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Molecular characterization of cancer-associated fibroblasts isolated from human colorectal cancer as a major stromal cell component promoting metastasis

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Dr. Pravin D. Potdar's present interest is to study molecular profiling of circulating tumor cells, circulating tumor DNA, cancer associated fibroblasts and cancer stem cells involved in metastatic process of cancers, and to see how this process can be reverted back to normal by using innovated technologies which include nanotechnology and nanomedicine.

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ABSTRACT

Aim: Colorectal cancer (CRC) remains a disease with poor prognosis and limited overall 5-year survival rate, which highlights a clear clinical need for novel treatment strategies. The stromal cancer-associated fibroblasts (CAFs) strongly dictate the metastatic potential of CRC and hence warrant investigation for clinical outcome. **Methods:** The authors established primary cultures of CAFs from metastatic CRC patients and performed cellular characterization using phase contrast microscopy and histological staining followed by light microscopy. The isolated CAFs were further explored for the molecular characterization of genes which promote the process of metastasis. **Results:** The stromal CAFs maintain their fibroblastic phenotype *in vitro* and manifested high proliferation potential. To explore the gene expression profile, total RNA was isolated from the primary culture of CAFs and qualitative RT-PCR was performed. The cultured CAFs exhibited gene signatures associated with cancer stemness, epithelial to mesenchymal transition induction and inflammatory cytokines/chemokines favoring metastasis. These genes play a pivotal role in chemotherapy resistance and are also associated with poor prognosis in CRC. **Conclusion:** This study further delineates the role of CAFs as a part of the "corrupted" stromal cells within the tumor microenvironment in establishing and orchestrating the metastatic fate of CRC. The gene expression profile clearly indicates that CAFs represent a potential target for improved therapy of CRC.

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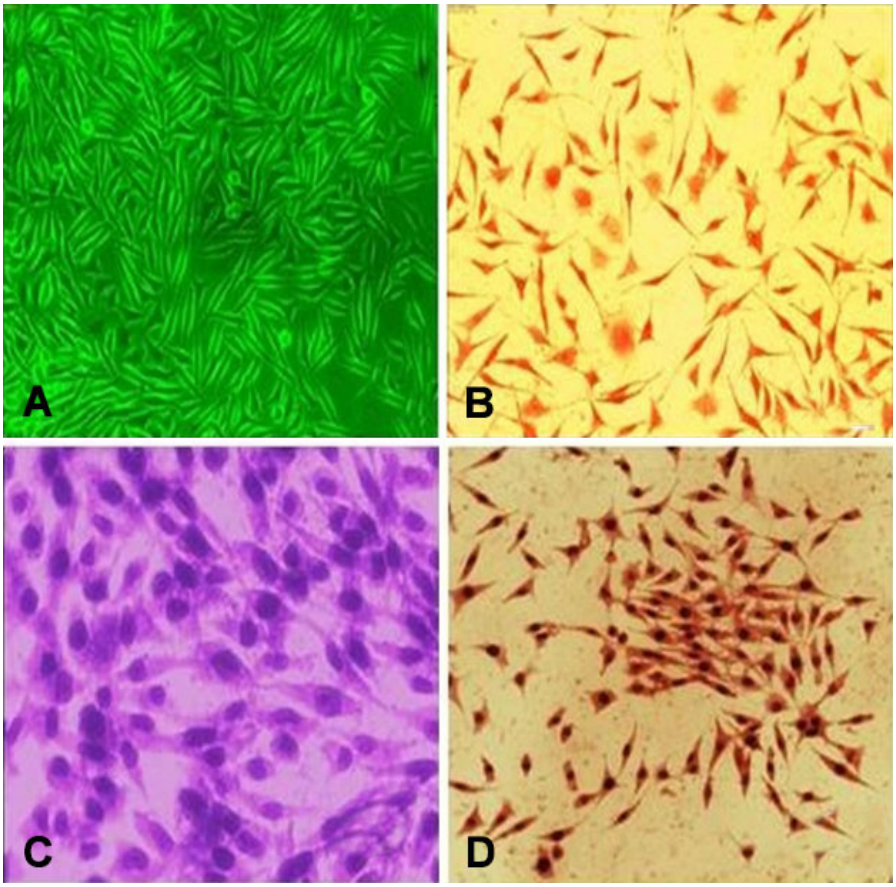

Figure 1: Phenotypic characterization of primary culture of CAFs isolated from metastatic CRC patient (×20). (A) CAFs isolated from the CRC patient cultured *in vitro* and exhibit spindle shaped morphology. Light microscopy of cultured CAFs for (B) Oil Red, (C) Giemsa stain and (D) Alzarin. CAFs: cancer associated fibroblasts; CRC: colorectal cancer

dictating the fate of carcinoma cells, it is most likely that the genes expressed by stromal cells may also dynamically influence the metastatic potential of CRCs. We have shown that stromal CAFs indeed express matrix metalloproteinase genes such as MMP1, MMP2, and MMP9 [Figure 6].

