

Knockdown of CD-74 in the Proliferative and Apoptotic Activity of Breast Cancer Cells

Hussain Al Ssadh¹, Waleed Al Abdulmonem^{2*}, Zafar Rasheed³, Inamul Hasan Madar⁴, Jamila Alhoderi¹, Samah K. Nasr Eldeen^{5,6}, Ali Alradhwan⁷, Noura Alasmael⁸, Abdullah Alkhamiss², Nelson Fernández¹

¹School of Biological Sciences, University of Essex, Colchester, UK; ²Department of Pathology, College of Medicine, Qassim University, Qassim, Saudi Arabia; ³Department of Medical Biochemistry, College of Medicine, Qassim University, Saudi Arabia; ⁴Department of Biotechnology and Genetic Engineering, Bharathidasan University, Tiruchirappalli, India; ⁵Clinical Laboratory Sciences, Inaya Medical College, Riyadh, Saudi Arabia; ⁶Central Laboratories, Egyptian Ministry of Health, Tanta, Egypt; ⁷Biochemistry Department, College of Medicine, Imam Abdulrahman Bin Faisal University, Saudi Arabia; ⁸College of Medicine, King Saud University, Saudi Arabia

Abstract

Citation: Al Ssadh H, Al Abdulmonem W, Rasheed Z, Madar IH, Alhoderi J, Eldeen SKN, Alradhwan A, Alasmael N, Alkhamiss A, Fernández N. Knockdown of CD-74 in the Proliferative and Apoptotic Activity of Breast Cancer Cells. Open Access Maced J Med Sci. 2019 Oct 15; 7(19):3169-3176.
<https://doi.org/10.3889/oamjms.2019.354>

Keywords: CD 74; Knockdown; Proliferation; Apoptosis; Breast cancer; CAMA-1; MDA-MB-231 cells

***Correspondence:** Waleed Al Abdulmonem. Department of Pathology, College of Medicine, Qassim University, Qassim, Saudi Arabia. E-mail: waleedmonem@qumed.edu.sa

Received: 01-Jul-2019; **Revised:** 01-Aug-2019; **Accepted:** 02-Aug-2019; **Online first:** 14-Oct-2019

Copyright: © 2019 Hussain Al Ssadh, Waleed Al Abdulmonem, Zafar Rasheed, Inamul Hasan Madar, Jamila Alhoderi, Samah K. Nasr Eldeen, Ali Alradhwan, Noura Alasmael, Abdullah Alkhamiss, Nelson Fernández. This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International License (CC BY-NC 4.0)

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

BACKGROUND: The cluster of differentiation (CD) 74 is known for its immunological functions and its elevated level was reported in various cancer cells.

AIM: The aim of the present study was to investigate the expression and potential roles of CD74 in the proliferative and apoptotic activity of breast cancer.

METHODS: Expression of CD74, macrophage migration inhibitory factor (MIF) and CD44 was assayed in CAMA-1 and MDA-MB-231 cell lines using flow cytometry. CD74 was knocked down using CD74 siRNA-transfection in CAMA-1, and MDA-MB-231 cells and proliferation and apoptosis were determined in the transfected breast cancer cells.

RESULTS: The data showed that CD74, MIF and CD44 were expressed in breast cancer cell lines and were associated with cell proliferation and apoptosis. Correlation analysis revealed that CD74 was positively correlated and colocalised with MIF on the cell-surface of CAMA-1 and MDA-MB-231. The knockdown of CD74 significantly reduced CAMA-1 and MDA-MB-231 cell proliferation and increased the level of apoptotic cells.

CONCLUSION: We concluded that the interactions of CD74 with MIF and CD74 with CD44 could be a potential tumour marker for breast cancer cells. Moreover, the level of co-expression of MIF and CD74 or CD44 could be a surrogate marker for the efficacy of anti-angiogenic drugs, particularly in breast cancer tumours. In short, the study revealed the potential roles of CD74 in the proliferation and apoptosis of breast cancer which may serve as a potential therapeutic target for breast cancer.

Introduction

The role of a cluster of differentiation (CD) – 74 is a transmembrane glycoprotein, and its role has recently been reported in the pathogenesis of several cancers including breast cancer [1], [2]. Several studies have suggested that a small proportion of intracellular CD74 is modified by the addition of chondroitin sulfate (CD74-CS), a form of CD74 and chondroitin sulfate is a sulfated glycosaminoglycan usually found attached to proteins as part of a proteoglycan [3], [4]. CD74-CS is expressed on the surface of immune cells and can bind MIF, mediating MIF's signalling pathway [3], [4]. Cell-surface

expression of CD74 is not strictly dependent on the expression of class II MHC molecules in term of antigen presentation [5], [6] and numerous non-class II positive cells express CD74 which functions as a receptor for the initiation of different signalling cascades [7], [8]. MIF is the natural ligand of CD74 and binds to the extracellular domain of CD74 with high affinity (KD = 1.40 Å ~ 10-9 M) and initiates a signalling cascade [9]. When bound to the extracellular domain of CD74, MIF promotes signalling pathways including cell proliferation and apoptosis [9], [10], [11], [12], [13]. The short cytoplasmic tail of CD74 lacks an intracellular signal-transducing domain, although serine phosphorylation takes place in the P35 variant of CD74, requiring CD44, a

polymorphic transmembrane protein with kinase activating properties [14], [15]. CD74 forms a complex with CD44 which is essential for the MIF-induced signalling cascade [10], [16]. This cascade induces phosphorylation of ERK1 and ERK2 and activates various effector proteins involved in inflammatory processes and cell proliferation. ERK1 and ERK2 remain phosphorylated for many hours and hence this cascade continues for up to 2 to 3 hours [17], [18], [19], [20], [21]. Concurrently, MIF binding to CD74 activates the P13K-Akt pathway leading to phosphorylation of BAD and BAX proteins which are involved in apoptosis [22]. In addition, this cascade augments Bcl-2 expression, further supporting cell survival [23], [24], [25]. Thus, the binding of MIF to the CD74 / CD44 complex initiates a pathway resulting in the proliferation of the mature B cell population and their rescue from cell death. In addition to activating the P13K-Akt pathway, MIF binding to CD74 also induces a signalling pathway which involves Syk tyrosine kinase [6], [16] and induces cleavage of intramembrane CD74 regional releases intracellular domain (CD74-ICD) [26], [27]. CD74-ICD translocates to the nucleus where it induces activation of transcription mediated by the NF- κ B p65 / RelA homodimer and its co-activator, TAFII105, resulting in regulation of transcription of genes that control B cell proliferation and survival [3], [6], [16]. Therefore, the CD74-MIF-CD44 complex initiates a pro-survival signal leading to the increase of proliferation and inhibition of apoptosis.

