RESEARCH ARTICLE

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Identification of AOC3 and LRRC17 as Colonic Fibroblast Activation Markers and Their Potential Roles in Colorectal Cancer Progression

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Abstract

Background: Accumulation of cancer-associated fibroblasts (CAFs) in the tumor stroma is linked to poor prognosis in colorectal cancer (CRC). CAF-cancer cell interplay, facilitated by secretomes including transforming growth factor-beta 1 (TGF-β1), supports fibroblast activation, drives colorectal carcinogenesis, and contributes to CRC aggressive phenotypes. Although widely used, traditional CAF biomarkers are found to have heterogeneous and non-specific expression. Amine oxidase copper containing 3 (AOC3) and leucine-rich repeat-containing 17 (LRRC17) have been reported to be emerging markers of myofibroblasts. **Aim:** Our objective was to investigate the potential of AOC3 and LRRC17 as biomarkers for fibroblast activation thus predicting their roles in CRC progression. **Methods:** Immunofluorescence (IF) staining of AOC3 and LRRC17 was performed on myofibroblast line (CCD-112CoN), primary fibroblasts from colorectal tumor (CAFs), and adjacent normal tissue (normal fibroblasts-NFs). SW620 (epithelial CRC cell line) was used as a control. Conventional CAF biomarker (alpha-smooth muscle actin - α-SMA) was included in the IF analysis. Fluorescence intensity was compared between groups using ImageJ software. Proliferation and contractility of treated cells were assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and collagen gel contraction assays, respectively. Fibroblast contraction under TGF-β1 treatment was compared to those treated with complete medium (addition of 10% serum) and serum free (SF) medium. **Results:** Positive AOC3, LRRC17, and α-SMA expression were observed in colonic fibroblasts, more prominent in CAFs, whereas negative staining was found in SW620. Significant downregulation of AOC3, and upregulations in LRRC17 and α-SMA expression was found in TGF-β1-treated fibroblasts compared to SF medium treatment (p-value<0.05). All fibroblasts exhibited higher proliferation in complete medium and under treatment with conditioned medium from SW620 than SF medium. Significant contraction of NFs was recorded in complete medium and TGF-β1 (p-value<0.01). **Conclusion:** Our results demonstrate AOC3 and LRRC17 as the potential markers of CAF activation which promote CRC progression.

Keywords: Colon cancer- activated fibroblast- biomarker

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Introduction

Colorectal cancer (CRC) is the second leading cancer with high morbidity and mortality, affecting males and females worldwide. Despite the significant advancement in cancer therapy, the prognosis of CRC patients remains poor (Sahai et al., 2020). Several approaches have been studied including the targeted treatment against cancer-associated fibroblasts (CAFs). CAFs, prominent cellular components of the tumor microenvironment (TME), drive cancer progression. Accumulation of activated CAFs is linked to poor prognosis in CRC cases and recurrence of cancer (Takatsuna et al., 2016). The CAF activation is mediated by secretomes including soluble growth factors (e.g., transforming growth factor-beta 1-TGF-β1), secreted by neoplastic cells and this promotes CRC heterogeneity, evolution, and progression (Ahmad Zawawi et al., 2022).

CAFs are highly heterogeneous, which may arise or trans-differentiate from different cell types. Particularly,

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highly contractile CAFs with myofibroblast-like phenotype has been revealed to promote desmoplastic tumors via extracellular matrix (ECM) deposition (Ueno et al., 2017). Desmoplastic tumors often manifest advanced stages of invasion and metastasis which is correlated with worse prognosis in CRC patients (Son et al., 2019). This CAF phenotype can be studied using conventional markers like alpha-smooth muscle actin (α-SMA), vimentin and fibroblast-specific protein 1 (FSP1). However, these markers' expression is found to be heterogeneous and non-specific to CAF (Öhlund et al., 2017). The lack of robust CAF markers has led to confusing definitions of CAFs thus limiting further research on CAF-cancer cell crosstalk and their role in CRC carcinogenesis. This highlights the need to discover novel markers of CAF for further investigation on the underlying mechanism which correspond to cancer aggressiveness and to design more targeted therapy for CRC.

Thereafter, amine oxidase copper containing 3 (AOC3) and leucine-rich repeat-containing 17 (LRRC17) expression have been reported previously for their potential as CAF markers (Hsia et al., 2016). AOC3 which encodes vascular adhesion protein-1 (VAP-1), is expressed on the endothelial cell surface and myofibroblast. Functionally, AOC3 is shown to regulate pulmonary fibrosis with its oxidase activity (Marttila-Ichihara et al., 2017). Low stromal AOC3 level is linked with poor prognosis in breast and lung cancer patients (Cha et al., 2018; Sun et al., 2017). As for other proposed marker, LRRC17, that acts as a negative regulator for osteoporosis in post-menopausal women (Hong et al., 2016), also shows potential functionality in cancer, as such in ovarian cancer via interaction with receptor activator of nuclear factor-κB ligand (RANKL) (Bapat et al., 2010). LRRC17 upregulation is associated with low survival in ovarian cancer patients (Oh et al., 2020).