The inflammatory cytokine, TNF-α, which is directly involved in tumor invasion and metastasis, is also expressed by CAFs [Figure 4]. Another hallmark of tumor metastasis involves angiogenesis which is potentially mediated by vascular endothelial growth factor (VEGF). Interestingly, we have reported that CAFs express high levels of VEGF [Figure 6] and thus orchestrate metastatic progression of CRCs. In addition, we have shown that CAFs express chemokine receptors such as CXCR4 and CXCR7 which are involved in tumor progression [Figure 4]. Thus, the isolated CAFs in our study expressed genes that play a significant role in tumor metastasis.

DISCUSSION

This study for the first time reported gene expression profiling of primary stromal fibroblast cells isolated from a patient with metastatic CRC. Although the stromal compartment in CRCs contains heterogeneity of cells,[11] we have performed prior separation of

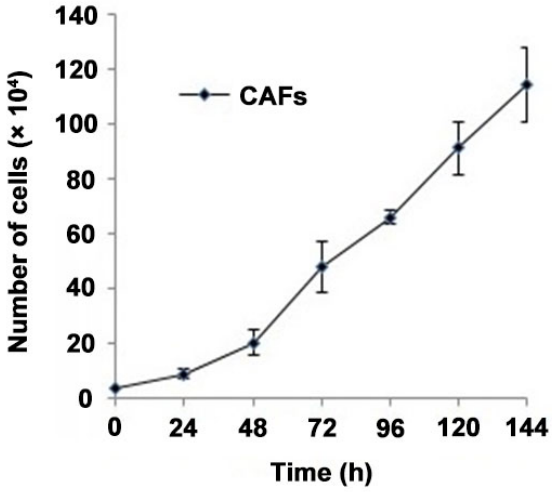

Figure 2: Cell kinetic of CAFs isolated from CRC patient. Nearly 40,000 of cells were seeded and cell count was done at different time interval. CAFs: cancer associated fibroblasts; CRC: colorectal cancer

stromal cells from tumor cells and explored the gene profiling only on RNA isolated from stromal primary cultured fibroblasts. Interestingly, we have reported that isolated CAFs maintained their tumor-promoting phenotype and genotype *in vitro* even in absence of carcinoma cells which is suggestive of hereditable changes in CAFs.[10,11] However, CAFs show an increase in the proliferation capacity from passage 18 thereby indicating independence from the *in-vivo* tumor microenvironment for growth signals.

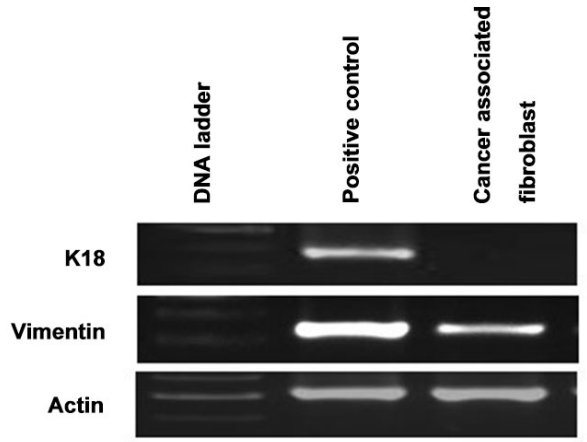

Figure 3: Gene expression of epithelial and myofibroblastic markers in CAFs isolated from CRC patient as measured by RT-PCR. CAFs: cancer associated fibroblasts; CRC: colorectal cancer

Another significant finding from this study is the molecular attributes of pluripotent gene expression which may be an inherent characteristic of stromal cells. A previous study has reported that the transcriptome of CRCs is also enriched with Oct4, Sox2, Nanog, and Klf4.[12] These cells, also termed cancer-initiating cells, represent a highly aggressive CRC which plays a significant role in chemoresistance and has been associated with poor prognosis.[13,14] We have reported that CAFs expressed similar pluripotent genes (Oct4, Sox2, Nanog, and Klf4) which suggests that both stromal cells and CRCs exhibit similar cancer stem cell gene expression and thus hypothesized that stromal cells may also regulate the cancer-stem cell properties in CRCs.

