

## RESEARCH ARTICLE

## Cancer-associated fibroblast (CAF) secretomes-induced epithelial-mesenchymal transition on HT-29 colorectal carcinoma cells associated with hepatocyte growth factor (HGF) signalling

Septelia Inawati Wanandi,<sup>1</sup> Noza Hilbertina,<sup>2</sup> Nurjati Chairani Siregar,<sup>3</sup> Murdani Abdullah,<sup>4</sup> Wifanto Saditya Jeo<sup>5</sup>

### Abstract

**Objective:** The aim of this study was to investigate the effect of cancer-associated fibroblasts (CAF) secretomes on the epithelial-mesenchymal transition (EMT) of colorectal carcinoma (CRC) cells and its association with hepatocyte growth factor (HGF) signalling focussing on the HGF receptor, c-Mesenchymal epithelial transition (c-Met), and the EMT markers, vimentin and e-cadherin, in CRC cells.

**Methods:** Conditioned mediums (CM) containing secretomes from colorectal CAFs and their counterpart normal fibroblasts (NFs) of three CRC patients were collected and supplemented to the HT-29 CRC cells. The mRNA levels of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and HGF in both fibroblasts, as well as c-Met, vimentin, and e-cadherin in HT-29 cells after supplemented with CAF- and NF-CM were determined using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). HGF protein level in the CM of CAFs and NFs was measured using enzyme-linked immunosorbent assay (ELISA). Vimentin and e-cadherin protein expressions were observed in HT-29 cells using immunofluorescent (IF) staining.

**Results:** Compared to the non-cancerous colon, fibroblasts from cancerous area of CRC substantially expressed higher mRNA levels of  $\alpha$ -SMA, a CAF marker. The HGF mRNA expressions in CAFs and NFs were in line with the HGF protein level in the secretomes of both cells. CAF-CM increased c-Met and vimentin mRNA levels in HT-29 cells. Surprisingly, e-cadherin mRNA level in HT-29 cells was increased following CAF-CM supplementation. We also demonstrated the co-localization of e-cadherin and vimentin in the HT-29 cell cytoplasm.

**Conclusion:** CAF secretomes of CRC promote a hybrid type of EMT associated with HGF signalling.

**Keywords:** Cancer-associated fibroblasts, Epithelial-mesenchymal transition, Hepatocyte growth factor, c-Met. (JPMA 71: S-18 [Suppl. 2]; 2021)

### Introduction

Cancer-associated fibroblast (CAFs) is the major cellular component of tumour microenvironment (TME) and serves as key players to promote growth and invasion of cancer cells by the various mechanisms.<sup>1</sup> The crosstalk between cancer cells and CAFs is mediated either by secretomes, such as cytokines, chemokines, growth factors, and enzymes, or by the multifaceted functions of the adjacent extracellular matrix.<sup>2,3</sup>

One of the most studied CAF-secreting factors is hepatocyte growth factor (HGF). HGF is known to contribute to the progression of cancer. In cancer cells, HGF activates tyrosine kinase pathways by binding to its receptor, c-Met, which in turn mediate tumour growth, angiogenesis, invasion, and metastases.<sup>1,4</sup> Previous studies have reported that HGF was the most significant

up-regulated secreted factor in the CAFs of breast cancer compared to its normal fibroblasts, which is positively correlated with the pro-tumourigenic effect.<sup>5,6</sup> HGF released from the CAFs of gastric cancer has also a tumour-promoting function.<sup>6</sup>

Epithelial-mesenchymal transition (EMT) is the critical process for cancer cells that invade adjacent tissues and metastases to a distant organ. During the EMT process, cancer cells lose cell adhesion markers, gain mesenchymal markers, and increase migration and invasion properties. Different signals received from TME such as transforming growth-beta (TGF- $\beta$ ), epidermal growth factor (EGF), Wntless-related integration site (WNT), Notch, may activate the EMT process.<sup>7,8</sup> The EMT process of colorectal carcinoma (CRC) is associated with adverse prognosis such as higher tumour, nodes, and metastases (TNM) stages, elevated tumour grade, presence of lymph vascular invasion and subsequently lymph node and distant metastases.<sup>9</sup>

Until now, the mechanism by which CAF secretomes affect the EMT process of CRC remains unclear. The aim of

<sup>1</sup>Department of BioChemistry and Molecular Biology, <sup>2,3</sup>Department of Anatomic Pathology, <sup>4</sup>Department of Internal Medicine, <sup>5</sup>Department of Surgery, Universitas Indonesia, Jakarta, Indonesia.