Recently, we quantified colocalization of CD74 and CD44 in breast cancer cells through non-invasive and validated bioimaging procedure [28] and also determined several novel biomarkers involved in the pathogenesis of breast cancer [29]. Not only have these, but we also showed that treatment of human breast cancer cells with interferon- γ up-regulates the expression of CD74 along with MIF and CD44 [2], [30]. In continuation of these studies, the present study was hypothesised to find out the potential roles of CD74 in the proliferative and apoptotic activity in breast cancer. This was achieved by studying the colocalization of CD74 and MIF as well as CD74 and CD44 by two different techniques confocal microscopy and immunoprecipitation. The cells proliferation and apoptosis in CD74 siRNA transfected cells were also studied to address the hypothesis that blocking CD74 or MIF would affect apoptosis and cell proliferation.

Methods

Cell lines and cell culture

Two human mammary gland cell lines, CAMA-1 and MDA-MB-231, were used, which were derived from a malignant pleural effusion. The CAMA-1 cell lines were maintained in RPMI 1640 medium

(LONZA-Belgium), supplemented with 10% (v / v) fetal calf serum (FCS; Imperial Laboratories, Andover, UK). The MDA-MB-231 cell line was maintained in D-MEM (high glucose), supplemented with 10% FCS. Raji cells (human negroid Burkitt's lymphoma) and HeLa cells (human cervical cancer), expressing high levels of CD74, MIF, and CD44, respectively, served as additional positive controls. Raji and HeLa cells were cultured in RPMI 1640 (LONZA-Belgium) containing 10% FCS and cultured in a humidified atmosphere of 5% CO₂ at 37°C. All media used for this study were purchased from PAA Laboratories GmbH (Pasching, Austria).

Flow cytometry analysis

Cell lines were lifted with Accutase (Sigma-Aldrich), and 1 x 10⁶ cells were used per sample. Monoclonal primary antibodies, By2 (anti-CD74), ab55445 (anti-MIF) and 156-3c11 (anti-CD44) were employed in indirect immunofluorescence staining. Cells were preincubated with saturating concentrations of primary antibody, followed by washing and labelling with FITC-conjugated goat anti-mouse IgG (Bio-legend). For cell surface staining, cells were fixed with 4% formaldehyde solution and washed with 1X phosphate-buffered saline (PBS). The cells were then blocked with blocking buffer (PBS / 0.1% BSA, bovine serum albumin) and washed in PBS. Primary and secondary antibodies were diluted with 0.1% BSA in PBS. Cells were sorted on a BD FAS Aria and analysed by FlowJo 8.8.6.

Immunofluorescence

In preparation for confocal immunofluorescence microscopy for studying colocalization between CD74 and MIF, CAMA-1 and MDA-MB-231 cells were cultured in LabTek 8-well chambers (Thermo Fisher Scientific) at a density of 6 x 10³ cells per well for two days. Following this, they were seeded. The cells were fixed with 4% paraformaldehyde for 20 min on ice. Cells were then blocked with 2% (w / v) BSA prepared in 1X PBS for 1 h at room temperature. For single staining of each antigen, cells were incubated with anti-CD74 (clone: By2) at a concentration of 1:500, (anti-MIF) (clone: ab55445) and 156-3c11 (anti-CD44) at a concentration of 1:400 for 1 h, followed by three washes with PBS. Secondary antibody, anti-mouse IgG conjugated with Alexa Fluor® 488 or Alexa Fluor® 555 (Invitrogen, Carlsbad, CA, USA), was used at a dilution of 0.25 μ g / 100 ml for 1 h. For double staining, cells were blocked again with 2% BSA and the staining process was repeated for each desired pair. Cells were thoroughly washed with PBS, the chambers removed, and the slide was mounted with anti-fade mounting medium (Vector Shield) covered with a coverslip (Chance proper LTD, West Midlands, England) and sealed with rubber cement (Fixogum

Rubber Cement, Marabu, Germany). Cells were incubated with a primary antibody followed by a secondary antibody. CD74 was labelled with FITC Alexa Fluor 488 (green) and CD44 was labelled with Alexa Fluor 555 (red). Colocalization of CD74 with CD44 was assessed by Pearson's correlation coefficient was used to analyze the degree of colocalization. The scale lay between -1 and 1, where 1 stand for colocalization, -1 stands for negative colocalization and 0 stands for no colocalization. 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) counterstain (Vector Laboratories, Burlingame, CA, USA) was used at a 1:250 dilution.

Quantitative colocalization analysis of confocal fluorescence microscopy images

To investigate whether CD74 and MIF colocalize, a high-precision single-cell bioimaging protocol was employed, previously developed by our research group [31]. The Pearson correlation coefficient (PCC) was used for quantitative analysis of colocalization [31], [32]. PCC provides the overall association of two probes in an image, statistically. It also indirectly measures the quantity, i.e. the fraction of one protein that colocalises with another protein. A Nikon A1Si confocal microscope (Nikon Instruments Inc.) with a plan-apochromatic VC1.4 N.A. 60X magnifying oil-immersion objective was used for image acquisition. Images were acquired in three channels, using one-way sequential line scans. DAPI was excited at 398.7 nm with laser power 1.6 arbitrary units, and its emission was collected at 450 nm with a PMT gain of 86. Alexa Fluor 488 was excited at 488 nm with laser power 5.8; its emission was collected at 525 nm with a PMT gain of 117. Alexa Fluor 555 was excited at 560.5 nm with laser power 3.7, and its emission was collected at 595 nm with a PMT gain of 98. The scan speed was $\frac{1}{4}$ frames/s (Galvano scanner). The pinhole size was 35.76 μm , approximating 1.2 times the Airy disk size of the 1.4-NA objective at 525 nm. Scanner zoom was centred on the optical axis and set to a lateral magnification of 60 nm/pixel. Axial step size was 105 nm, with 80-100 image planes per z-stack.

