The Regulatory Role of Syndecan-1 on Human MiR-222-3p Expression in Breast Cancer Cell Lines

Original Article

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ABSTRACT

Introduction: MicroRNAs (miRNAs), small non-coding endogenous RNA molecules whose length ranging from 18-25 nucleotides, are implicated in regulating many physiological and pathological processes, including cell proliferation and apoptosis, adhesion, migration, invasiveness, epithelial-to-mesenchymal transition and the cancer stem cell properties. These molecules regulate gene expression at the posttranscriptional level by inducing mRNA degradation or translational repression. It was reported in previous work that miR-222-3p expression and Syndecan-1 (SDC-1) silencing regulate the aggressiveness of primary breast cancer and its metastasis. SDC-1, a cell surface heparan sulphate proteoglycan acting as a co-receptor for many growth factor receptors, is known to regulate the expression of many miRNAs. However, its impact on miR-222-3p expression in breast cancer is still unclear.

Aim of the Work: To investigate the effect of SDC-1 silencing on the expression of hsa-miR-222-3p in the human breast cancer cell lines.

Materials and Methods: We used the human breast cancer cell lines MCF-7 (low invasive) and MDA-MB-231 (highly invasive), which were transfected with 20 nM control and SDC-1 siRNA.hsa-mir-222-3p expression was analyzed by quantitative PCR (qPCR) in control and SDC-1-silenced cells 48h post transfection. Moreover, the expression of β-catenin protein72h post SDC1 knockdown was assessed by Western blotting.

Results: No significant change was observed for the expression levels of hsa-miR-222-3p and β-catenin protein after SDC-1 knockdown neither in MCF-7 nor MDA-MB-231 cell lines.

Conclusion: SDC-1 is not a regulator for miR-222-3p expression and the altered cell behavior mediated by SDC-1 knockdown in MCF-7 and MDA-MB-231 breast cancer cells is miR-222-3p-independent.

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Key Words: β-catenin, breast cancer, hsa-miR-222-3p, SDC-1.

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INTRODUCTION

GLOBCAN 2018 reported that breast cancer is the second cause of mortality related to cancer worldwide after lung cancer by 11.5%[1]. It is considered the most commonly diagnosed cancer and the first leading cause of cancer death among females. In 2018 about 2,088,849 cases were diagnosed with breast cancer (11.6% of all cases diagnosed with cancer) and about 626,679 cases were recorded to die because of breast cancer (6.6% of death cases by cancer[1]. Although the survival rate is improved dramatically due to the advanced screening and early treatment, but it is still a major threaten for females in developing countries. It was estimated that breast cancer ranks the second cancer incidence by 32.0% among Egyptian females and that proportion will be elevated by 3-fold by 2050 relative to its incidence in 2013[2].

Syndecan-1/CD138 (SDC-1) is a cell surface heparan sulphate proteoglycan that is intensively expressed by plasma and epithelial cells[3]. SDC-1 can bind to many of angiogenic and growth factors through its heparan sulphate chains and acts as a cell surface co-receptor for growth factor receptors promoting cell proliferation[4] .In addition, SDC-1 acts as a cell adhesion molecule through its interaction with various ligands in the extracellular matrix (ECM) and on the cell surface[5]. SDC-1 affects the expression of many microRNAs (miRNAs) in a wide range of cancer entities. This could be originated from its ability to regulate many transcription factors such as nuclear factor kappa B[6] and p53[7].

MiRNAs are small non-coding endogenous RNA molecules whose length from 18 to 25 nucleotides and act as post transcriptional regulators[8]. A single miRNA

