Original article

Cancer-associated fibroblasts up-regulate *CCL2*, *CCL26*, *IL6* and *LOXL2* genes related to promotion of cancer progression in hepatocellular carcinoma cells

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ABSTRACT

Impact of different cancer-associated fibroblast (CAF) cell lines on proliferation, migration, invasion and differential expressions of genes in different hepatocellular carcinoma (HCC) cell lines was investigated. Two human CAF cell lines (F26/KMUH, F28/KMUH) and two human HCC cell lines (HCC24/KMUH, HCC38/KMUH) were studied. Influence of F28/KMUH cells on expressions of genes in HCC38/KMUH cells was detected by microarray to select genes for further analysis. Both CAF cell lines promoted proliferation (all $P < 0.05$), migration (all $P < 0.05$) and Matrigel invasion (all $P < 0.0001$) of both HCC cell lines. F26/KMUH cells showed stronger promoted effects on, firstly, proliferation of HCC24/KMUH cells ($P = 0.0064$) and, secondly, migration of both HCC cell lines than F28/KMUH cells did (all $P < 0.002$). Ten up-regulated genes (*APLN*, *CCL2*, *CCL26*, *CXCR4*, *IL6*, *MUC1*, *LOXL2*, *PDGFA*, *PGK1*, *VEGFA*) related to proliferation, migration, invasion and angiogenesis of HCC detected by microarray were selected for quantitative reverse transcriptase-polymerase chain reaction analysis. Both CAF cell lines had same tendency of effects on differential expressions of genes in same HCC cell line, but expressions of genes between different HCC cell lines were not consistent. Only *CCL2*, *CCL26*, *IL6* and *LOXL2* genes were consistently up-regulated in both HCC cell lines. In conclusion, the effects of CAFs to promote proliferation, migration and invasion of HCC cells are influenced by the characteristics of both CAFs and HCC cells. Up-regulations of *CCL2*, *CCL26*, *IL6* and *LOXL2* genes in cancer cells are part of the common effects of CAFs on HCC cells.

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1. Introduction

The tumor microenvironment plays active roles in determining the malignant phenotype. 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 crosstalk between cancer cells and CAFs is via a large variety of soluble factors [3]. Chemokines have been proposed as key players in the crosstalk 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 *CCL2, CCL5, CCL7, CXCL8, CXCL12*, and *CXCL14*. These chemokines then act back on the cancer cells to promote their proliferative, migratory, and invasive properties [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 crosstalk of HCC-CAF interactions can help us to

design a new strategy in the treatment of HCC [4]. Recent study showed that lysophostatidic acid secreted from HCC cells could accelerate HCC progression by recruiting peritumoral tissue fibroblasts and promoting their transdifferentiation into CAFs [5]. However, the influence of CAFs on differential expressions of genes in HCC cells to promote cancer progression still needs to be clarified. On the other hand, the characteristics of CAFs have significant individual/intrinsic differences [8] which may have different influences on different HCC cells. The purpose of this study was to investigate the influence of different CAF cell lines on proliferation, migration, invasion and gene expressions of different HCC cells. All gene names are according to the official symbols from the HUGO Gene Nomenclature Committee.

2. Materials and methods

2.1. Cell lines

Two human HCC cell lines (HCC24/KMUH, HCC38/KMUH) used in our previous studies [9–11], and two CAF cell lines (F26/KMUH, F28/KMUH) newly established from patients with HCC in our institution were investigated. CAF cell lines were verified by

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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 CXCL12 (R & D Systems, Inc., Minneapolis, MN, USA). All procedure 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% CO_2 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 units/mL penicillin and 100 μg/mL streptomycin (HyClone, Logan, Utah, USA).