To date, AOC3 and LRRC17's roles in the activation of CAFs and CRC development (Ward et al., 2016), remain poorly understood. Henceforth, we investigated the AOC3 and LRRC17 expression in colonic fibroblasts to characterize their expression and roles in colorectal carcinogenesis. This may provide novel insights into the underlying mechanisms and verify their potential as reliable CAF markers for future prognostic and therapeutic purposes.

Materials and Methods

Cell culture

Normal colonic myofibroblast cell line derived from a 22-week-old female, CCD-112CoN (ATCC CRL-1541) were cultured in Dulbecco Modified Eagle Medium (DMEM) with 15% fetal bovine serum (FBS) (cat:10437010) and 1% 10,000 U/ml penicillin-streptomycin (Pen/Strep) (cat:15140122) (ThermoFisher Scientific, USA) till reached confluency.

Primary fibroblast cultures were established from surgical tissue samples of a CRC patient collected with informed consent at Hospital Universiti Sains Malaysia. Ethical approval for the study was acquired prior to sample collection (code no: USM/JEPeM/20120685). Primary fibroblasts were isolated from the fresh CRC tumor (denoted as CAFs) and normal adjacent colon tissue (normal fibroblasts, denoted as NFs) using Collagenase Type IV (ThermoFisher Scientific, USA) and cultured in DMEM with 10% FBS and 3% Pen/Strep (complete medium). Human epithelial CRC cell line, SW620 (ATCC CCL-227) was included as negative control and cultured in DMEM with 10% FBS and 1% Pen/Strep. All cells were maintained in a humidified incubator at 37°C, 5% CO_2.

Immunofluorescence (IF) analysis

IF staining was conducted to study the expression of AOC3 and LRRC17 in various cell types. Conventional fibroblast activation marker, α-SMA was included in the experiment.

CCD-112CoN, isolated CAFs and NFs, and SW620 were seeded at 3 x 10^4 on a round glass coverslip in 24-well plates, cultured for 24 hrs to allow cell attachment. Next, the cells were treated with different conditions; a) serum free (SF) medium (DMEM alone), b) complete medium (DMEM + 10% FBS + 1% Pen/Strep), c) conditioned medium of SW620 (CM-SW620), and d) DMEM + 10 ng/ml TGF-β1, for 72 hrs. CM-SW620 was prepared by incubating 85% confluence SW620 with SF medium for 48 hrs before it was collected.

The cells were then fixed with 4% paraformaldehyde (Sigma-Aldrich, S6019215027, Merck KGaA, Germany) for 1 hr. Next, cells were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, STBJ7635, USA) in phosphate buffered saline (PBS) and blocked with PBS + 2% FBS for 30 mins. No fixation or permeabilization was applied for AOC3 live staining. The cells were subsequently immunostained with primary antibodies; VAP-1 antibody (100ng/ml TK8-14) (Santa Cruz Biotechnology, sc-33670, Europe), LRRC17 polyclonal antibody (200ng/ml VK3114774A, Invitrogen, Sweden), and mouse anti-α-SMA antibody IgG2a (200ng/ml ab 7817, Abcam, USA) at 4°C overnight. The cells were then incubated with fluorochrome-conjugates Alexa Fluor 488 Goat Anti-mouse, IgG (H+L) and Alexa Fluor 555 Donkey Anti-rabbit IgG (H+L), (Thermo Fisher Scientific, USA) in the dark at room temperature for 1 hr. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole hydrochloride (DAPI) (1ng/ml, Santa Cruz Biotechnology, Europe) for 15 mins. Coverslips were mounted on the microscopic slides. Images were taken using a fluorescence microscope (Olympus, BX41). Fluorescence signal intensity was analyzed via ImageJ 1.53k software. The experiments were performed in duplicates.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

MTT assay was performed to study fibroblast proliferation. CAFs and NFs were seeded at three different concentrations (1 x 10^4, 3 x 10^4, and 5 x 10^4) in 96-well plates, cultured for 24 hrs, and treated under different conditions for 72 hrs as mentioned in the previous section. Cells were subsequently incubated with MTT reagent and solvent for 3 hrs and 15 mins. The optical density (OD) was recorded by reading absorbance at a wavelength 590 nm using a Varioskan Flash (Thermo

Fisher Scientific, USA) spectrophotometer. A higher OD value indicates greater cell proliferation. The experiments were performed in triplicates.

Collagen Gel Contraction Assay

The contractile ability of CAF and NF under different conditions was analysed using a collagen gel contraction assay. Collagen matrix contractility is an indicator of CAF-myofibroblast which is enriched with ECM products (Kalluri et al., 2016).