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can target several mRNAs that are involved in the tumorgenesis[9]. Fuji et al. showed that the silencing of SDC-1 resulted in a decrease in the expression of hsa-mir-149-5p and hsa-mir-126 in prostate cancer[10]. Moreover, SDC-1 knockdown was shown to decrease the expression of mature hsa-mir-331-3p via controlling Dicer expression which in turn mediated the epithelial- mesenchymal transition (EMT) in prostate cancer[11]. MiR-221/222 is a miR cluster that influences metastasis by regulating tumor growth, invasion and EMT in many cancers, such as breast cancer[11-15], lung[16], liver cancer[16], pancreatic[17] and colorectal cancer[18]. Over expression of mir-222-3p induces the polarization of tumor associated macrophage via targeting SOCS3 in epithelial ovarian cancer[19]. Moreover, upregulation of mir-222-3p enhances the proliferation and invasion of endometrial carcinoma cell lines (RL95-2, AN3CA) via targeting estrogen receptor alpha (ERα) [20]. MiR-222 is also involved in the promotion of the aggressive basal-like phenotype in breast cancer promoting EMT in breast cancer via targeting trichohinophalangeal 1 (TRPS1)[21]. Over expression of mir-222-3p results in estrogen-independent growth and fulvestrant resistance in estrogen receptor-α (ERα)-positive cell lines via β–catenin activation[13]. The aim of this study was to reveal the effect of SDC-1 silencing on the expression of hsa-miR-222-3p in the human low invasive MCF-7 and high invasive MDA-MB-231 breast cancer cell lines.

MATERIAL AND METHODS

Cell culture

Human breast cancer cell lines were purchased from ATCC/LGC Promochem (Wesel, Germany).The hormone receptor positive MCF-7 and the triple negative MDA-MB-231 cell lines weremaintained in RPMI-1640 and DMEM media (Sigma Aldrich, Deisenhofen, Germany), respectively. Media were supplemented with1% Glutamine, 10% FBS (BiochromGmbH, Berlin, Germany) 1% penicillin- streptomycin antibiotic (SIGMA) and maintained in a condition of 37 °C, 5 % CO2, and 95% relative humidity.

SDC-1 Knockdown by cell transfection with SDC-1 siRNA

For siRNA transfection, the cells were plated into a six-well plate for 24 hours to reach 60-70% confluency prior to transfection. transfection was performed using Dharmafect reagent (Dharmacon, Lafayette,CO., USA) in OPTI-MEM medium (Gibco BRL , Karlsruhe, Germany),20 nMSDC-1 siRNA (Ambion, Taxis, USA)and 20 nM negative control siRNA (Negative control #1, Ambion, Taxis, USA). Transfection was performed according to manufacturer's instructions and after 24 hours of the transfection the media was changed with the fresh corresponding media supplemented with 10% FBS .

Quantitative Real-Time PCR Analysis

Total cellular RNA was isolated after 48hours of transfection using InnuPREP RNA mini kit (analytic Jana, Jana, Germany) according to manufacturer's instructions. RNA concentration and purity were assessed at 260 nm/280 nm. For cDNA preparation, 1μg oftotal cellular RNA was reverse transcribed into cDNA using High capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) according to manufacturer's instructions and the cDNA was stored at -20°Cfor further usage. For miRNA quantification, 100ng of total cellular RNA was converted into micro-cDNA using the TaqMan MicroRNA Reverse Transcription Kit with included specific primers for the individual microRNA as described by the manufacturer (ABI). Quantitative PCR (qPCR) was performed using the ABI PRISM 7300 Sequence Detection System (Applied biosystem) using the default thermal cycling conditions as follows; initial activation for 15 minutes at 95ºC followed by 40 cycles of denaturation for 15 seconds at 94ºC then annealing/extension steps for 30 seconds at 60ºC. Data analysis were performed using the $2^{-\Delta\Delta Ct}$ method. This quantification method measures the relative change in the expression level of SDC-1 after normalization to 18S rRNA. SDC-1 Taqman probe (Hs00174579_m1) and 18SrRNATaqman probe (Hs99999901_s1) were used.

Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting (Western blot)

MCF-7 and MDA-MB-231 negative control siRNA and SDC-1 siRNA transfected cells were washed twice with ice cold PBS and lysed using blue loading buffer (Cell Signaling Technology, Inc., Beverly, MA, United States) according to the manufacturer's instruction. Then, the total cell lysates were sonicated for 10-15 seconds on ice then the lysates were heated for 5 minutes at 95C° and centrifuged for 2-5 minutes A total of 20μg of the cell lysate were loaded onto SDS-PAGE gel and blotted with anti-human-β-Catenin antibody (Cell Signaling) overnight at 4C°. Proteins were visualized with horseradish peroxidase-conjugated secondary antibody followed by chemiluminescence detection. Tubulin was used as a loading control.

