl oxidase-like 2 (LOXL2) genes in cancer cells were parts of the common effects of CAFs on hepatocellular carcinoma (HCC) cells to promote proliferation, migration and invasion of cancer cells. However, the subject of how HCC cells to influence the gene expressions of CAFs still needs to be clarified. The purpose of this study was to investigate this issue. Two human HCC (HCC24/KMUH, HCC38/KMUH) and two human CAF cell lines (F26/KMUH, F28/KMUH) were studied. Influence of HCC38/KMUH cancer cells on differential expressions of genes in F28/KMUH CAFs was detected by microarray to select target genes for further analysis. Both HCC cell lines increased proliferation (all p < 0.005) and migration (all p < 0.0001) of two CAF cell lines. HCC24/KMUH cancer cells had stronger ability to promote migration of F26/KMUH CAFs than HCC38/KMUH cancer cells did (p < 0.0001). Eleven up-regulated cancer-promoting genes, including apelin (APLN), CCL2, CCL26, fibroblast growth factor 1 (FGF1), fibroblast growth factor 2 (FGF2), IL6, mucin 1 (MUC1), LOXL2, platelet-derived growth factor alpha polypeptide (PDGFA), phosphoglycerate kinase 1 (PGK1), and vascular endothelial growth factor A (VEGFA) detected by microarray showed good correlation with results of quantitative reverse transcriptase-polymerase chain

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Introduction

Invasion and metastasis of malignancy are determined by the characteristics of cancer cells and the interactions between the cancer cells and the tumor microenvironment. Cancer-associated fibroblast (CAF) is one of the most crucial components of the tumor microenvironment to promote the growth and invasion of cancer cells [1–5]. The interactions between cancer cells and CAFs are bidirectional and are initiated from secretion of soluble factors from cancer cells to enhance the ability of CAFs to secrete a variety of tumor-promoting factors [3]. Hepatocellular carcinoma (HCC) is the most common primary liver cancer and ranks globally as the third to fourth leading cause of cancer-related death [6,7]. Understanding the HCC–CAF interactions may help us to target the tumor microenvironment and thus to improve the prognosis of HCC [4]. Our recent study showed that up-regulations of chemokine (C-C motif) ligand 2 (CCL2), C-C motif ligand 26 (CCL26), inter-leukin 6 (IL6), and lysyl oxidase-like 2 (LOXL2) genes in cancer cells were parts of the common effects of CAFs on HCC cells to promote proliferation, migration, and invasion of cancer cells [8]. Although lysophosphatidic acid secreted from HCC cells had been demonstrated to accelerate HCC progression by recruiting peritumoral tissue fibroblasts and promoting their transdifferentiation into CAFs [5], the subject of how HCC cells to influence the gene expressions of CAFs still need to be clarified. Moreover, the characteristics of CAFs have significant individual/intrinsic differences [9]. This suggests that the responses of different CAFs to different HCC cells stimulations may be variable. The purpose of this study was to investigate whether different human CAF cell lines had different responses to different human HCC cell lines. All gene names are according to the official symbols from the HUGO Gene Nomenclature Committee.

Methods

Cell lines

Two CAF cell lines (F26/KMUH, F28/KMUH) newly established from patients with HCC in our institution and two human HCC cell lines (HCC24/KMUH, HCC38/KMUH) used in our previous studies [10–12] were investigated. CAF cell lines were verified by positive stain for fibroblast activation protein (ENZO Life Sciences International, Inc., Butler Pike, Plymouth Meeting, PA, USA), α-smooth muscle actin (Sigma-Aldrich, St. Louis, MO, USA) and chemokine (C-X-C motif) ligand 12 (CXCL12; R & D Systems, Inc., Minneapolis, MN, USA). Both CAF cell lines were also verified to have capacities to penetrate the Matrigel. All procedures to establish these cell lines were approved by the Institutional Review Board of our hospital and patients were given informed consent. All cultures were maintained at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. The serum-containing culture medium consisted of 10% fetal bovine serum, 90% DME/HIGH glucose, supplemented with 20 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (HyClone, Logan, UT, USA).