Small interfering (si) RNA transfection

CAMA-1 and MDA-MB-231 cell lines were seeded in six-well plates at a density of 2×10^5 per well in 2 ml normal growth medium supplemented with 10% FCS. The cells were then allowed to grow until they reached 60-80% confluency. For each transfection, 4 μl of CD74 siRNA duplex at dose of 80 pmols (sc-35023) (Santa Cruz Biotechnology, USA) was diluted and 4 μl of siRNA transfection reagent was added at dose of 80 pmols (sc-29528) (Santa Cruz Biotechnology, USA) into 100 μl of siRNA transfection medium (sc-36868) (Santa Cruz Biotechnology, USA) separately without serum or

antibiotics. Both diluents were mixed and incubated for 15-45 minutes at room temperature. Cells were then washed with 2 ml of siRNA transfection medium. The siRNA transfection reagent mixture was then added to each well, and the volume made up to 1 ml by adding 800 μl of siRNA transfection medium (Santa Cruz Biotechnology, USA). In the same manner, this was applied for negative control siRNA (sc-44230) (Santa Cruz Biotechnology, USA). The cells were then incubated overnight at 37°C in a CO₂ incubator for 18-24 hr. Following incubation, the medium was aspirated and replaced with fresh 1X normal growth medium. Cells were assayed using the appropriate manufacturer's protocol 24-72 hours after the addition of fresh medium in the step above. Transfection efficiency was confirmed by western blot and microscopy. Once the transfection was confirmed, the effect of CD74 siRNA on the proliferation and apoptosis of CAMA-1 and MDA-MB-231 could then be studied.

Proliferation and Apoptosis assay

CAMA-1 and MDA-MB231 cell lines were cultured in 6 well plates at a density of (15×10^3 cell/well) or in 96 well plates at a density of (15×10^2 cell/well) at 37°C and then transfected with CD74 siRNA duplex as explained previously. The cells were then washed twice with 1X PBS and incubated with 2 μl of Annexin V-FITC (BioLegend, UK) at room temperature for 20 minutes in the dark. Cells were then thoroughly washed and then fixed with 4% PFA followed by washing steps in PBS. Finally, the samples were read using BD FACSAria and analysed by FlowJo 8.8.6. In the same manner, the MTT assay was then used to assess cell proliferation. Briefly, 20 μl of MTT solution (5 mg/ml in PBS) and 100 μl was added per well, and cells were incubated at 37°C with 5% CO₂ in a humidified chamber for 4 hr for colour development. The resultant Formazan crystals were dissolved in dimethyl sulfoxide (100 μl) and the absorbance intensity measured at 595 nm using a microplate reader (Versamax). The percentage of cell proliferation was calculated relative to the rate of proliferation in untreated cells.

Results

Cell-surface expression of CD74, MIF and CD44

The cell-surface expression of CD74, MIF and CD44 were analysed in CAMA-1, MDA-MB-231. Non-permeabilized were stained with an appropriate concentration of By2 (anti-CD74), ab55445 (anti-MIF) and 156-3C11 (anti-CD44) antibodies followed by 1 μl RAM-FITC secondary antibody. Cells without staining and isotype cells, stained with only secondary

antibody, were used as a negative control. CD74, MIF and CD44 expression were detected on the cell surface and cytoplasmic of CAMA-1 and MDA-MB-231. Monocytes, Raji cells, cervical cancer HeLa cells, and lymphocytes, (Jurkat) cells, were used as a positive control as they express high levels of CD74, CD44, and MIF respectively. This is displayed in Figure 1 in where empty histograms show CD74, MIF or CD74 protein grey filled the histogram.

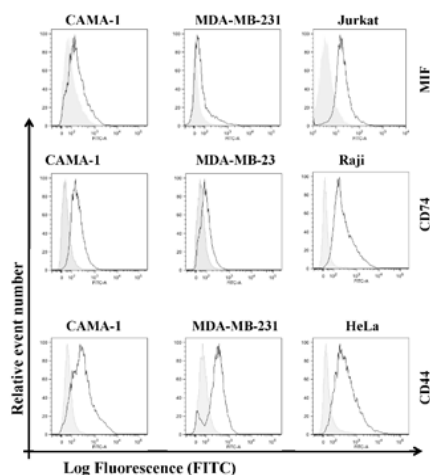


Figure 1: Flow cytometric analysis for cell surface expression of CD74, MIF and CD44 in the breast cancer cells displayed; Empty histograms represented the expression of CD74, MIF, and CD44; Expression in Raji, Jurkat, and HeLa cells are used positive controls. Whereas, grey-filled histograms were shown as a negative control obtained from isotype-matched with control antibody; The data are representative of three independent assays

Colocalization of MIF with CD74 and CD44

To investigate whether MIF colocalised with CD74 or CD44 on CAMA-1 and MDA-MB-231 cells, all cell lines were immunostained with an appropriate primary antibody followed by a secondary antibody (Figure 2 and Figure 3).