Statistical analysis

Data were presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS V0.16 software (Chicago, IL, USA).An unpaired t-test was used to compare between 2 groups and a two sided *p-value* of <0.05 was considered statistically significant,. All calculations were performed on the means of triplicate measurements of at least three independent experiments.

RESULTS

Confirmation of successful SDC-1 Knockdown by qPCR

Prior to any experiment, SDC-1 knockdown was verified by qPCR. Our results showed that relative to control SDC-1mRNA expression was significantly

(*P*<0.0001) downregulated by 96% and91%in SDC-1 siRNA-transfected MDA-MB-231 and MCF-7cells, respectively (Figure 1).

Hsa-mir-222-3 pexpression is not regulated by SDC-1 expression in MCF-7 and MDA-MB-231cells

The impact of SDC-1 knockdown on expression of hsa-miR-222-3p was investigated. Our TaqMan probe-based qPCR results showed nochangeinmiR-222-3p expression (only increased by 10%, *p*>0.05) in both MCF-7 and MDA-MB-231cell lines following SDC-1 depletion (Figure 2).

SDC-1 knockdown has no impact on β-catenin expression in MCF-7 and MDA-MB-231 cells

We next tested the effect of SDC-1 silencing on β-catenin expression by western blot. Our data showed no change of β-catenin expression in MCF-7 and MDA-MB-231 cell lines (Figure 3).

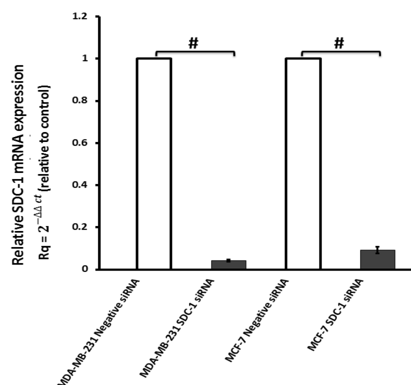

Fig. 1: qPCR for SDC-1 mRNA expression in control and SDC-1-depleted cells. SDC-1 mRNA was successfully knockdown in MDA-MB-231and MCF-7 breast cancer cell lines using siRNA approach. Total cellular RNA was isolated and reverse transcribed into cDNA and SDC-1 mRNA expression was checked using Taqman qPCR. The 2^(-ΔΔ ct)method was used to determine relative SDC-1 expression after normalization to 18S rRNA. Data are mean± SEM. #*P* <0.0001 as determined by student t- test.

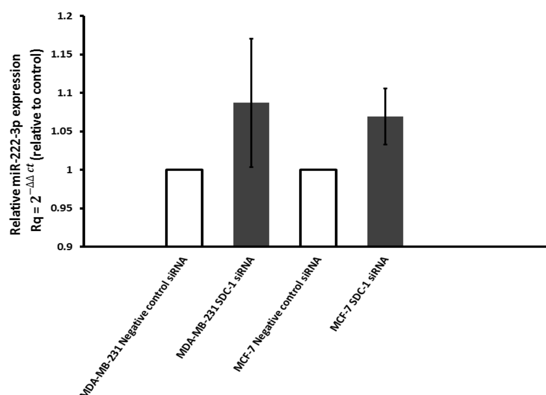

Fig. 2: SDC-1 knockdown has no effect on hsa-mir-222-expression in the MCF-7 and MDA-MB-231 cell lines. Total cellular RNA of control and SDC-1 siRNA transfected cells was isolated and reverse transcript to micro-cDNA. The 2^(-ΔΔ ct)method was used to determine relative miR-222levels after normalization to 18S rRNA. Data are mean ± SEM.

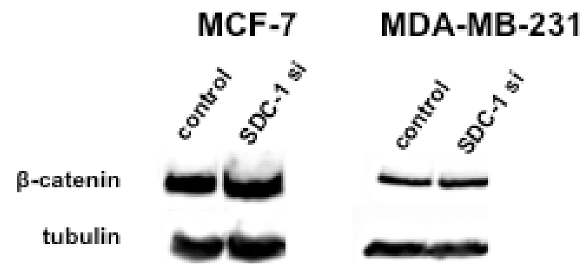

Fig. 3: No alteration in β- catenin protein expression post SDC-1 silencing in MCF-7 and MDA-MB-231 cells. Total cell lysate of control and SDC-1-silenced MCF-7 and MDA-MB-231 cells was subjected into western blot and probed with β-catenin and tubulin (loading control) antibodies. Data shown are duplicates from a single experiment as representative of three independent experiments.