EMT represents one of the major events in CRCs and has been associated with high aggressiveness of the carcinoma with a concomitant increase in metastatic potential.[15-17] The inflammatory cytokine IL6 has been previously reported to be involved in expanding the

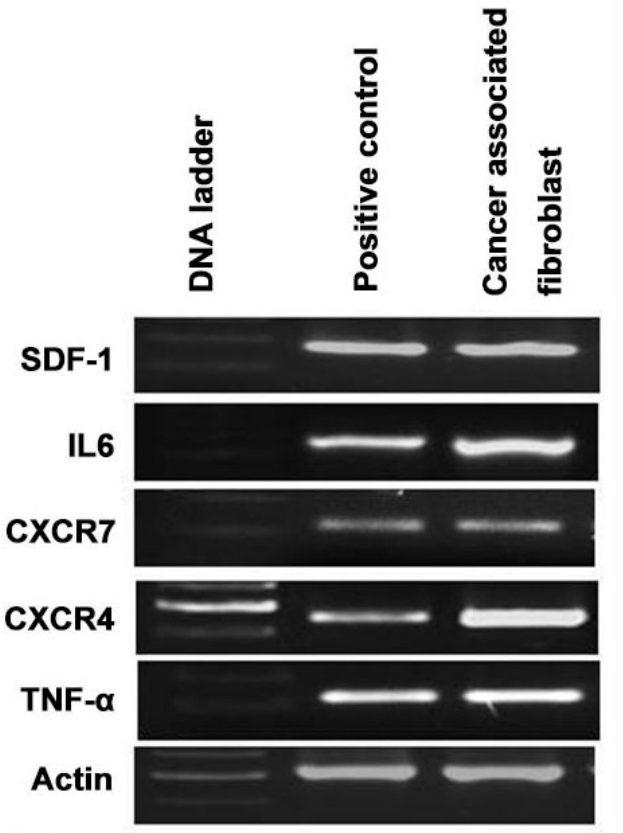

Figure 4: Gene expression of EMT inducer (SDF-1, IL6), chemokines and cytokines isolated in CAFs isolated from CRC patient as measured by RT-PCR. EMT: epithelial to mesenchymal transition; CAFs: cancer associated fibroblasts; CRC: colorectal cancer

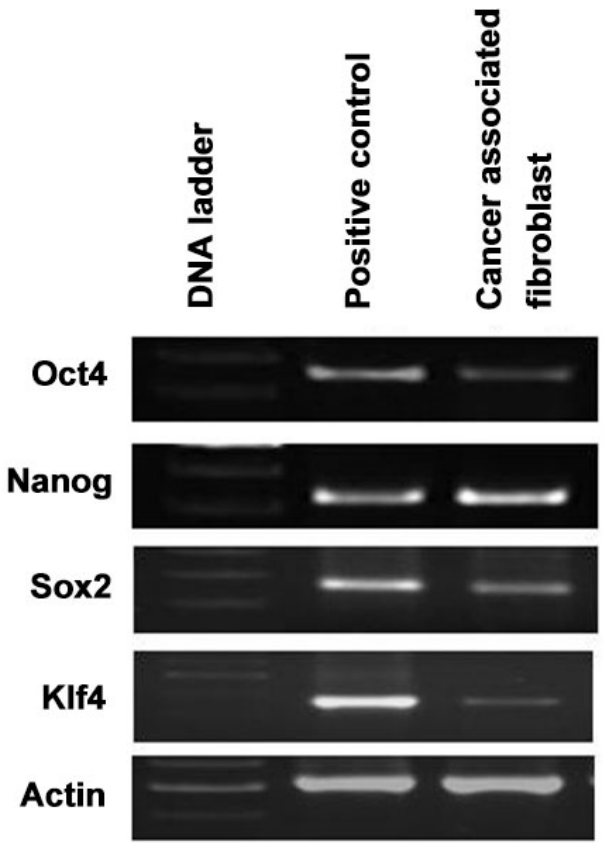

Figure 5: Gene expression of pluripotency stem markers in CAFs isolated from CRC patient as measured by RT-PCR. CAFs: cancer associated fibroblasts; CRC: colorectal cancer