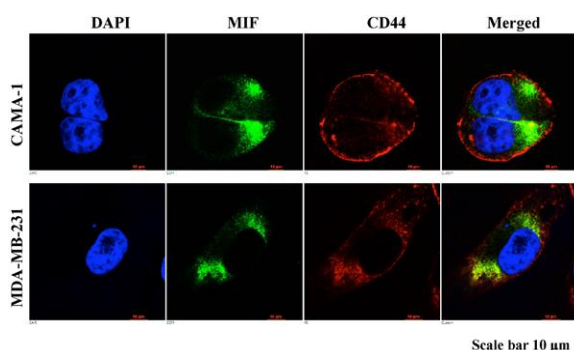


Figure 2: Colocalization of MIF and CD44 on the cell surface of CAMA-1 and MDA-MB-231 cells, determined by confocal microscopy analysis; CAMA-1 and MDA-MB-231 and MDA-MB-435 cells were cultured in LabTek 8-well chambers at a density of 10×10^3 cells per well overnight; The cells were stained with MIF labelled with Alexa Fluor® 488 (green) or CD44 labelled with Alexa Fluor® 555 (red); Cell nuclei were stained with 4', 6-diamidino-2-phenylindole (blue); Fluorochromes were acquired separately to evaluate the expression of CD44 and MIF; Yellow/orange fluorescence reveals the potential colocalization of two antigens. The images represent three different experiments

CD74 or CD44 was labelled with Alexa Fluor® (red) and MIF Alexa Fluor® (green). CAMA-1 and MDA-MB-231 cells showed clear expression of MIF, CD74, and MIF. Merging green and red channels assessed the colocalization and Pearson's product-moment correlation coefficient (PCC) was used to analyse the degree of colocalization.

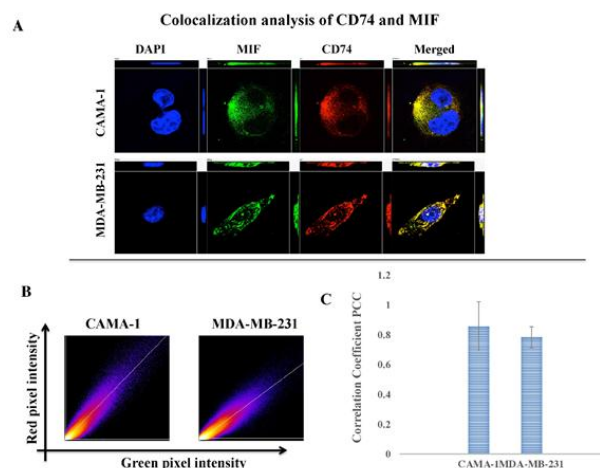


Figure 3: Colocalization of CD74 and MIF on the cell surface of CAMA-1 and MDA-MB-231 cells, determined by confocal microscopy analysis; A) CAMA-1 and MDA-MB-231 cells were cultured in LabTek 8-well chambers at a density of 10×10^3 cells per well overnight; Cells were stained with MIF labeled with Alexa Fluor® 488 (green) or CD74 labeled with Alexa Fluor® 555 (red); Cell nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI) (blue); Fluorochromes were acquired separately to evaluate the expression of CD74 and MIF; Yellow / orange fluorescence reveals the potential colocalization of the two antigens; 3D images were acquired in the stack, with z-direction step size $0.14 \mu\text{m}$ using NIS element; Single-plane of z-stack is shown in three directions as xy, yz and zx; Data represent three different experiments; B) Each pixel in the image was plotted in the scatter diagram based on its intensity level in each channel; The color in the scatterplot represents the number of pixels plotted in that region; In this example, green is shown on the x-axis and red is shown on the y-axis; The scatterplot shows high colocalization and no bleed through either green or red channels; The scatter plot provides the rate of the area of association of two fluorochromes, calculated by linear regression; The scatter plot comprised of dots, appearing as cloud, indicates complete colocalization; B) Graphical representation of colocalization analysis based on the Pearson product-moment correlation coefficient (PCC) on each cell; The value for PCC ranges from +1 and -1 inclusive; A value of +1 would mean the total positive correlation, every pixel that contains Alexa Fluor® 488 (FITC) also contains Alexa Fluor® 555 (TRITC), while a value of -1 would mean the total negative correlation, every pixel that contains Alexa Fluor® 488 does not contain Alexa Fluor® 555 and vice versa; The PCC was calculated based on different images and indicates strong colocalization of CD74 and MIF on CAMA-1 and MDA-MB-231 Data represents three different experiments

Knockdown of CD74 expression in CAMA-1 and MDA-MB-231 cells by CD74 siRNA

Prior studies have reported that CD74 is over-expressed in human breast adenocarcinomas, and has a role in tumour progression along with MIF and CD44. The expression of CD74 in CAMA-1 and MDA-MB-231 cells was therefore evaluated. The expression of CD74 was found to be highest in CAMA-1 cells compared to MDA-MB-231 cells (Figure 4). In pilot experiments, it was found that a

concentration of 80 pmol/ml for 24 hr of specific CD74 siRNA was optimal for disrupted expression of CD74. Therefore, a dose of 80 pmol/ml was selected for optimal transfection of CAMA-1 and MDA-MB-231 cells with siRNA for all subsequent experiments.

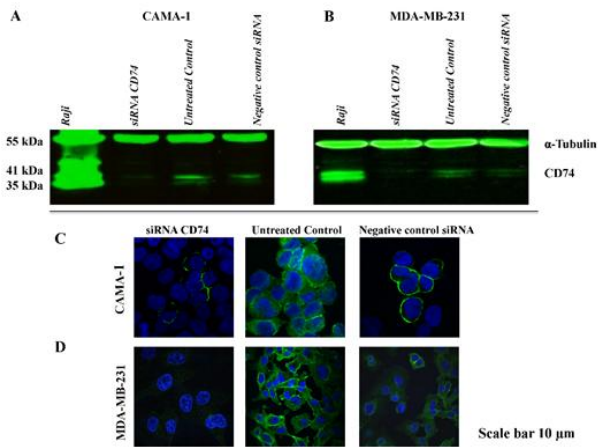


Figure 4: Figure 4: CD74 knockdown by CD74 siRNA transfection reagent in CAMA-1 and MDA-MB-231 cells; A) and B) siRNA-mediated knockdown of CD74 expression in CAMA-1 and MDA-MB-231 cells were detected by Western blot; An approximately two to five (siRNA) fold weaker signal of CD74 protein expression is apparent, as compared to the negative control siRNA group normalized to the expression of α -Tubulin; C) and D) Confocal images of CAMA-1 and MDA-MB-231 cells transfected with CD74 siRNA, untreated control and negative control siRNA; Data represent three different experiments

Knockdown of functional CD74 expression in CAMA-1 and MDA-MB-231 cells promotes apoptosis

In the light of the observations indicating apoptotic modes of cell death in CAMA-1 and MDA-MB-231 cells treated with CD74 siRNA next, multi-parameter flow cytometric analysis of siRNA-transfected CAMA-1 and MDA-MB-231 cells was pursued to obtain more sensitive and quantitative details of a possible apoptotic mode of cell death. Following 24 hr of a culture of CD74 siRNA-transfected CAMA-1 and MDA-MB-231 cells, flow cytometry was used to detect the expression of annexin V in the absence of PI staining.