In this experiment, CCD-112CoN and isolated CAFs and NFs were resuspended at 3 x 10^4 cells/ml with SF medium and immediately mixed with 3 mg/ml Collagen I, rat tail (Santa Cruz Biotechnology, sc-136157, Europe). The cell mixture was prepared on ice and immediately transferred into a 24-well plate. The collagen and cell mixture were solidified in the incubator (37°C, 5% CO_2) for 20 mins. 500 µl of; a) SF medium, b) complete medium, and d) DMEM+10ng/ml TGF-β1 was added to each well. The gels were dissociated by running a 200 µl pipette tip along gel edges to keep the gels free from the well. Subsequently, the 24-well plate was incubated in the humidified incubator and the gel contraction was recorded at the four time points (24, 48, 72, 96 hrs). The digital images of gel were taken using a digital camera and diameter (*d*) of the gel was measured by using a ruler. The contractility of different groups was calculated using the following formula: [(*d*control/*d*control)100%]- [(*d*group/*d*control)100%].

Greater fibroblast contractility is indicated by smaller diameter of the gel. The assay was repeated thrice.

Statistical Analyses

All data(s) for different analyses are presented as mean±SEM and IBM SPSS Statistics 27 was employed for all statistical analyses. The Kruskal Wallis test with post-hoc Bonferroni correction was adopted for comparison between groups. The paired two-tailed t-test was used for comparison between two respective groups. The p-value<0.05 shows statistical significance and p<0.01 indicated higher statistical significance.

Results

Positive expression of AOC3, LRRC17, and α-SMA in fibroblasts

The patient's clinicopathological characteristics are shown in Table 1. The IF analysis displayed striking differences in protein fluorescence intensity staining between all fibroblast groups and SW620 (Figure 1) for selected biomarkers in this study. All fibroblasts were positively stained with AOC3, LRRC17, and α-SMA but negative staining of these markers was detected in SW620. Interestingly, stronger IF staining of AOC3, LRRC17, and α-SMA was observed in CAFs compared to NFs and CCD-112CoN. AOC3 expression was pretty much scattered around the plasma membrane and other intracellular structures like endosome and nucleus while LRRC17 expression was concentrated in the extracellular and prominently nucleus, whereas α-SMA localized in cytoskeletal part, including stress fibers, suggesting a difference in staining localization. Different localization of these proteins in the present study is supported by information stated in the GeneCards database (http://www.genecards.org/).

Downregulation of AOC3, upregulation of LRRC17 and α-SMA signify more activated state of fibroblasts/ CAF phenotype

To determine the effect of fibroblast activation on the expression of AOC3 and LRRC17, all fibroblasts were treated under different conditions; a) SF medium, b) complete medium, c) CM-SW620, and d) DMEM + 10 ng/ml TGF-β1. Lower fluorescence intensity of AOC3 staining was observed in complete medium (addition of 10% FBS), CM-SW620, and TGF-β1 when compared to SF medium. However, a significant difference in the staining is found in the TGF-β1-treated group in comparison to SF medium (Figure 2(a-c)). In contrast, LRRC17 and α-SMA co-expression were greater in complete medium and TGF-β1 in comparison to SF medium. LRRC17 higher expression in TGF-β1-treated group was significant in CAFs, NFs, and CCD-112CoN. Interestingly, higher expression of α-SMA, indicative of activated or CAF characteristic, only revealed significant differences in TGF-β1 to SF medium in NFs and CCD-112CoN, not CAFs. We observed a difference in morphology in TGF-β1-treated CAFs, NFs, and CCD-112CoN where these cells presented with slightly thicker elongated bodies and more prominent indented nuclei, mimicking the CAF-myofibroblast phenotype different to that in SF medium (Figure 3).

Influence of different factors on fibroblast proliferative and contractility capacities

MTT assay result of CAFs and NFs treated with different conditions; a) SF medium, b) complete medium, c) CM-SW620, and d) DMEM + 10 ng/ml TGF-β1 is shown in Figure 4(a-b). Both CAFs and NFs were grown in complete medium and CM-SW620 exhibited higher proliferation (higher absorbance) than SF medium. No

Table 1. Clinicopathological Characteristics of the Selected CRC Patient

Features	Patient characteristics
Sex	Female
Age at resection	64
Anatomical site of CRC	Sigmoid colon
Histological subtype and tumor differentiation (WHO Classification 5th Edition)	Moderately differentiated adenocarcinoma
TNM Stage	pT4a N0 (Stage IIB) [a]
Pattern of growth	Cribriform
Peritumoral lymphocytic, lymph node, lymphovascular infiltration	Moderate, Absent, Absent
Surgical Margin	Not involved
CEA	188.2 ng/ml

World Health Organization,WHO; Tumor Node Metastasis,TNM; Carcinoembryonic antigen, CEA; a UICC AJCC Cancer Staging Manual 8th Edition