**Correspondence:** Septelia Inawati Wanandi. Email: septelia.inawati@ui.ac.id

this study was to investigate the effect of CAF secretomes on the EMT of CRC cells and its association with HGF signalling. In this study, we focussed on the HGF receptor, c-Met, and the EMT markers, vimentin and e-cadherin, in CRC cells.

## Material and Methods

This experimental study was performed from January 2018 to May 2019 at the Molecular Biology and Proteomics Core Facilities Laboratory, Indonesian Medical Education and Research Institute (IMERI), and the Institute for Human Virology and Cancer Biology (IHVCB), Faculty of Medicine Universitas Indonesia. Cancerous and non-cancerous colorectal tissues were obtained from three CRC patients who underwent tumour resection at Cipto Mangunkusumo General Hospital, Jakarta. The patients had not been treated with preoperative chemotherapy or radiotherapy. The research protocol was approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia (No. 433/UN2.F1/ETIK/2017). Written informed consents have been signed by the patients prior to specimen collection.

**Cell isolation and culture:** CAFs were isolated from the cancerous area of CRC, while their counterparts, normal fibroblasts (NFs), were isolated from the non-cancerous area of the same CRC patients at least five centimetres from the tumour margin. All specimens were transported in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% foetal bovine serum (FBS) (Invitrogen), 3% Penicillin-Streptomycin and kept at 4°C prior to use. Specimens were processed according to the protocol for colon CAF establishment and culture growth.<sup>10</sup>

Fresh specimens were excised to 2-3mm<sup>3</sup> and attached to tissue culture dishes for fibroblast culture. For the first ten days, tissue was preserved in FBS supplemented with 2% of Penicillin-Streptomycin and 1% Fungizone. On day 12 of tissue culture, the medium was replaced by the Fibroblast Growth Medium-2 (FGM-2) bullet kit (Lonza, Switzerland).

The colorectal cancer cell line, HT-29 (ATCC® HTB-38), was grown in DMEM (Invitrogen, USA), supplemented with 10% FBS. Cells were incubated at 37°C in a humidified incubator with 5% CO<sub>2</sub>.

**Collection of CAF- and NF-Conditioned Medium (CM):** CAFs and NFs cells were seeded on a six-well plate of 2x10<sup>5</sup> cells/well and grown under standard conditions. CM was collected after cell confluency reached 80-90%. CM was filtered using a 0.22-mm filter and preserved at -

80°C for further analysis. For the CM supplementation, HT-29 cells were seeded on a 12-well plate at 1x10<sup>5</sup> cells/well and grown for 24 hours in a serum-free medium under standard conditions. Subsequently, HT-29 cells were supplemented with 50% (v/v) CAF-CM or NF-CM and incubated for 72 hours. All experiments were performed in triplicates.