Annexin V is a non-quantitative probe used to detect phosphatidylserine expressed on the cell surface, an indication of apoptosis. CAMA-1 and MDA-MB-231 cells treated with CD74 siRNA displayed significantly higher levels of annexin V staining ($\pm 55\%$ and 58% respectively) compared with negative control siRNA-treated counterparts ($\pm 8\%$ and $\pm 13\%$ respectively) (Figure 5A). These observations indicate that CD74 might play important regulatory roles in apoptosis.

Effects of CD74 knockdown on CAMA-1 and MDA-MB-231 cell proliferation

CAMA-1 and MDA-MB-231 cell proliferation and viability were determined using the MTT metabolic and viability assay (Figure 5B). CAMA-1 and MDA-MB-231 cells treated with CD74 siRNA displayed significantly reduced proliferation compared to cells treated with the negative control siRNA control sequence.

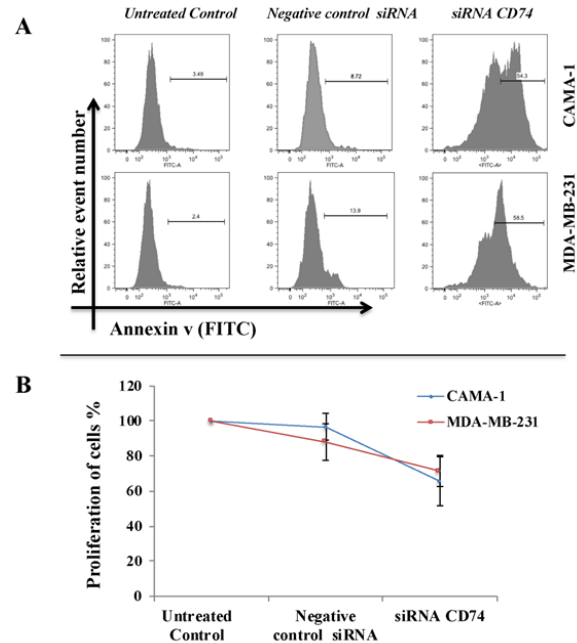


Figure 5: Figure 5: Effect of CD74 siRNA on the apoptosis and proliferation of CAMA-1 and MDA-MB-231 cells; A) Flow cytometric determination of the effect of CD74 siRNA on apoptosis of CAMA-1 and MDA-MB-231 cells; Cultured CAMA-1 and MDA-MB-231 cells were divided into three groups: nontransfected cells, cells transfected with negative control siRNA and cells transfected with CD74 siRNA; After a 24 hr treatment, the cells were harvested for quantitation of apoptosis by determining changes in the cell-surface expression of annexin V; Displayed is also a description of the observed frequency of cells undergoing apoptosis, which was found to be much higher in the CD74 siRNA treated cells than in the negative control-treated group of CAMA-1 and MDA-MB-231 cells, respectively; B) Effect of CD74 siRNA on the proliferation of CAMA-1 and MDA-MB-231 cells; MTT assay showed that treatment of CAMA-1 and MDA-MB-231 cells with CD74 siRNA inhibited their proliferation; Each point in the curve represents the arithmetic mean OD values \pm SD from representative experiments that were performed in triplicate

Discussion

The present study aimed to investigate the role of CD74 and its interrelation to MIF in breast cancer cells. This was achieved by studying the expression and colocalization of MIF with CD74 and CD44 molecules in the breast cancer cell lines CAMA-1 and MDA-MB-231 cells. The results obtained from confocal microscopy demonstrated that CD74 and MIF are highly colocalized on the cell-surface of all

breast cancer cells. Pearson's correlation coefficient and scatter plot analysis (Figure 3) also gave rise to the colocalization of CD74 and MIF [33], which accurately depicts the percentage of colocalization of CD74 and MIF molecules. Several groups have studied the association of CD74 with MIF and CD44 in cancers since it was reported that CD74 and CD44 are involved in signalling with MIF [10], [15], [16]. We also showed that CD74 and CD44 colocalise in breast cancer cells using a non-invasive and validated bioimaging procedure [28]. Also, it was shown that the formation of a molecular complex between MIF, CD74 and CD44 in prostate carcinoma cells lines (DU-145) could mediate signal transduction including (gene regulation, apoptosis, and cell proliferation) in prostate cancer [34].

Previous studies by immunofluorescence have confirmed the colocalization of MIF and CD74 in non-small cell lung cancer [35]. Additionally, using correlation analysis, Zheng *et al.* identified a positive correlation between MIF and CD74 in gastric cancer cells [36]. Correspondingly, Starlets *et al.* showed that, in malignant B cells obtained from patients with chronic lymphocytic leukaemia (CLL), MIF binds to the extracellular domain of CD74 to initiate a signalling cascade leading to cell proliferation and survival [6]. The interaction of MIF with CD74 and CD44 has been reported, suggesting that MIF in association with CD74 and CD44, as a complex, plays a significant role in bladder cancer cell proliferation [37]. Similarly, Meyer-Siegler *et al.*, reported that the interaction between MIF and CD74 activates the ERK1 and ERK2 signalling pathway, presumably through interaction with CD44, in the prostate cancer cell lines DU-145 and LNCaP, but not in normal human prostate epithelial cells (PrEC) or benign prostate epithelial cells (BPH-1) [34]. However, human benign prostate hyperplasia epithelial cells (BPH-1) and PrEC prostate cancer cells do not express CD74 on the cell surface, so for this reason, both cells do not interact with MIF and CD44 [34]. Correspondingly, Shi *et al.* showed that mammalian COS-7 cells do not bind MIF unless engineered to express the extracellular domain of CD74 [10].