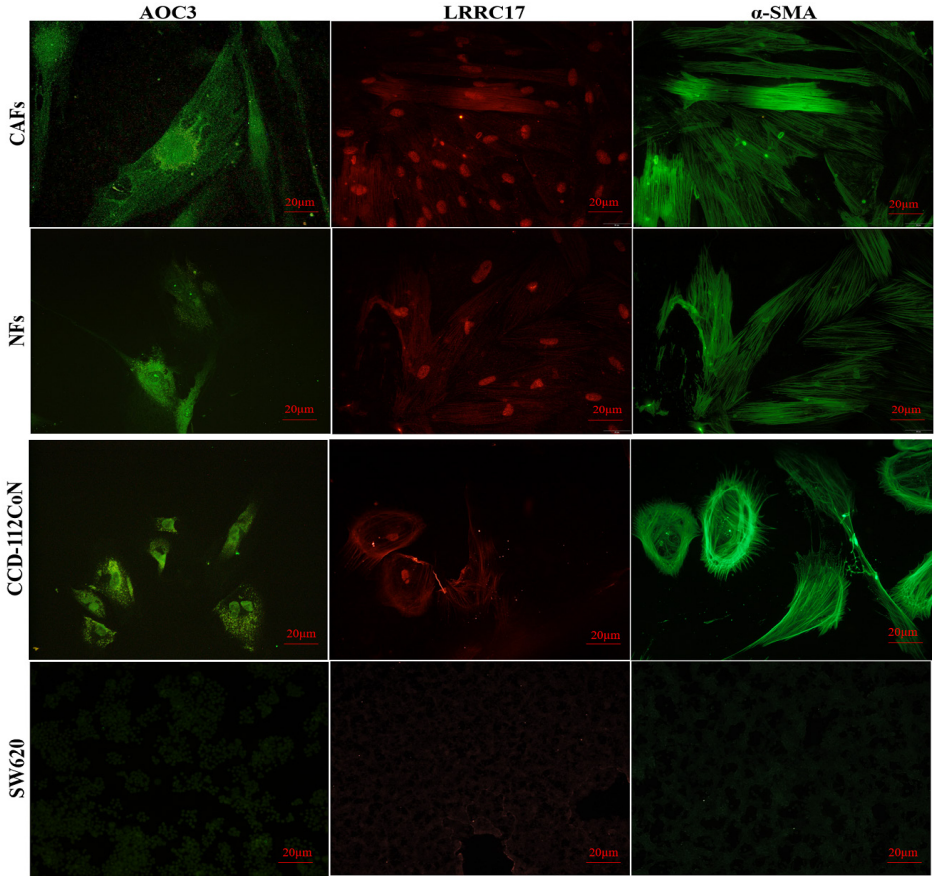

Figure 1. IF of AOC3, LRRC17 and α-SMA on CAFs, NFs, CCD-112CoN, and SW620 (Magnification: 400x).