## Quantitative Reverse Transcription-PCR (qRT-PCR):

The relative mRNA expression of  $\alpha$ -SMA and HGF in fibroblasts (CAF and NF), as well as c-Met, vimentin, and e-cadherin in HT-29 cells were determined using qRT-PCR. Total RNA was isolated according to the manufacturer's protocol (TriPure isolation reagent; Merck, Germany). One step qRT-PCR was performed according to manufacturer's protocol (SensiFASTTMSYBR®No-ROX One-Step Kit; Bioline, UK). Primers used for qRT-PCR analysis were as follows:  $\alpha$ -SMA 5'TCA ATG TCC CAG CCA TGT AT3'(F), 5'CAG CAC GAT GCC AGT TGT3'(R); HGF 5'CTG GTT CCC CTT CAA TAG CA3'(F), 5'CTC CAG GGC TGA CAT TTG AT3'(R); c-Met 5'CAT CTC AGA ACG GTT CAT GCC3'(F), 5'TGC ACA ATC AGG CTA CTG GG3'(R); vimentin 5'ACA TTG AGA TTG CCA CCT ACA G3'(F), 5'ACC GTC TTA ATC AGA AGT GTC C3'(R); e-cadherin 5'CCT GGG CAG AGT GAA TTT TG3'(F), 5'GGC GTA GAC CAA GAA ATG GA3'(R); 18S rRNA 5'AAA CGG CTA CCA CAT CCA AG3'(F), 5'CCT CCA ATG GAT CCT CGT TA 3'(R). The relative mRNA expressions of  $\alpha$ -SMA and HGF in both fibroblasts, as well as c-Met, vimentin, and e-cadherin in HT-29 cells were calculated using the Ct values normalized to those in the control and to 18S rRNA as a reference gene, according to Livak's formula.<sup>10</sup> All experiments were performed in triplicates.

**Enzyme-linked immunosorbent assay (ELISA):** 100 $\mu$ L of CAF-CM and NF-CM respectively were subjected to enzyme-linked immunosorbent assay for HGF (Human HGF ELISA Kit, RAB0212 from Sigma-Aldrich, USA) according to the manufacturer's instruction.

**Immunofluorescence (IF) staining:** Protein expressions of vimentin and e-cadherin in HT-29 cells after supplemented with CAF-CM were analysed with IF staining. The primary antibodies were mouse monoclonal anti-vimentin (GTX629744; Genetex; dilution 1:200) and rabbit polyclonal anti e-cadherin (ab15148; Abcam; dilution 1:50), respectively. Secondary antibodies were goat anti-mouse conjugated with Fluorescein isothiocyanate (FITC) and mouse anti-rabbit conjugated with Rhodamine with the dilution of 1:500, respectively.

HT-29 cells were seeded on the coverslip and grown for

72 hours in DMEM supplemented with 50% CAF-CM. Cells then fixed with 3.7% paraformaldehyde for 20 min. After washing with Phosphate-buffered saline (PBS), cells were treated with 0.2% Triton X-100 for 5 min to permeabilize, then washed with PBS. Cells were incubated with 2% PBS-Bovine Serum Albumin (BSA) for 1 hour, followed by incubated with primary antibodies cocktail overnight at 4°C. Subsequently, cells were washed with PBS-BSA 2% and incubated with FITC- and Rhodamine-conjugated secondary antibodies, respectively. Protein expressions were visualised using a confocal microscope.