To investigate the role of CD74 in apoptosis and proliferation, siRNA that targeted CD74 was used. Western blot results (Figure 4A and 4B) showed that CD74 expression was strongly knocked down in CAMA-1 and MDA-MB-231 cells in comparison with the control and CD74 siRNA. Microscopic results also confirmed that CD74 expression was strongly knocked down in both cell lines (Figure 4C and 4D). When CD74 expression was knocked down, apoptosis was observed in CAMA-1 and MDA-MB-231 cells. Both cell lines, when treated with CD74 siRNA, displayed significantly higher levels of annexin V staining ($\pm 55\%$ and $\pm 58\%$ respectively) compared to negative control siRNA-treated counterparts ($\pm 8\%$ and $\pm 13\%$ respectively). In the same manner, it was found that in CAMA-1 and MDA-MB-231 cells treated

with CD74 siRNA, a significantly reduced proliferation was observed compared to cells treated with the negative control siRNA control sequence and untreated cells. Likewise, it has been reported that knockdown of MIF or CD74 expression by RNA interference inhibits DU-145 cell proliferation and downstream MIF signalling [34], [38]. It is also reported that knockdown of the functional expression of MIF markedly decreased H460 cell proliferation and induced apoptosis, as seen by augmented expression of annexin A5 following treatment of H40 cells by MIF siRNA [39]. In particular, Verjans *et al.* showed that anti-MIF and anti-CD74 antibodies potently blocked cell proliferation of non-invasive MDA-MB-468 and invasive MDA-MB-231 breast cancer cells; however, this was not observed in non-tumorous MCF-12A cells [40]. This could be explained by the absence of the cell-surface portion of CD74 in MCF-12A cells. It is also reported that CD74 regulates Fas death receptor signaling in lymphomas by decreasing the levels of Fas receptors on the cell surface [41]. In the same manner, Liu *et al.*, have shown that CD74 promotes tumor growth, angiogenesis, and cancer cell metastasis *in vivo* [42]. The effect of CD74 in tumor growth and cell proliferation was studied by blocking the activity of MIF or CD74 in HEK / CD74 or a renal cell carcinoma (Caki-1) cells. The data showed that CD74-upregulated vascular endothelial growth factor D (VEGF-D) positively regulates the expression of cyclin D and E, which results in the promotion of cell cycle progression [42]. It was reported that G1 / S phase proteins cyclin D and cyclin E, were upregulated by CD74 and promoted cell cycle progression [6]. The recent finding also showed that the expression of CD74 was associated with MIBC / high grade of the UCB, while the knockdown of CD74 attenuated the proliferation, invasion, and angiogenesis of HT-1376 [18]. Figure 6 shows the proposed signal transduction pathway of MIF with CD74 and CD44.

In conclusion, it was observed that the interaction of MIF with CD74 CD44 could be a potential tumor marker for breast cancer cells. Moreover, level of co-expression of MIF and CD74 could be a surrogate marker for the efficacy of anti-angiogenic drugs, particularly in breast cancer tumors. Also, knockdown of CD74 by CD74 siRNA significantly reduced CAMA-1 and MDA-MB-231 cell proliferation and increased the level of apoptotic cells.

References

1. Wang P, Shi Q, Zuo T, He X, Yu J, Wang W. Expression of cluster of differentiation 74 in gallbladder carcinoma and the correlation with epithelial growth factor receptor levels. *Oncol Lett.* 2016; 11:2061-2066. <https://doi.org/10.3892/ol.2016.4191> PMID:26998122 PMCID:PMC4774522
2. Al Ssadh H, Alabdulmenaim W. Immunophenotyping of the