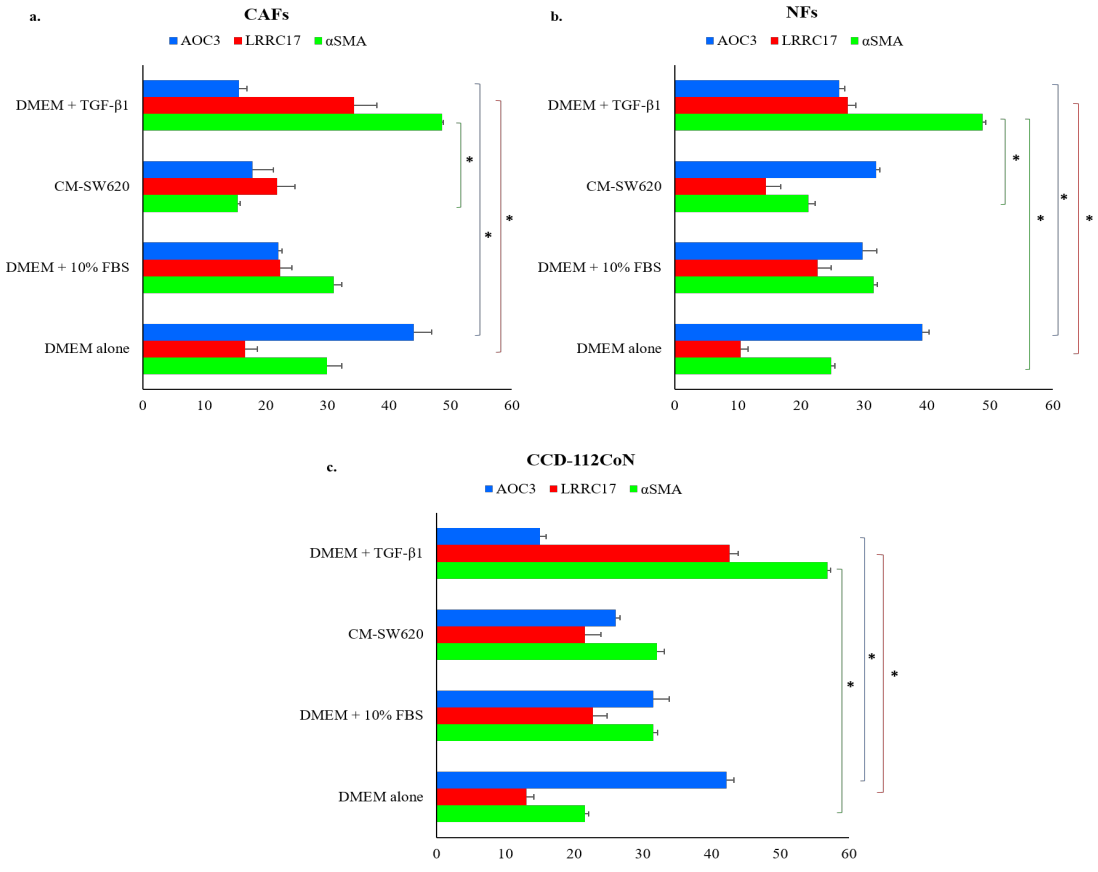

Figure 2. AOC3, LRRC17 and α-SMA Expression under Different Conditions in (a) CAFs, (b) NFs, and (c) CCD-112CoN. Data are presented as mean±SEM from three separate experiments. *p-value<0.05; **p-value<0.01, Kruskal Wallis test with post-hoc Bonferroni correction.

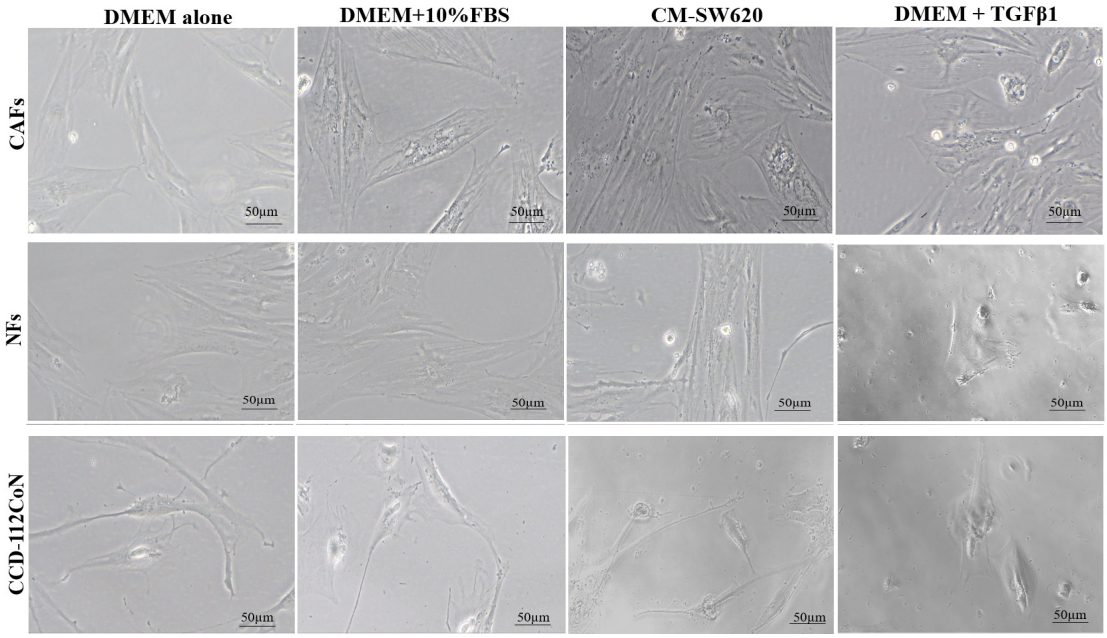

Figure 3. Representative Images of Morphology of CAFs, NFs, and CCD-112CoN Treated under Different Conditions (Magnification: 200x).

significant difference in proliferation was found between these two conditions compared to SF medium.

For collagen gel contraction assay, different rates of collagen gel contraction were observed across CAFs, NFs, and CCD-112CoN groups. The greatest diameter of gel was found in CAFs in SF and complete medium at 24 hrs while the smallest diameter of the gel was recorded in NFs in complete medium and TGF-β1 at 96 hrs. Generally, gel contraction is directly proportional to treatment duration where highest fibroblast contractility was seen at 96 hrs post-treatment for all groups. Differences in contractile ability were observed between CAF, NFs, and CCD-11CoN groups. The addition of 10% serum in complete medium triggered greater contraction in NFs and CCD-112CoN with a significant difference to that of SF medium (Figure 5(a-c)). We also found that TGF-β1 caused a very minimal effect in cellular proliferation but significant increase in contraction in NFs and CCD-112CoN compared to SF medium. Minimal effect of CAFs proliferation is more apparent than NFs.