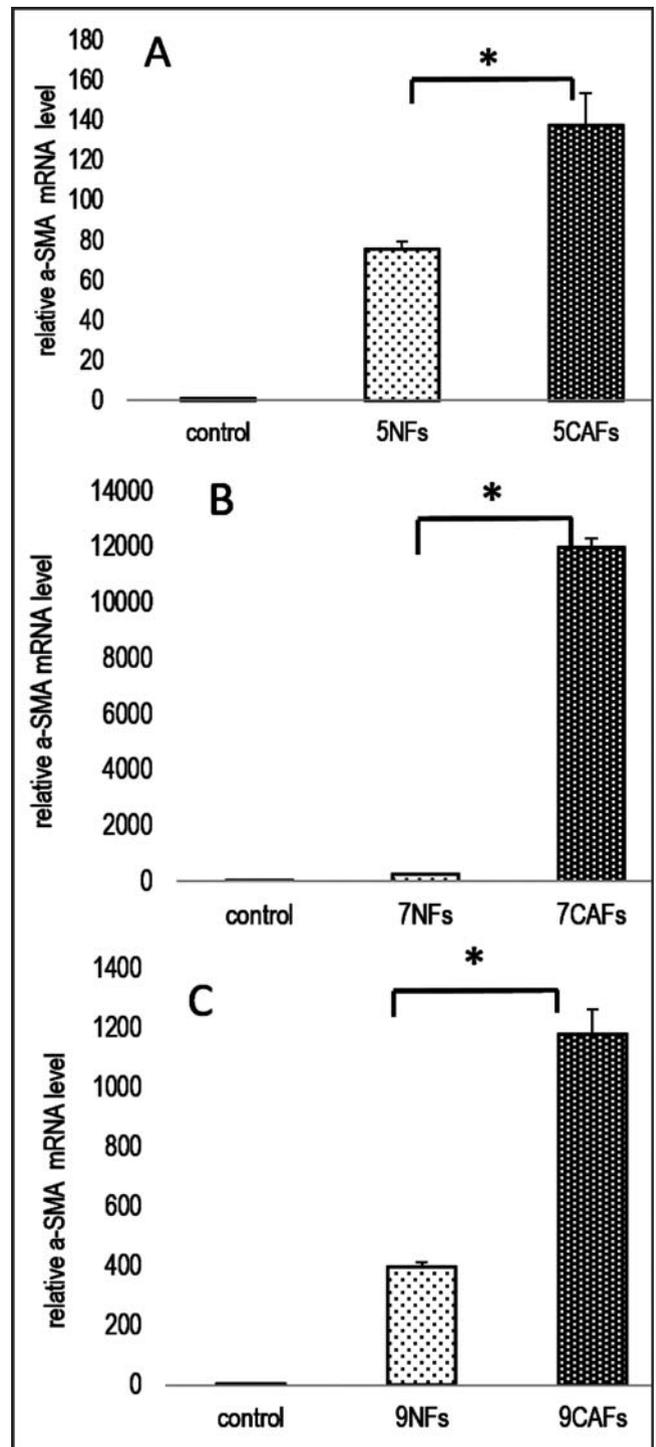
**Statistical analysis:** Data were expressed as mean standard error (SE) and statistically analysed using Student's t-test. Pearson test was used to analyse the correlation between data. Statistical significance was considered if  $p < 0.05$ .