Influence of HCC cells on proliferation of CAFs

CAFs from each cell line were seeded in three 24-well cell culture insert companion plates (18 wells/plate, 1 × 10^5 cells/well). Cancer cells from each cell line were seeded in 36 culture inserts (2 × 10^5 cells/insert) with transparent polyethylene terephthalate membrane (pore size: 0.4 μm, BD Falcon, Cell Culture Inserts, BD Biosciences, Mississauga, Ontario, Canada) using two new 24-well companion plates. Both CAFs and cancer cells were incubated with serum-containing medium for 24 hours, then all medium was replaced with serum-free medium and the inserts were transferred to CAFs containing wells. The cells were incubated for another 48 hours. Then the inserts were removed and CAFs in each well were analysis. The premixed WST-1 cell proliferation reagent (Clontech Laboratories, Inc., A Takara Bio Company, Mountain View, CA, USA) was applied. The experimental procedures were carried out following the manufacturer’s protocols. The cells were incubated with reagent for 4 hours at 37°C in a humidified atmosphere of 5% CO2 and 95% air. After that, 0.1 mL suspension in each well was transferred to 96-well plate for automated microplate reader (MRX, Dynex Technologies, Inc., Chantilly, VA, USA) analysis. The absorbance was measured at 450 nm wavelength (reference wavelength, 630 nm).
Influence of HCC cells on migration of CAFs

For each CAF cell line, cancer cells from each cell line seeded in eight wells (1 × 10^5 cells/well) of 24-well cell culture insert companion plate were incubated with serum-containing medium for 24 hours. Then the medium of each well was replaced with serum-containing medium again and the CAFs containing insert (3000 cells/insert, with transparent polyethylene terephthalate membrane, pore size: 8 μm, BD Falcon Cell Culture Inserts, BD Biosciences) with serum-free medium was transferred to cancer cells containing wells. An additional eight CAFs containing inserts with serum-free medium (3000 cells/insert) were transferred to wells with serum-containing medium but without cancer cells as control group. The cells were incubated for further 18 hours. Then the cells inside the insert were wiped with cotton-swabs and removed. The cells that migrated through the pores and adhered onto the outer side of the insert were stained by the technique of Liu-stain [13]. Whole migrated cells in each insert were counted at 100× magnification.

Influence of HCC cells on differential expressions of genes in CAFs

Microarray experiment

CAFs from each cell line were seeded in two six-well cell culture insert companion plates (2 × 10^5 cells/well) and cancer cells from each cell line were seeded in 6 culture inserts (2 × 10^5 cells/insert, with transparent polyethylene terephthalate membrane, pore size: 0.4 μm, BD FalconCell Culture Inserts, BD Biosciences) using a new six-well companion plate. Both cancer cells and CAFs were incubated with serum-containing medium for 24 hours, then all medium was replaced with serum-free medium and the inserts were transferred to CAFs containing wells. The wells without insert were used as control group. The cells were incubated for further 48 hours. Then total RNA in each well were extracted by RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). Purified RNA was quantified by OD260 nm by a ND-1000 spectrophotometer (Nanodrop Technology, Wilmington, DE, USA) and qualified by Bioanlyser 2100 (Agilent Technology, Santa Clara, CA, USA). To select candidate genes for further quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis, influence of HCC38/KMUH cells on differential expressions of genes in F28/KMUH cells was investigated by microarray (Agilent SurePrint G3 Human GE 8 × 60 k, Agilent Technologies, Santa Clara, CA, USA). Microarray experimental procedures were carried out following the manufacturer’s protocols and were the same as in our previous study [10]. Control group was labeled with Cy3-CTP and the experimental group was labeled with Cy5-CTP (CyDye, PerkinElmer, Waltham, MA, USA) during the in vitro transcription process. The A value representing the average signal of two channels (Cy5 and Cy3) of the spot is calculated by the formula \( \log_{2} \frac{\text{Cy5 Background Signal} + \log_{2} \text{Cy3 Background Signal}}{2} \). The Cy5 (or Cy3) Background Signal was calculated by the formula: mean signal intensity of Cy5 (or Cy3) — mean background light intensity of Cy5 (or Cy3). The Cy5 (or Cy3) BgSubSignal indicated the mean background intensity of Cy5 (or Cy3) channel. The M-value was calculated by the formula: \( \log_{2} \frac{\text{Cy5 Background Signal/Cy3 Background Signal}}{2} \). If one gene was detected by multiple probes, the average A and M values were calculated for analysis. Genes with both Cy3 BgSubSignal/Cy3 BgUsed and Cy5 BgSubSignal/Cy5 BgUsed values less than one or the average A value < 6 were excluded in the selection of differential expressions of genes because of the high probability of false results in these groups. Selection of differentially expressed genes was based on the absolute M value (log₂ gene expression fold change) \( \geq 1 \).