Discussion

The AOC3 and LRRC17 expressions in CAFs, NFs, and CCD-112CoN from IF staining have provided the characterization specific for activated state of fibroblast that transdifferentiates into CAF-myofibroblast under those differing conditions. We managed to replicate results from Hsia et al., (2016) whereby AOC3 was utilized to verify the potential characterization of a subgroup CAF with myofibroblast phenotype. Additionally, we managed to elucidate LRRC17 expression in this subgroup. This CAF subgroup is thought to mediate cancer invasion and metastasis by increasing ECM deposition and stiffness (Mellone et al., 2016), hence suggesting AOC3 and LRRC17 expression in regulating tumor biology. The soluble AOC3 has been detected in CRC, though little is described on its function (Ward et al., 2016; Toiyama et al., 2009). Few reports on other cancers suggested a correlation of downregulated AOC3 expression with lymph node metastasis in lung cancer, and LRRC17 as

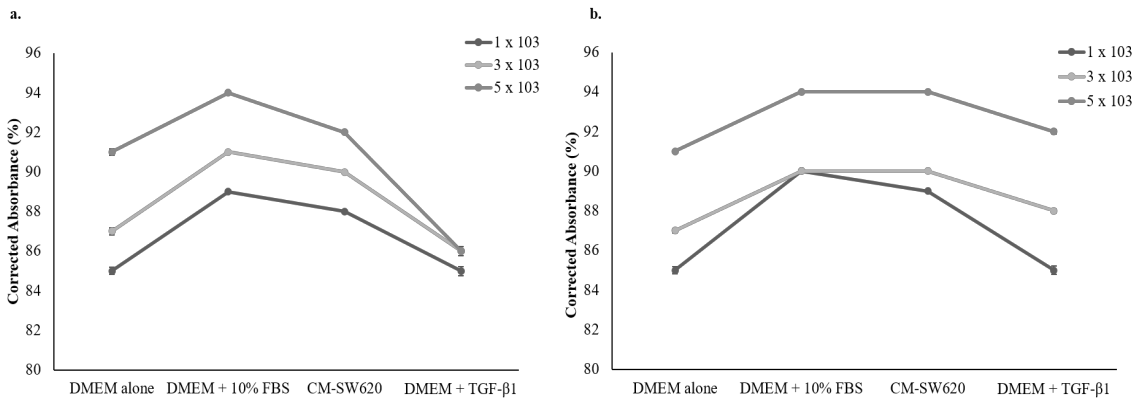

Figure 4. Proliferation of (a) CAFs and (b)NFs were assessed via MTT assay at OD 590 nm. The plots are presented as mean (corrected absorbance (%))±SEM. Computed using Kruskal Wallis for statistical significance difference.