- cluster of differentiation 74, migration inhibitory factor, and cluster of differentiation 44 expression on human breast cancer-derived cell lines. *International J Health Sci.* 2019; 13:17-24.
3. Matza D, Kerem A, Shachar I. Invariant chain, a chain of command. *Trends in Immunology.* 2003; 24:264-268. [https://doi.org/10.1016/S1471-4906\(03\)00073-5](https://doi.org/10.1016/S1471-4906(03)00073-5)
 4. Binsky I, Lantner F, Grabovsky V, Harpaz N, Shvidel L, Berrebi A, Goldenberg DM, Leng L, Bucala R, Alon R, Haran M, Shachar I. TAp63 regulates VLA-4 expression and chronic lymphocytic leukemia cell migration to the bone marrow in a CD74-dependent manner. *J Immunology.* 2010; 184:4761-4769. <https://doi.org/10.4049/jimmunol.0904149> PMID:20357260 PMCid:PMC3129539
 5. Henne C, Schwenk F, Koch N, Moller P. Surface expression of the invariant chain (CD74) is independent of concomitant expression of major histocompatibility complex class II antigens. *Immunology.* 1995; 84:177-188.
 6. Starlets D, Gore Y, Binsky I, Haran M, Harpaz N, Shvidel L, Becker-Herman S, Berrebi A, Shachar I. Cell-surface CD74 initiates a signaling cascade leading to cell proliferation and survival. *Blood* 2006; 107:4807-4816. <https://doi.org/10.1182/blood-2005-11-4334> PMID:16484589
 7. Stumpner-Cuvelette P, Benaroch P. Multiple roles of the invariant chain in MHC class II function. *Molecular Cell Res.* 2002; 154:1-13. [https://doi.org/10.1016/S0167-4889\(01\)00166-5](https://doi.org/10.1016/S0167-4889(01)00166-5)
 8. Maharshak N, Cohen S, Lantner F, Hart G, Leng L, Bucala R, Shachar I. CD74 is a survival receptor on colon epithelial cells. *World J Gastroenterology.* 2010; 16:3258. <https://doi.org/10.3748/wjg.v16.i26.3258> PMID:20614481 PMCid:PMC2900717
 9. Leng L, Metz CN, Fang Y, Xu J, Donnelly S, Baugh J, Delohery CY, Mitchell RA, Bucala R. MIF signal transduction initiated by binding to CD74. *Journal Exp Med.* 2003; 197:1467-1476. <https://doi.org/10.1084/jem.20030286> PMID:12782713 PMCid:PMC2193907
 10. Shi X, Leng L, Wang T, Wang W, Du X, Li J, McDonald C, Chen Z, Murphy JW, Lolis E, Noble P, Knudson W, Bucala R. CD44 is the signaling component of the macrophage migration inhibitory factor-CD74 receptor complex. *Immunity.* 2006; 25:595-606. <https://doi.org/10.1016/j.immuni.2006.08.020> PMID:17045821 PMCid:PMC3707630
 11. Bach JP, Deuster O, Balzer-Geldsetzer M, Meyer B, Dodel R, Bacher M. The role of macrophage inhibitory factor in tumor igenesis and central nervous system tumors. *Cancer* 2009; 115:2031-2040. <https://doi.org/10.1002/cncr.24245> PMID:19326434
 12. Fan H, Hall P, Santos LL, Gregory JL, Fingerle-Rowson G, Bucala R, Morand EF, Hickey MJ. Macrophage migration inhibitory factor and CD74 regulate macrophage chemotactic responses via MAPK and Rho GTPase. *Journal Immunology* 2011; 186:4915-4924. <https://doi.org/10.4049/jimmunol.1003713> PMID:21411731 PMCid:PMC3388798
 13. Tillmann S, Bernhagen J, Noels H. Arrest functions of the MIF ligand/receptor axes in atherogenesis. *Frontiers Immunology.* 2013; 4:1-20. <https://doi.org/10.3389/fimmu.2013.00115> PMID:23720662 PMCid:PMC3655399
 14. Zernecke A, Bernhagen J, Weber C. Macrophage migration inhibitory factor in cardiovascular disease. *Circulation.* 2008; 117:1594-1602. <https://doi.org/10.1161/CIRCULATIONAHA.107.729125> PMID:18362243
 15. Borghese F, Clanchy FI. CD74: an emerging opportunity as a therapeutic target in cancer and autoimmune disease. *Expert Opinion Therapeutic Targets.* 2011; 15:237-251. <https://doi.org/10.1517/14728222.2011.550879> PMID:21208136
 16. Gore Y, Starlets D, Maharshak N, Becker-Herman S, Kaneyuki U, Leng L, Bucala R, Shachar I. Macrophage migration inhibitory factor induces B cell survival by activation of a CD74-CD44 receptor complex. *J Biological Chem.* 2008; 283:2784-2792. <https://doi.org/10.1074/jbc.M703265200> PMID:18056708
 17. Mitchell RA, Metz CN, Peng T, Bucala R. Sustained mitogen-activated protein kinase (MAPK) and cytoplasmic phospholipase A2 activation by macrophage migration inhibitory factor (MIF) Regulatory role in cell proliferation and glucocorticoid action. *J Biological Chem.* 1999; 274:18100-18106. <https://doi.org/10.1074/jbc.274.25.18100> PMID:10364264
 18. Gai JW, Wahafu W, Song L, Ping H, Wang M, Yang F, Niu Y, Qing W, Xing N. Expression of CD74 in bladder cancer and its suppression in association with cancer proliferation, invasion, and angiogenesis in HT-1376 cells. *Oncology Letters.* 2018; 15:7631-7638. <https://doi.org/10.3892/ol.2018.8309> PMID:29731899 PMCid:PMC5920967
 19. Lue H, Kapurniotu A, Fingerle-Rowson G, Roger T, Leng L, Thiele M, Calandrad T, Bucalae R, Bernhagen J. Rapid and transient activation of the ERK MAPK signaling pathway by macrophage migration inhibitory factor (MIF) and dependence on JAB1/CSN5 and Src kinase activity. *Cellular Signaling.* 2006; 18:688-703. <https://doi.org/10.1016/j.cellsig.2005.06.013> PMID:16122907
 20. Wortzel I, Seger R. The ERK cascade distinct functions within various subcellular organelles. *Genes Cancer.* 2011; 2:195-209. <https://doi.org/10.1177/1947601911407328> PMID:21779493 PMCid:PMC3128630
 21. Roskoski R. ERK1/2 MAP kinases: structure, function, and regulation. *Pharmacological Res.* 2012; 66:105-143. <https://doi.org/10.1016/j.phrs.2012.04.005> PMID:22569528
 22. Lue H, Thiele M, Franz J, Dahl E, Speckgens S, Leng L, Fingerle-Rowson G, Bucala R, Lüscher B, Bernhagen J. Macrophage migration inhibitory factor (MIF) promotes cell survival by activation of the Akt pathway and role for CSN5/JAB1 in the control of autocrine MIF activity. *Oncogene.* 2007; 26:5046-5059. <https://doi.org/10.1038/sj.onc.1210318> PMID:17310986
 23. Lantner F, Starlets D, Gore Y, Flaishon L, Yamit-Hezi A, Dikstein R, Leng L, Bucala R, Machluf Y, Oren M, Shachar I. CD74 induces TAp63 expression leading to B-cell survival. *Blood.* 2007; 110:4303-4311. <https://doi.org/10.1182/blood-2007-04-087486> PMID:17846227
 24. Gordin M, Tesio M, Cohen S, Gore Y, Lantner F, Leng L, Bucala R, Shachar I. c-Met and its ligand hepatocyte growth factor/scatter factor regulate mature B cell survival in a pathway induced by CD74. *Journal Immunology.* 2010; 185:2020-2031. <https://doi.org/10.4049/jimmunol.0902566> PMID:20639480 PMCid:PMC3646513
 25. Cohen S, Shoshana OY, Zelman-Toister E, Maharshak N, Binsky-Ehrenreich I, Gordin M, Hazan-Halevy I, Herishanu Y, Shvidel L, Haran M, Leng L, Bucala R, Harroch S, Shachar I. The cytokine midkine and its receptor RPTP ζ regulate B cell survival in a pathway induced by CD74. *Journal Immunology* 2002; 188:259-269. <https://doi.org/10.4049/jimmunol.1101468> PMID:22140262 PMCid:PMC3244541
 26. Matza D, Lantner F, Bogoch Y, Flaishon L, Hershkoviz R, Shachar I. Invariant chain induces B cell maturation in a process that is independent of its chaperonic activity. *Proceedings National Academy Sci.* 2002; 99:3018-3023. <https://doi.org/10.1073/pnas.052703299> PMID:11867743 PMCid:PMC122465
 27. Schneppenheim J, Dressel R, Huttl S, Lullmann-Rauch R, Engelke M, Dittmann K, Wienands J, Eskelinen E, Hermans-Borgmeyer I, Fluhrer R, Saftig P, Schröder B. The intramembrane protease SPPL2a promotes B cell development and controls endosomal traffic by cleavage of the invariant chain. *J Experimental Med.* 2003; 210:41-58. <https://doi.org/10.1084/jem.20121069> PMID:23267015 PMCid:PMC3549707
 28. Al Ssadh H, Spencer PS, Alabdulmenaim W, Alghamdi R, Madar IH, Miranda-Sayago JM, Fernández N. Measurements of heterotypic associations between a cluster of differentiation CD74 and CD44 in human breast cancer-derived cells. *Oncotarget.* 2017; 8:92143. <https://doi.org/10.18632/oncotarget.20922> PMID:29190904 PMCid:PMC5696170
 29. Al Ssadh H, Alabdulmenaim W. Novel predication of protein biomarkers in interferon-gamma-stimulated breast cancer cells.