## Results

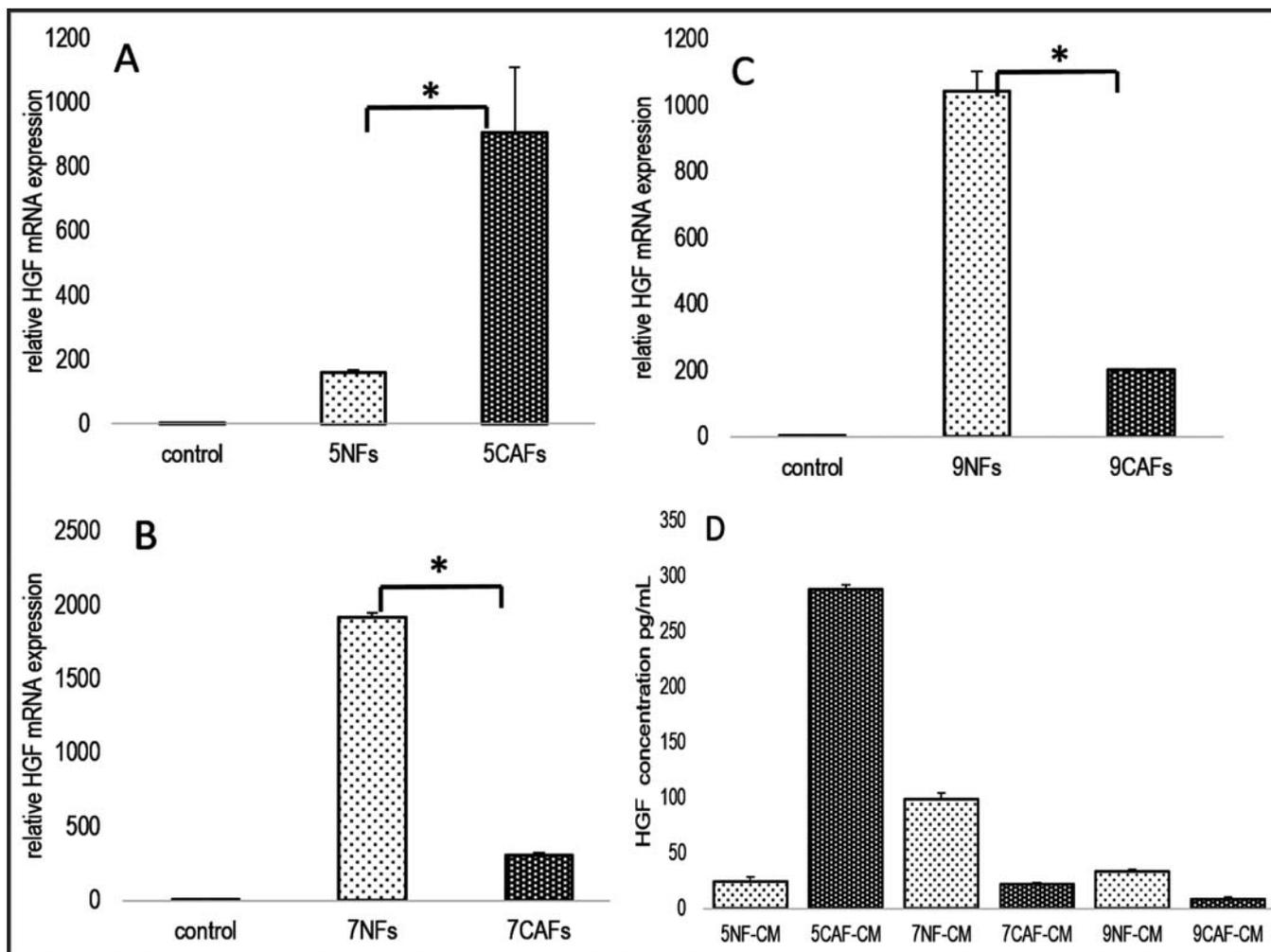
**Identification of CAFs in colorectal adenocarcinoma:** In this study, fibroblasts from cancerous (CAFs) and non-cancerous (NFs) area of three CRC patients were isolated as described previously.<sup>11</sup> To identify CAFs in the cancerous area and non-cancerous area of colorectal carcinoma, we determined the mRNA level of CAF-marker  $\alpha$ -SMA using qRT-PCR. We found that the  $\alpha$ -SMA mRNA levels in the cancerous area of all samples were significantly higher than that in their counterparts from non-cancerous of CRC. Therefore, we confirmed that the fibroblasts from cancerous area were CAFs while from the non-cancerous area were NFs (Figure-1A-C).

**Analysis of HGF mRNA and protein expression:** To compare the expressions of HGF in CAFs and NFs, we performed qRT-PCR for mRNA expression in CAFs and NFs, and ELISA for protein level in CAF- and NF-CM. In spite of differential HGF expressions between CAFs and NFs, and among three samples, we highlighted that the mRNA expressions of HGF in both fibroblasts (Figure 2A-C) were in line with its protein expression in both CMs (Figure-2D).

**Effect of CAF-CM on c-Met, vimentin, and e-cadherin mRNA expression in HT-29 cells:** All CAF-CMs induced higher expression of c-Met mRNA in HT-29 cells compared to the NF-CMs (Figure-3A). These results were associated with the higher vimentin mRNA expression in HT-29 cells after CAF-CM compared to NF-CM supplementation (Figure-3B). Unexpectedly, the mRNA levels of the epithelial marker e-cadherin were also significantly higher in HT-29 cells following CAF-CM supplementation compared to NF-CM (Figure-3C). Moreover, Figure 3D-F demonstrated the co-localized expressions of e-cadherin



**Figure-1:** Analysis of  $\alpha$ -SMA mRNA relative expressions in CAFs and NFs using qRT-PCR. Three CAFs (5CAFs, 7CAFs, 9CAFs) and NFs (5NFs, 7NFs, 9NFs) were isolated from the cancerous and non-cancerous areas of three CRC patient specimens (A-C), respectively. The relative mRNA expressions of  $\alpha$ -SMA were calculated using the Ct values normalized to those in HT-29 cells as a control and to 18S rRNA as a reference gene, according to Livak's formula. All experiments were performed in triplicates. Statistical differences were indicated as \* symbol for  $p < 0.05$  compared to control.