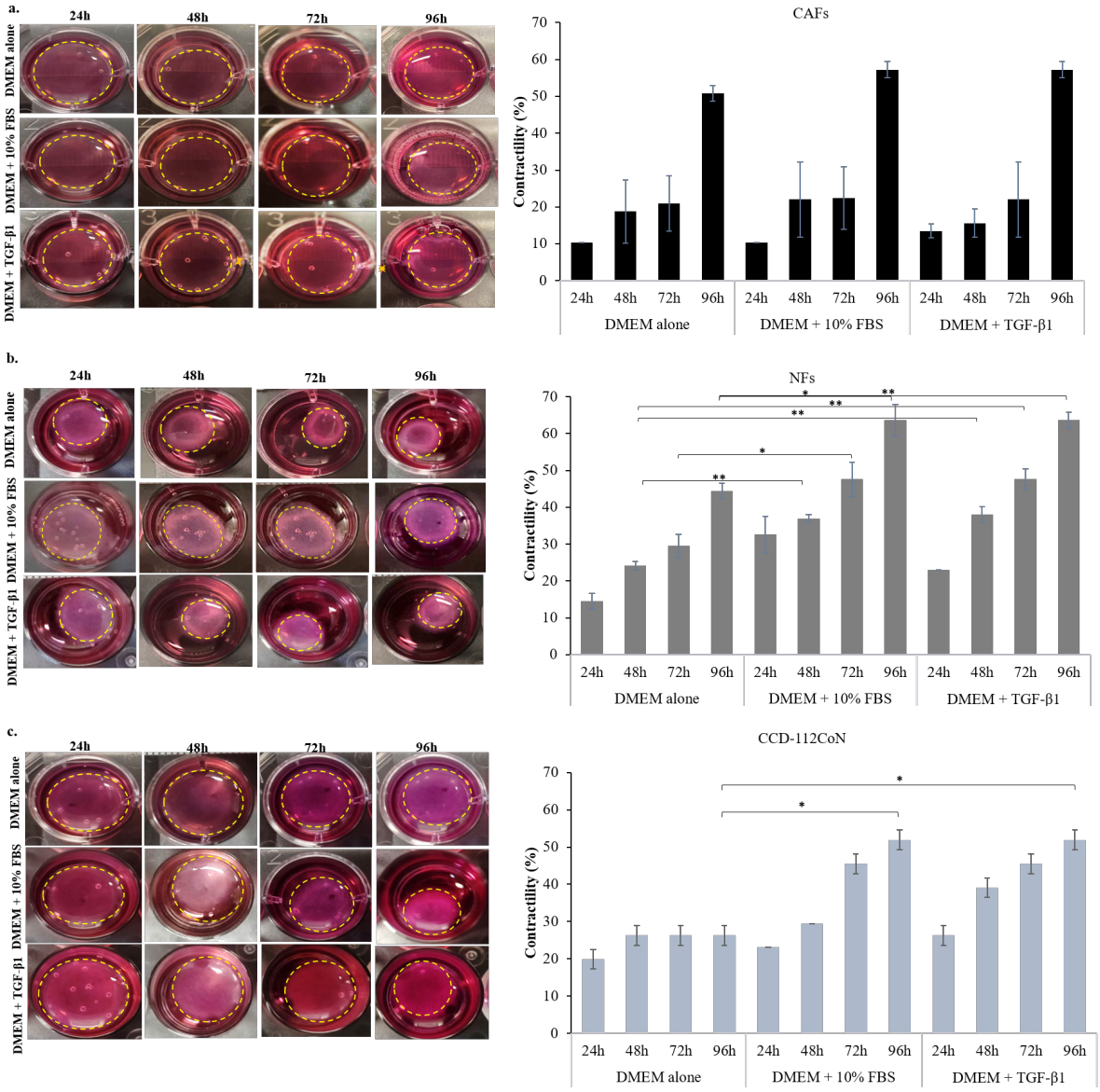

Figure 5. Contractility (%) of (a) CAFs, (b) NFs, and (c) CCD-112CoN under different conditions were assessed over different time points using contraction gel assay. Data are presented as mean±SEM from triplicate experiments. *p-value<0.05; **p-value<0.01, two-tailed paired t-test

a negative regulator of cell viability via p53 pathway in ovarian cancer (Chang et al., 2021; Oh et al., 2020). These potential protumorigenic properties of AOC3 and LRRC17 might explain the CAF phenotype from AOC3- and LRRC17-expressed fibroblasts. However, both proteins' functional role in the context of colonic CAF remains to be elucidated. Further downstream analysis of the protein expression in CAF may provide information on the underlying mechanism.

Primarily, α-SMA is not only the hallmark of CAF activation, but also used for identification of myofibroblasts. Trans-differentiation of fibroblasts into myofibroblasts and/or CAFs was proposed previously but this notion is highly debated still, considering multiple reports on differences in the definition and classification of heterogeneous fibroblast populations in the TME. Nevertheless, α-SMA expression was shown to be non-specific and heterogeneous as found in smooth muscle cells and the differential expression within a tumor (Öhlund et al., 2017). This sheds light on the CAF heterogeneity which may also reflect different hierarchies or stages of fibroblast activation (Kwa et al., 2019). Indeed, NFs in response to TGF-β1 may resemble the activated phenotype different to that of its origin condition in SF medium as evidenced by the significant α-SMA upregulation whereas CAFs are likely at more activated stage in its origin comparable to NFs, hence small differences of α-SMA expression between CAFs in TGF-β1 and SF medium. These are further corroborated by a significant increase in contractility and proliferation in NFs different to that of CAF in complete medium and TGF-β1. Additionally, the significant increase in CCD-112CoN contractility observed only after prolonged 96 hrs treatment in complete medium and TGF-β1 may indicate different stages of activation to that of NFs. Given that, size and growth rate of these cells may vary from the different stages of activation and affect the above results despite the constant cell density seeded.

Investigation into factors and pathways that are essential in CAF-regulated phenotype allows precise CAF characterization (Guillén Díaz-Maroto et al., 2019). The serum supplementation is often used for human fibroblast expansion and cultures (Rittié and Fisher, 2005). The changes in selected proteins' expression in complete medium relative to SF medium which in line with previous finding (Hsia et al., 2016) indicate the cellular activation, presumably activated by components in FBS. This may include growth factors such as TGF-β1, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF) (Lee et al., 2022). However, since little information is available on the exact constituents of FBS, this remains inconclusive. The significant increase of collagen contractility in NFs and CCD-112CoN upon treatment of FBS correlates to higher α-SMA expression. Supported by previous studies, it is noteworthy that FBS exhibit more profound effect in cellular proliferation than trans-differentiation. We observed the typical fibroblast morphology was maintained in CAFs, NFs, and CCD-112CoN upon treatment with complete medium (addition of 10% FBS), in agreement with previous report on corneal fibroblast phenotype (Maltseva et al., 2001). Increased proliferation of CAFs and NFs seem to mimic CCD-18Co and myofibroblasts isolated from CRC patients dramatic growth in complete medium compared to SF medium (Musa et al., 2020). Plus, present results are in line with Berglund et al. (2017) where mesenchymal stem cells displayed better growth in TGF-β1-suplemented with FBS compared to TGF-β1 alone which correlate with PDGF and FGF in FBS. However, no significant results in the current work may suggest indirect effect of FBS that lead to non-rapid stimuli of proliferation.