- International J Health Sci. 2019; 13:35-43.
30. Alabdulmonem W, Alhomaidan HT, Rasheed Z, Madar IH, Alasmael N, Alkhatib S, Al Ssadh H. CD74 a Potential Therapeutic Target for Breast Cancer Therapy: Interferon Gamma Up-regulates its Expression in CAMA-1 and MDA-MB-231 Cancer Cells. International J Cancer Res. 2018; 14:58-69. <https://doi.org/10.3923/ijcr.2018.58.69>
31. Obara B, Jabeen A, Fernandez N, Laissue PP. A novel method for quantified, superresolved, three-dimensional colocalisation of isotropic, fluorescent particles. Histochem Cell Biol. 2013; 139:391-402. <https://doi.org/10.1007/s00418-012-1068-3> PMID:23381680
32. Bolte S, Cordelieres FP. A guided tour into subcellular colocalization analysis in light microscopy. J Microsc-Oxford. 2006; 224:213-32. <https://doi.org/10.1111/j.1365-2818.2006.01706.x> PMID:17210054
33. Bolte S, Cordelieres FP. A guided tour into subcellular colocalization analysis in light microscopy. Journal of microscopy. 2006; 224(3):213-232. <https://doi.org/10.1111/j.1365-2818.2006.01706.x> PMID:17210054
34. Meyer-Siegler KL, Iczkowski KA, Leng L, Bucala R, Vera PL. Inhibition of macrophage migration inhibitory factor or its receptor (CD74) attenuates growth and invasion of DU-145 prostate cancer cells. J Immunology. 2006; 177:8730-8739. <https://doi.org/10.4049/jimmunol.177.12.8730> PMID:17142775
35. McClelland M, Zhao L, Carskadon S, Arenberg D. Expression of CD74, the receptor for macrophage migration inhibitory factor, in non-small cell lung cancer. The american journal of pathology. 2009; 174(2):638-646. <https://doi.org/10.2353/ajpath.2009.080463> PMID:19131591 PMID:PMC2630571
36. Zhang B, Shen M, Xu M, Liu LL, Luo Y, Xu DQ, Wang YX, Liu ML, Liu Y, Dong HY, Zhao PT, Li ZC. Role of macrophage migration inhibitory factor in the proliferation of smooth muscle cell in pulmonary hypertension. Mediators Inflamm. 2012; 2012:840737. <https://doi.org/10.1155/2012/840737> PMID:22363104 PMID:PMC3270469
37. Meyer-Siegler KL, Leifheit EC, Vera PL. Inhibition of macrophage migration inhibitory factor decreases proliferation and cytokine expression in bladder cancer cells. BMC Cancer. 2004; 4:34-46. <https://doi.org/10.1186/1471-2407-4-34> PMID:15248897 PMID:PMC481073
38. Ren Y, Chan HM, Fan J, Xie Y, Chen YX, Li W, Tam PKH. Inhibition of tumour growth and metastasis in vitro and in vivo by targeting macrophage migration inhibitory factor in human neuroblastoma. Oncogene. 2006; 25:3501-3508. <https://doi.org/10.1038/sj.onc.1209395> PMID:16449971
39. Guo Y, Hou J, Luo Y, Wang D. Functional disruption of macrophage migration inhibitory factor (MIF) suppresses proliferation of human H460 lung cancer cells by caspase-dependent apoptosis. Cancer Cell International. 2013; 13:28-37. <https://doi.org/10.1186/1475-2867-13-28> PMID:23522304 PMID:PMC3695853
40. Verjans E, Noetzel E, Bektas N, Schütz AK, Lue H, Lennartz B, Hartmann A, Dahl E, Bernhagen J. Dual role of macrophage migration inhibitory factor (MIF) in human breast cancer. BMC Cancer. 2009; 9:230. <https://doi.org/10.1186/1471-2407-9-230> PMID:19602265 PMID:PMC2716369
41. Berkova Z, Wang S, Ao X, Wise JF, Braun FK, Rezaeian AH, Sehgal L, Goldenberg DM, Samaniego F. CD74 interferes with the expression of fas receptor on the surface of lymphoma cells. J Experimental Clinical Cancer Res. 2014; 33:80-90. <https://doi.org/10.1186/s13046-014-0080-y> PMID:25304249 PMID:PMC4210479