**Figure-2:** Analysis of HGF mRNA relative expression and protein levels. The HGF mRNA relative expressions in CAFs and NFs from three CRC patient specimens (A-C) were determined using qRT-PCR, whereas the HGF protein levels in CAF and NF secretomes (D) were measured using ELISA. The relative mRNA expressions of HGF were calculated using the Ct values normalized to those in HT-29 cells as a control and to 18S rRNA as a reference gene, according to Livak's formula. All experiments were performed in triplicates. Statistical differences were indicated as \* symbol for  $p < 0.05$  compared to control.

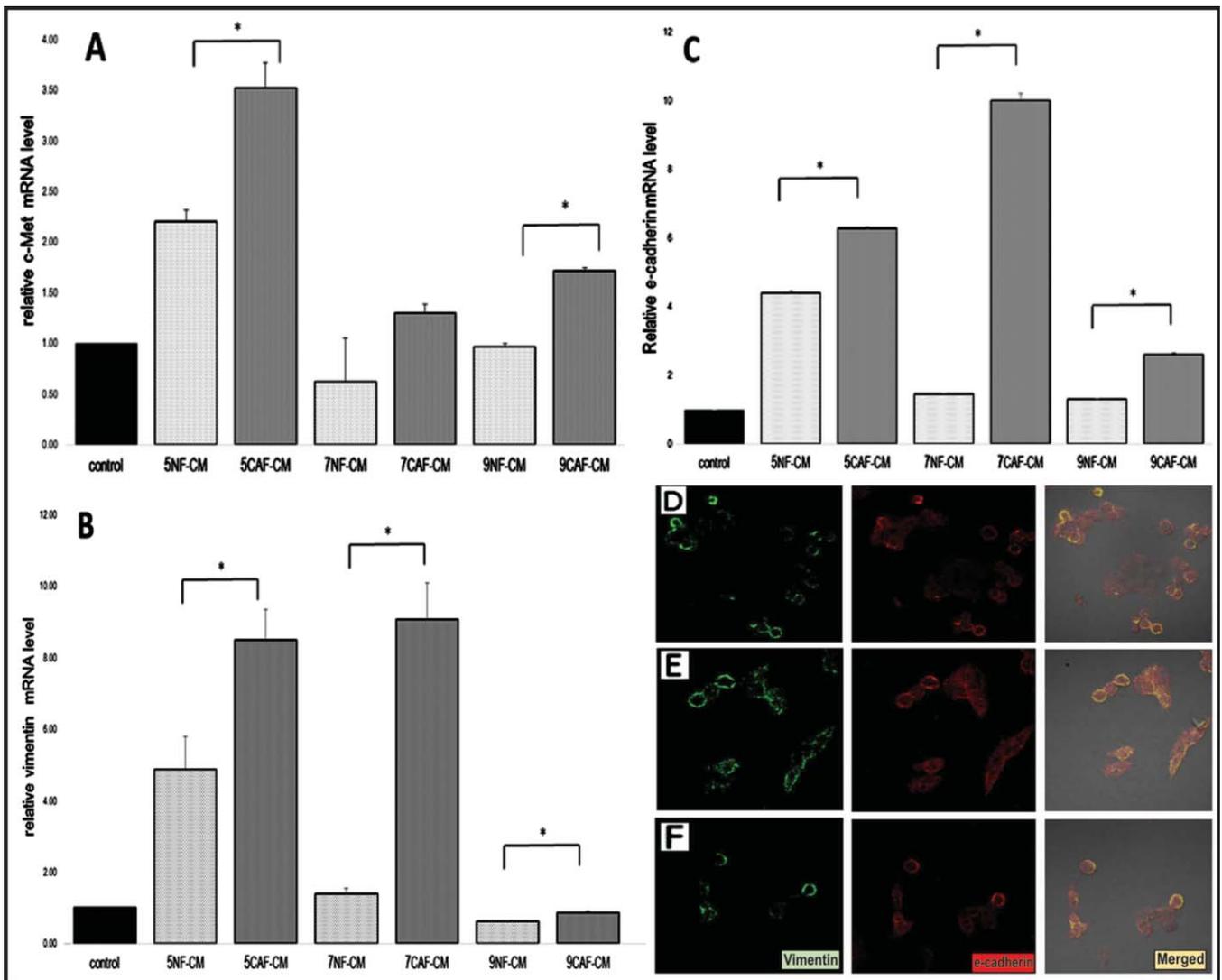
and vimentin in the cytoplasm of HT-29 cells supplemented with CAF-CM from three samples (5CAF-CM, 7CAF-CM, and 9CAF-CM).