Quantitative RT-PCR experiment

For quantitative RT-PCR study, specific oligonucleotide primer pairs were selected from Roche Universal ProbeLibrary, Roche Diagnostics Ltd., Taipei, Taiwan and used for real-time PCR. The procedures for real-time PCR reactions were the same as in our previous studies [10–12]. At each real-time PCR run, the data were automatically analyzed by the system and an amplification plot was generated for each cDNA sample. From each of these plots, the LightCycler3 Data analysis software automatically calculates CP value (crossing point, the turning point corresponds to the first maximum of the second derivative curve), which implies the beginning of exponential amplification. The fold expression or repression of the target gene relative to reference gene in each sample was calculated by the formula: gene expression fold change = \( 2^{-\Delta \Delta CP} \), where \( \Delta \Delta CP = CP_{\text{target gene}} - CP_{\text{reference gene}} \), and \( \Delta \Delta CP = CP_{\text{test sample}} - CP_{\text{control sample}} \). The housekeeping gene TATA box binding protein (TBP) was used as reference gene. Eleven genes were selected for quantitative RT-PCR study. The PCR primers used were 5′-AATCCAGCCCACTTACAGGTTTTC-3′ sense primer and 5′-TAGCAAGAAGACCCCAAAGG-3′ antisense primer for apelin (APLN), 5′-AGTCGCTCGCCCTTCT-3′ sense primer and 5′-GTGACTGGCCCAGTATTG-3′ anti-sense primer primer for CCL2, 5′-CTGACCTGTGTTGGCGAAGC-3′ sense primer and 5′-TGAGTTGTTGATCAGACCTTC-3′ anti-sense primer for CCL2, 5′-CAGCAGGACACATATAAGGACAG-3′ sense primer and 5′-GGTTAGTTGGAGGTCAGGTGAGG-3′ anti-sense primer for fibroblast growth factor 1 (FGF1), 5′-TACAGCAGCAGCTTACAACTC-3′ sense primer and 5′-TTTGGCAACACAGCA-3′antisense primer for fibroblast growth factor 2 (FGF2), 5′-TTTACACAAACCGGCTTTC-3′ sense primer and 5′-AGCAGCAACACGGAGG-3′antisense primer for IL6, 5′-GACAGCAGCTTCTTACAAAC-3′sense primer and 5′-AGAACCTTGTGGGAGTGAAGG-3′antisense primer for mucin 1 (MUC1), 5′-AGTGACTCTCCCTTTGAC-3′sense primer and 5′-GACCTGTCCTTATAGCAGCCTTG-3′antisense primer for PDGFRA, 5′-TGGTAAAGGAGGAGGCTTCTTCTC-3′antisense primer for platelet-derived growth factor alpha polypeptide (PDGFα), 5′-GCCGCAACACTCACTCGCTCTCTACAAATCC-3′antisense primer for phosphoglycerate kinase 1 (PGK1), 5′-GGTTAGTGGACCTTGCTCTTCTTCTCTACTC-3′antisense primer for vascular endothelial growth factor A (VEGFα), and 5′-CAATTAGTATGTTACCGCAGG-3′sense primer and 5′-TTCTGCTCTGATTTAGCACA-3′antisense primer for vascular endothelial growth factor A (VEGFα), and 5′-CAATTAGTATGTTACCGCAGG-3′sense primer and 5′-TTCTGCTCTGATTTAGCACA-3′antisense primer for vascular endothelial growth factor A (VEGFα), and 5′-CAATTAGTATGTTACCGCAGG-3′sense primer and 5′-TTCTGCTCTGATTTAGCACA-3′antisense primer for vascular endothelial growth factor A (VEGFα), and 5′-CAATTAGTATGTTACCGCAGG-3′sense primer and 5′-TTCTGCTCTGATTTAGCACA-3′antisense primer for vascular endothelial growth factor A (VEGFα), and 5′-CAATTAGTATGTTACCGCAGG-3′sense primer and 5′-TTCTGCTCTGATTTAGCACA-3′antisense primer for vascular endothelial growth factor A (VEGFα), and 5′-CAATTAGTATGTTACCGCAGG-3′sense primer and 5′-TTCTGCTCTGATTTAGCACA-3′antisense primer for vascular endothelial growth factor A (VEGFα), and 5′-CAATTAGTATGTTACCGCAGG-3′sense primer and 5′-TTCTGCTCTGATTTAGCACA-3′antisense primer for vascular endothelial growth factor A (VEGFα), and 5′-CAATTAGTATGTTACCGCAGG-3′sense primer and 5′-TTCTGCTCTGATTTAGCACA-3′antisense primer for vascular endothelial growth factor A (VEGFα), and 5′-CAATTAGTATGTTACCGCAGG-3′sense primer and 5′-TTCTGCTCTGATTTAGCACA-3′antisense primer for vascular endothelial growth factor A (VEGFα), and 5′-CAATTAGTATGTTACCGCAGG-3′sense primer and 5′-TTCTGCTCTGATTTAGCACA-3′antisense primer for...
Statistical analysis

The two-tailed unpaired t test was used to analyze the significant difference between two means. The statistical significance was defined as $p < 0.05$.