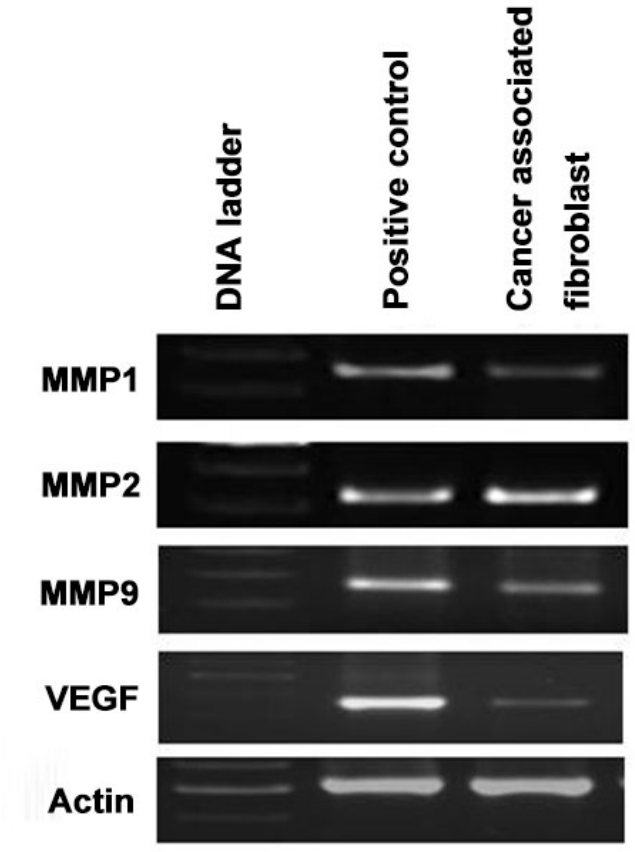

Figure 6: Gene expression of metastatic genes in CAFs isolated from CRC patient as measured by RT-PCR. CAFs: cancer associated fibroblasts; CRC: colorectal cancer

cancer stem cell populations as well as inducing EMT. Both of these have implications in tumor metastasis and resistance to chemotherapeutic drugs.[18,19] Our result is in agreement with this study which has also reported that IL6 secreted by CAFs is a potent inducer of an EMT phenotype in breast cancer characterized by loss of e-cadherin expression and robust induction of a vimentin gene with increased proliferative indices.[20] Robust evidence has reported the role of chemokines in cancer.[21] In our study, we have shown that SDF-1 and CXCR4 are prominently expressed by the CAFs. There are reports which suggest that radiation could also induce EMT[22] and therefore we suggest that CAFs could be associated with tumor-regrowth in CRC after pre-operative chemoradiotherapy. A study by Onoue *et al*.[23] has demonstrated that EMT transition is also induced in oral squamous cell carcinoma by the SDF-1/CXCR4 axis. We believe that expression of the SDF-1 gene, as reported in our study, can also strongly contribute to EMT. An elevated level of another SDF-1 receptor, CXCR7, has been reported in CRC brain metastasis as well as in certain classes of aggressive colon tumor.[24] Thus, the expression of CXCR7 in our study could reflect the metastatic status of the CRC patient.

We have also shown that CAFs express MMP1, MMP2, and MMP9, which are proteases involved in tumor invasion and metastasis. Specifically, the collagenase MMP1 is a key mediator of primary tumor invasion and the use of neutralizing antibodies against MMP1 completely abolished colon cancer invasion.[25] Moreover, the polymorphism in the MMP1 gene has been associated with increased susceptibility to CRCs.[26] Thus, MMP1 secreted by the CAFs can act as a circulating tumor cell attractant for primary invasion of CRCs. The expression of gelatinases MMP2 and MMP9 has been associated with the worst outcome in many subsets of CRCs patients.[27] Angiogenesis is one of the hallmarks of metastatic cancer and is essential for the spread of CRCs. Clinical studies have reported that VEGF is the predominant proangiogenic factor in CRC and is overexpressed in 50% of CRCs, associated with distant metastasis.[28] We have reported similar expression of VEGF in the stromal CAFs from a CRC patient. Although mRNA of MMP1, MMP2, MMP9, and VEGF was confirmed in this study, more investigations are warranted in order to isolate and confirm proteins from the same genes.

Tumor necrosis factor alpha (TNF-α) plays a role in tumor growth by upregulating colony stimulating factor one (CSF-1) which in turn regulates the secretion of VEGF. Silencing of TNF-α in colon cancer cells with small-interfering RNAs inhibits the expression of CSF-1, associated with a decrease in the expression of VEGF and MMP2 mRNAs.[29] Our study has demonstrated similar expression of TNF-α with a concomitant expression of VEGF and MMP2 by stromal CAFs. Thus, the results clearly support and are in agreement with previous observations that CAFs indeed orchestrate the metastatic program required for primary tumor invasion and spread of cancer at secondary sites. More investigation should be undertaken in order to evaluate the positive expression of Alfa-SMA and FAP and negative expression of CK and CD45 that are considered as markers for CAFs in CRC. In addition, other experiments should be conducted that include the evaluation of CAFs, such as multiple colon CAF-derived factors that sustain proliferative signaling in CRC cells and support the cancer cells to resist cell death and evade growth suppressors including EGF, hepatocyte growth factor (HGF), IGF1/2, PGE-2, PDGF, fibroblast growth factor (FGF)-1, and VEGF.

In conclusion, the present study is the first report of the isolation of CAFs from PBMCs of metastatic CRC. These were fast growing cells and showed all the characteristics of metastatic tumor cells. Molecular profiling of these cells prominently expressed metastatic genes such as MMP1, MMP2, MMP9, and