## Discussion

The activated fibroblasts, CAFs, are the major cellular component of TME secreting a variety of factors that are lower or not expressed by the normal fibroblasts.<sup>12,13</sup> CAFs develop contractile phenotype during tumour progression and are different from normal fibroblasts in many aspects, including highly active endoplasmic reticulum, high expression  $\alpha$ -SMA and vimentin, and the formation of intricately organized stress fibre and fibrous adhesion complexes.<sup>6</sup> Our study verified that CAFs were higher concentrated in the cancerous compared to

that in the non-cancerous area of CRC.

It has been widely reported that CAFs mediate the crosstalk between cancer cells and TME either by cell-cell contact or through secretion of soluble mediators such as cytokines, chemokines, growth factors, and enzymes, which in turn induces the EMT process of cancer cells.<sup>14</sup> Here, we aimed to elaborate the role of HGF, one of the main CAF secretomes, on the EMT process of CRC. HGF has been known to contribute to cancer progression by binding to its receptor c-Met that primarily expressed on epithelial and endothelial cells.<sup>15-17</sup> Based on the cytokine antibody array it is suggested that HGF was the most significant up-regulated secreted factor in breast cancer compared to normal fibroblast.<sup>6</sup> Woo et al. have shown



**Figure-3.** Analysis of c-Met, vimentin, and e-cadherin expressions in HT-29 CRC cells after CAF- and NF-CM supplementation. HT-29 cells were supplemented with 50% (v/v) CM from three CAFs (5CAF, 7CAF, 9CAF) and NFs (5NF, 7NF, 9NF), respectively and incubated for 72 hours. The mRNA relative expressions of c-Met (A), vimentin (B), and e-cadherin (C) were determined using qRT-PCR. The relative mRNA expressions were calculated using the Ct values normalized to those in HT-29 cells supplemented with 50% (v/v) medium (DMEM/FGM) as a control and to 18S rRNA as a reference gene, according to Livak's formula. All experiments were performed in triplicates. Statistical differences were indicated as \* symbol for  $p < 0.05$  compared to control. To analyse the vimentin and e-cadherin protein expressions and their cellular localization, IF stainings of HT-29 cells after supplemented with 5CAF-CM (D), 7CAF-CM (E), and 9CAF-CM (F) was performed using antibody anti-vimentin, anti-e-cadherin, and 'merged' (anti-vimentin and anti-e-cadherin). All experiments were performed in triplicates. Statistical differences were indicated as \* symbol for  $p < 0.05$  compared to control.

that HGF was only found in the secretomes of fibroblasts but not in cancer cells or culture medium.<sup>18</sup> In addition, Deying et al. and Tyan et al. have reported that HGF mRNA and protein levels in CAFs were significantly higher than in NFs.<sup>5,16</sup>

Our result showed that the expressions of HGF mRNA in CAFs and NFs corresponded to its protein levels in the secretomes of both cells. It should be noted that the expression of HGF mRNA in both CAFs and NFs were

much higher than that in the epithelial cells HT-29 cells. Although HGF mRNA in CAFs and NFs, and HGF protein in CAF- and NF-secretomes are differentially expressed among CRC patients, we revealed that all CAF secretomes increased the expression of c-Met higher than NF secretomes, indicating the activation of HGF/c-Met signalling. Moreover, the increased mRNA expression levels of c-Met in HT-29 cells supplemented with CAF-CM significantly and positively correlated with those of  $\alpha$ -

SMA (Pearson test,  $r=0.735$ ;  $p=0.024$ ) and HGF (Pearson test,  $r=0.818$ ;  $p=0.007$ ) in CAFs.