Results

Proliferative and migratory experiments

Both HCC cell lines significantly increased proliferative (all $p < 0.005$; Fig. 1) and migratory abilities (all $p < 0.0001$; Fig. 2) of both CAF cell lines. There was no significant difference between different HCC cell lines to increase the proliferation of CAFs (all $p > 0.3$). HCC24/KMUH cancer cells had stronger ability to promote migration of F26/KMUH CAFs than HCC38/KMUH cancer cells did ($p < 0.0001$).

Microarray and quantitative RT-PCR experiments

HCC38/KMUH cancer cells up-regulated 232 genes and down-regulated 356 genes in F28/KMUH CAFs. Among these gene, three chemokine genes (CCL2, CCL26, CXCL1) were up-regulated. Eleven up-regulated genes (APLN, CCL2, CCL26, FGF1, FGF2, IL6, MUC1, LOXL2, PDGFA, PGK1, VEGFA) favored proliferation, migration, invasion, or angiogenesis of HCC were selected for quantitative RT-PCR analysis. There were good correlations between results from quantitative RT-PCR and microarray. Among these tested genes (Table1), HCC24/KMUH cancer cells had same tendency of effects as HCC38/KMUH cancer cells did on differential expressions of genes in F28/KMUH CAFs. However, the responses of F26/KMUH CAFs to both HCC cell lines were not consistent with F28/KMUH CAFs and were variable. Only PGK1 gene in F26/KMUH CAFs was consistently up-regulated caused by both HCC cell lines as in F28/KMUH CAFs. On the contrary, PDGFA gene in F26/KMUH CAFs was consistently down-regulated, which was caused by both HCC cell lines. For the other tested genes in F26/KMUH CAFs, HCC38/KMUH cancer cells up-regulated APLN, LOXL2, and VEGFA genes and down-regulated FGF1 and MUC1 genes, and HCC24/KMUH cancer cells up-regulated FGF2 gene and down-regulated CCL2 gene.

Discussion

The present study showed that HCC cells could promote proliferation and migration of CAFs. Since the characteristics of CAFs have significant individual/intrinsic differences [9], our results from migratory experiment and expressions of genes also showed that the effects of HCC cells on CAFs were influenced by the characteristics of CAFs. These results suggested that the mechanisms for HCC cells to promote proliferation and migration of CAFs were complex. Nevertheless, consistent up-regulation of PGK1 gene in both CAF cell lines may be one of the common
mechanisms for HCC cells to promote proliferation of CAFs. The protein encoded by \textit{PGK1} gene is a glycolytic enzyme that catalyzes the conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate that forms part of the glycolytic pathway. CAFs overexpression of \textit{PGK1} gene has been shown to have high proliferative index [14]. Besides \textit{PGK1} gene, concomitant up-regulation of \textit{FGF1}, \textit{FGF2}, \textit{IL6}, \textit{MUC1}, and \textit{PGDFA} genes in F28/KMUH CAFs may also be the explanations for HCC cells to promote proliferation of F28/KMUH CAFs and thus promote cancer progression. The proteins encoded by \textit{FGF1} and \textit{FGF2} genes are members of the fibroblast growth factor family. Up-regulation of \textit{FGF1} can inhibit p53-dependent apoptosis and cell cycle arrest via an intracellular pathway to promote cell survival and proliferation [15]. Overexpression of \textit{FGF1} in CAFs has been demonstrated to promote migration and invasion in colorectal cancer cells through \textit{FGF1}/\textit{FGFR-3} signaling [16]. \textit{FGF2} plays an important role in proliferation, differentiation, and survival of cells of almost all organ systems [16], and has capacity to promote proliferation of cancer cells [17]. \textit{FGF1} and \textit{FGF2} also can induce angiogenesis [17–20]. \textit{IL6} can promote survival and proliferation of cells through activating the STAT3 pathway [21–23]. \textit{IL6} can also stimulate circulating blood-derived endothelial progenitor cell angiogenesis [24]. Moreover, CAFs can promote tumor growth through the paracrine production of secreted \textit{IL6} [25]. The protein encoded by \textit{MUC1} gene is a transmembrane mucin glycoprotein that has been demonstrated to promote cell survival through STAT3 activation [26], and invasiveness and metastasis through induction of epithelial to mesenchymal transition [27]. The protein encoded by \textit{PGDFA} gene is a member of the platelet-derived growth factor family, which is a significant mediator in the proliferation of CAFs [28].