Our study also revealed the importance of crosstalk between epithelial cancer cells and CAFs from the coculture of fibroblasts with SW620 via treatment of CM-SW620. Cancer secretomes promote CAF phenotype that led to malignant progression. SW620 and SW480 cocultures greatly expressed ECM protein like matrix metalloproteinase 2 (MMP2) (Karagiannis et al., 2012). Increased ECM stiffness contributes myofibroblast trans-differentiation (Kalluri et al., 2016). Nonetheless, the SW620 secretomes may play selective role towards fibroblast activation as no significant effect in AOC3 and LRRC17 expression in CAFs, NFs, and CCD-112CoN in CM-SW620. This may propose variation in paracrine interaction between different type of cancer cells and fibroblasts. Bhome et al., (2022) has recently demonstrated the epithelial characteristic of SW620 which cause low TGF-β1 mediated CAF trans-differentiation. SW620 release of miR-200 inhibit TGF-β1 mediated CAF trans-differentiation as the downregulation of α-SMA. Similarly, CM-SW620 downregulated α-SMA in CAFs and NFs compared to SF medium. This also suggests different signalling pathway related to hosts response (i.e., colonic fibroblasts) in the coculture which cause a minimal effect in CAF trans-differentiation.

TGF-β is a main driver in CAF trans-differentiation (Jena et al., 2021). Unlike myofibroblast in fibrosis, CAF-myofibroblast subgroup negate apoptosis and continue to proliferate and secrete pro-tumorigenic factors. This activation stage might be induced by TGF-β1 prolonged exposure as demonstrated from the localized prostate cancer (Pidsley et al., 2018). Herein, 72 hrs of TGF-β1 treatment was shown to greatly promote LRRC17 but inhibit AOC3 expression in CAFs, NFs, and CCD-112CoN. This indicates more activated state of fibroblast mimicking CAF phenotype, as evidenced by the different morphology to that of cells in SF medium. TGF-β1 also leads to α-SMA upregulation with increased contractility in CAFs, NFs, and CCD-112CoN which are consistent with Hsia et al., (2016). Similar results were reported in CAFs of different tumor locations (Melling et al., 2018; Hinz et al., 2001). These characteristics are related to an increased ECM deposition that promote tumor invasiveness (Prasad et al., 2016). We demonstrated that TGF-β1-activated fibroblast resembles CAF phenotype as the differentiated morphology and increased ECM stiffness. Importantly, TGF-β1-signaling often relies on downstream factors for myofibroblast activation. This explains the minimal effect of TGF-β1 in cellular proliferation when compared to SF medium. Melling et al., (2018) proposed the putative role of miR-145 in activation of CAF isolated from oral squamous cell carcinoma by targeting TGF-β1. Specific to colonic CAFs, Ca2+/ calmodulin-dependent protein kinase II (CaMKII) has been shown to regulate the TGF-β1-induced CAF trans-differentiation and proliferation (Chen et al., 2021).

Some limitations in the current study must be addressed whereby the samples were extracted from similar entity of CRC (left-sided CRC). Given that, these findings may not be able to fully represent the heterogeneous nature of CRC TME.

Further comparative analysis between FBS and SF medium with selected factors may provide better clarification into the roles of serum on human fibroblast activation. Additionally, the coculture of primary colonic fibroblasts and SW620 may not be sufficient to inclusively highlight the cancer secretomes effect in CAF generation. Hence, further studies are warranted by including other CRC cell lines with different properties such as SW480, HT-29, and DLD-1. Also, future efforts into looking the downstream factors of TGF-β1 may provide critical insight of TGF-β1-induced colonic fibroblast activation mechanism.

In conclusion, our study acts as a preliminary finding, proposing AOC3 and LRRC17 as emerging biomarkers for colonic fibroblast activation which drive CRC carcinogenesis and demonstrated their correlations with cellular proliferation, and contractility. Further work such as single-cell analysis is needed to clarify the expression of AOC3 and LRRC17 in various CAF subgroups thus to further elucidate the potential of these novel markers in clinical use. We also demonstrated that FBS, SW620 secretomes, and TGF-β1 regulate fibroblast activation differently, depending on their origin and phenotype. TGF-β1-activated fibroblasts seems to mimic the CAF phenotype with differentiated morphology and strong contractile phenotype. Different pattern of expressions among the selected markers and characterization in CRC suggest different mechanisms underlying and potential

relevance as novel markers in CRC.

Author Contribution Statement

Sahira Syamimi Ahmad Zawawi (SSAZ) and Nur Azlien Shahira Mohd Azram (NASMA) performed the experiments. SSAZ and Marahaini Musa (MM) performed data analysis and manuscript writing. MM and Sarina Sulong (SS) involved in grant administration. MM conceptualized the study design and reviewed the manuscript. Andee Dzulkarnaen Zakaria (ADZ) and Lee Yeong Yeh (LYY) assisted in patient recruitment and collection of tumor tissues. Nur Asyilla Che Jalil (NACJ) assisted in the collection of clinicopathological data of patients.

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Ethical approval

Ethical approval for the study was acquired prior to sample collection from the Human Research Ethics Committee, USM (code no: USM/JEPeM/20120685).

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