HGF has always been secreted as pro-HGF, an inactive precursor. If it is not bound to the c-Met, the pro-HGF does not cause the c-Met activation and therefore functions as a receptor antagonist. A proteolytically inactive pro-HGF mutant has been confirmed to be the competitive antagonism between HGF and pro-HGF.<sup>15</sup> Conversion of pro-HGF into the active form of HGF is the rate-limiting step in the HGF/c-Met signalling pathway. Pro-HGF activator is the trypsin-like serine proteases, matriptase, hepsin, and HGF activator (HGFA) that are usually over-expressed in tumour cells. Furthermore, it has been reported that the expression of endogenous inhibitors of pro-HGF activation were decreased in tumour tissue.<sup>15,19</sup> The present study also suggested that the milieu of NFs is composed of more inhibitors and less activators of HGF compared to that of CAFs. As a consequence, the higher HGF mRNA expression and protein secretion in the NFs were not capable to activate the c-Met mRNA expression compared to CAFs. However, this argument needs to be further investigated.

In addition, the different expression of HGF mRNA and protein among three samples used in this study might be due to the differences in clinical characteristics of CRC patients such as age, tumour differentiation, CAFs, and stromal phenotype. Recent studies have revealed that the intratumoural heterogeneity exists within CRC primary tumours and cellular components of TME. Intratumoural heterogeneity originates from intrinsic factors such as cellular phenotype, and extrinsic factors such as tumour progression, treatments, and spatial distribution.<sup>2</sup>

The binding of HGF to c-Met receptor activates the expression of various target genes including those that are responsible for EMT process.<sup>20</sup> EMT is a critical process for cancer cells to invade to adjacent tissues and to spread toward distant organs, thus, a key step in the progression of cancer cells to more aggressive stages.<sup>21</sup> The phenotypes of EMT are the loss of cell adhesion and the increase of cell mobility related to mesenchymal cell characteristics.<sup>7,22</sup> Based on the expression of epithelial (e-cadherin) and mesenchymal (fibronectin,  $\alpha$ -SMA, and vimentin) markers, EMT phenotype can be divided into complete phenotype, i.e. loss of e-cadherin and positive for any mesenchymal markers, and incomplete phenotype or hybrid type, i.e. positive for both epithelial and mesenchymal markers.<sup>23</sup> Our study demonstrated an increase in mesenchymal marker (vimentin) along with the cell adhesion marker (e-cadherin). Therefore, we assumed that CAF secretomes provoked the incomplete or hybrid EMT phenotype of HT-29 CRC cells. This

phenotype represents the intermediate states in cells express epithelial and mesenchymal characteristics at the same time.<sup>21</sup> The hybrid EMT occurred in breast cancer stem cells is reflected in the highest invasive ability, suggesting that the transient EMT phenotype maintains cell plasticity and allows cancer cells to acquire stem cell traits.<sup>21</sup>

Our IF finding indicated that vimentin and e-cadherin were co-localized in the cytoplasm of HT-29 cells treated with CAF-CM. In contrast to vimentin that is classically located in cytoplasm of mesenchymal cells,<sup>22</sup> e-cadherin is normally found in cell membrane for cell-cell adhesion. Previous studies have indicated that the expression of e-cadherin in CRC does not always absent or decreased, but often redistribute from cell membrane to cytoplasm or nucleus.<sup>24,25</sup> The aberrant cytoplasmic location of e-cadherin found in this study is plausibly due to the ubiquitination of E-cadherin by Hakai which in turn induces the endocytosis of this protein leading to the disruption of cell-cell contact, as described by Fujita et al.<sup>26</sup>

## Conclusion

CAF secretomes of CRC facilitates a hybrid type of EMT, which likely associated with HGF/c-Met signalling. More studies are needed to elaborate the role of other CAF-secreting factors in the EMT of CRC linked to stemness properties.

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