Five genes (\textit{APLN}, \textit{CCL2}, \textit{CCL26}, \textit{LOXL2}, \textit{VEGFA}) favored HCC angiogenesis; invasion and metastasis were also consistently up-regulated in F28/KMUH CAFs under the stimulation of both HCC cell lines. Up-regulation of \textit{APLN} [29–31] and \textit{VEGFA} [32,33] genes can promote angiogenesis. The protein encoded by \textit{LOXL2} gene is a member of the lysyl oxidase family that functions as an amine oxidase for formation of lysine-derived cross-links found in collagen and elastin, and can induce epithelial to mesenchymal transition [34,35]. Overexpression of \textit{LOXL2} has been shown to promote tumor cell invasion [35–38]. Chemokines have been proposed as key players in the cross-talk interactions between cancer cells and CAFs [3]. The soluble factors released from cancer cells enhance the ability of the CAFs to secrete a variety of tumor-promoting chemokines such as \textit{CXCL12}, \textit{CCL2}, \textit{CCL5}, \textit{CCL7}, \textit{CXCL8}, and \textit{CXCL14}. These chemokines then act back on the cancer cells to promote their proliferative, migratory, and invasive properties [3]. The present study showed that \textit{CCL2} and \textit{CCL26} genes were consistently up-regulated only in F28/KMUH CAFs under the stimulations from both HCC cell lines. \textit{CCL2} has been shown to promote adhesion, migration and invasion of prostate cancer cells partially through its differential regulation of protein kinase C and \textit{MMP 9} signaling [39]. \textit{CCL2} may also promote progression of human esophageal and gastric carcinoma through its role in angiogenesis via macrophage recruitment and activation [40,41]. The product of \textit{CCL26} gene displays chemotactic activity for eosinophils, which contain preformed vascular endothelial growth factor in their secretory granules. Recruitment of eosinophils to tumor sites can promote angiogenesis [42].

For F26/KMUH CAFs, all tested genes except \textit{PGK1} gene were not consistently up-regulated caused by both HCC cell lines. However, HCC cells did promote proliferation and migration of F26/KMUH CAFs. This suggests that other mechanisms, which need to be further investigated, are involved in the interactions between F26/KMUH CAFs and HCC cells.

In conclusion, HCC cells can promote proliferation and migration of CAFs. However, the impact of HCC cells on differential expressions of cancer-promoting genes in CAFs is influenced by the characteristics of CAFs. This implies that blocking single or several particular cancer-promoting genes could reduce the potential of the CAFs to promote tumor growth.

\begin{table}[h]
\centering
\begin{tabular}{lcccccc}
\hline
Gene name & HCC24/KMUH influenced by & HCC38/KMUH influenced by & HCC24/KMUH influenced by & HCC38/KMUH influenced by \\
& F26/KMUH & F28/KMUH & F26/KMUH & F28/KMUH \\
\hline
\textit{APLN} & 1.20 & 9.76 & 10.31 & 9.54 \\
\textit{CCL2} & 0.54 & 0.81 & 1.53 & 4.14 \\
\textit{CCL26} & 1.00 & 0.95 & 4.21 & 4.51 \\
\textit{FGF1} & 0.75 & 0.54 & 1.73 & 1.24 \\
\textit{FGF2} & 1.58 & 1.19 & 2.29 & 2.21 \\
\textit{IL6} & 1.25 & 1.2 & 5.13 & 1.49 \\
\textit{MUC1} & 1.02 & 0.57 & 4.21 & 2.70 \\
\textit{LOXL2} & 1.12 & 1.78 & 3.71 & 2.89 \\
\textit{PGDFA} & 0.51 & 0.08 & 4.55 & 1.48 \\
\textit{PGK1} & 1.58 & 3.47 & 3.54 & 2.51 \\
\textit{VEGFA} & 1.04 & 2.31 & 3.15 & 2.79 \\
\hline
\end{tabular}
\caption{Influence of hepatocellular carcinoma cells on gene expression fold change of cancer-associated fibroblasts.}
\end{table}
genes in CAFs is unable to become a common stratagem for the treatment of HCC.

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References