2.2. Proliferative experiment

Cancer cells from each cell line were seeded in three 24-well cell culture insert companion plates (18 wells/plate, 6×10^3 cells/well). CAFs from each cell line were seeded in 36 culture inserts (8×10^3 cells/insert) with transparent polyethylene terephthalate membrane (pore size: 0.4 μm, BD Falcon™ Cell Culture Inserts, BD Biosciences, Mississauga, Canada) using two new 24-well companion plates. 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 cancer cells containing wells. Each well and insert contained 1 and 0.5 mL serum-free medium respectively. The cells were incubated for further 48 hours. Then the inserts were removed and HCC cells 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% CO_2 and 95% air. After then, 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).

2.3. Migratory experiment

For each HCC cell line, serum-free medium containing the mixture of cancer cells and CAFs from each cell line was transferred to eight 24-well cell culture inserts with transparent polyethylene terephthalate membrane (pore size of 8 μm, BD Falcon™ Cell Culture Inserts, BD Biosciences, Mississauga, Canada). Each insert contained 8×10^3 cancer cells and 2×10^3 CAFs. Additional eight inserts containing cancer cells alone (8×10^3 cells/insert) in serum-free medium were used as control group. Serum-containing medium was added to each well. The cells were incubated for 36 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 [12]. Whole migrated cells in each insert were counted at 100× magnification.

2.4. Matrigel invasive experiment

For each HCC cell line, serum-free medium containing the mixture of cancer cells and CAFs from each cell line was transferred to eight 24-well cell culture inserts with transparent polyethylene terephthalate membrane (pore size: 8 μm) coated with Matrigel (BD BioCoat™ Matrigel™ Invasion Chambers, BD Biosciences, Mississauga, Canada). Each insert contained 1×10^4 cancer cells and 1×10^3 CAFs. Additional eight inserts containing cancer cells alone (1×10^4 cells/insert) in serum-free medium were used as control group. Serum-containing medium was added to each well. The cells were incubated for 36 hours. Then the cells inside the insert were wiped with cotton-swabs and removed. The cells that adhered onto the outer side of the insert were stained by the technique of Liu-stain [12]. Whole HCC cells in each insert were counted at 100× magnification.

2.5. Microarray and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) experiments

Cancer cells from each cell line were seeded in two 6-well cell culture insert companion plates (1×10^5 cells/well) and CAFs from each cell line were seeded in six inserts (1×10^4 cells/insert) with transparent polyethylene terephthalate membrane (pore size: 0.4 μm, BD Falcon™ Cell Culture Inserts, BD Biosciences, Mississauga, Canada) using a new 6-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 cancer cells 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 Trizol® Reagent (Invitrogen™, Life Technologies Corporation, NY, USA), and followed by RNAeasy 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 Bioanalyser 2100 (Agilent Technology, Santa Clara, CA, USA). To select candidate genes for further quantitative RT-PCR analysis, influence of F28/KMUH cells on differential expressions of genes in HCC38/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 [9]. Control group was labeled with Cy3-CTP and the experimental group was labeled with Cy5-CTP (CyDye, PerkinElmer, 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 ($\log_2$ Cy5 BgSubSignal + $\log_2$ Cy3 BgSubSignal)/2. The Cy5 (or Cy3) BgSubSignal was calculated by the formula: mean signal intensity of Cy5 (or Cy3) – mean background light intensity of Cy5 (or Cy3). The Cy5 (or Cy3) BgUsed indicated the mean background intensity of Cy5 (or Cy3) channel. The M value was calculated by the formula: $\log_2$ (Cy5 BgSubSignal/Cy3 BgSubSignal). 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 less than 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 ($\log_2$ gene expression fold change) ≥ 1.

For quantitative RT-PCR study, specific oligonucleotide primer pairs were selected from Roche Universal ProbeLibrary and used for real-time PCR. The procedures for real-time PCR reactions were the same as in our previous studies [9–11]. 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 = $2^{-\geq\geq CP}$, where $\geq CP = CP_{target\ gene} - CP_{reference\ gene}$, and $\geq\geq CP = \geq CP_{test\ sample} - \geq CP_{control\ sample}$