DISCUSSION

It was reported that hsa-miR-222-3p regulates breast cancer metastasis and promotes the aggressive breast cancer phenotype which is resistant to some chemotherapy such as fulvestrant[13,21,22,]. In addition, it was proven that miR-222-3p inhibits many WNT/β-catenin suppressors such as APC/ AXIN/ GSK3β, resulting in WNT signaling activation in triple negative breast cancer (TNBC)[23]. On the other hand, it was found that SDC-1 regulates WNT/ β-catenin signaling via binding its HS side chain with R-spondin, leading to activation of LGR4 and resulting in suppression of Frizzled degradation and WNT/ β-catenin signaling activation in Multiple Myeloma[24]. In addition, it was proven that the SDC-1 HS chain is required for Wnt1-induced breast tumorigenesis in mice associated with an increase of cellular accumulated β- catenin[25]. Takentogether, the previous findings prompted us to investigate the regulatory role of SDC-1 on miR-222-3p expression in breast cancer.

Our qPCR revealed that SDC-1 silencing did not affect miR-222-3p expression in both MCF-7 and MDA-MB-231 cells. We have previously shown that SDC-1 knockdown resulted in enhanced migratory and invasive phenotype of MDA-MB-231 cells via modulation of Rho-GTPase dependent modulation of cytoskeletal function and downregulation expression of the EMT marker E-cadherin[3]. miR-221/222 promotes EMT in breast cancer cell lines and tissues[26.27]. This suggests that SDC1 may modulate E-cadherin expression in breast cancer in miR-222-indepenedent mechanism.

This conforms to our previous findings that SDC-1 depletion does not initiate but promote EMT once it is established in MCF-7 and MDA-MB-231 cells[28]. Our Western blot showed that β-catenin expression was not altered upon SDC-1 depletion. It was reported that miR-222 over expression enhances constitutive activation of β-catenin in glioma cells[29]. Furthermore,miR-222/221 activates β-catenin expression in the nucleus of MCF-7 and MDA-MB-231 cells via targeting many β-catenin

suppressors such as wIF1, DKK2, SFRP2, or AXIN2 leading to promotion of carcinogenesis[30] in addition, Wnt3a activation promote miR-221/222 expression in MDA-MB-231 cells which suggests that positive feedback loop between Wnt/β-catenin signaling and miR-221/222 may be present[23].Therefore, our results suggest that SDC-1 could modulate breast cancer tumorgenesis through an alternative mechanism independent of miR-222/222-β-catenin axis pathway. Overall, we suggest that SDC-1 regulates expression of a wide range of other miRNAs, which are involved in modulating cancer cell behavior.

CONFLICT OF INTEREST

There are no conflicts of interest.

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الملخص العربى

دورالسنديكان (١) المنظم لظهور الرنا-متناهي الصغر-٢٢٢-٣ بيفي الخلايا السرطانية لثدي الإنسان

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المقدمة: الرنا-متناهي الصغر هي جزيئات من الحمض النووي التي يتراوح طولها بين 25-18 من النيوكليوتيدات، وهي تشارك في تنظيم العديد من العمليات الفسيولوجية والمرضية. وأفادت الابحاث السابقة بأن الرنا-متناهي الصغر 3-222- بي، ينظم عدوانية سرطان الثدي في مراحلة الأولي ونموة الثانوي في اماكن اخرى. سنديكان (1) هو عبارة عن سلفات الكبريتيوجد على سطح الخلية و يعمل كمستقبل مساعد للعديد من مستقبلات عوامل النمو ، ومن المعروف أنه ينظم ظهور العديد من جزيئات الرنا-المتناهية الصغرولايزال تأثيره على الرنا-متناهي الصغر222- في سرطان الثدي غير واضح.

الهدف من البحث: للتحقق من تأثير سنديكان (1) على ظهورالرنا-متناهي الصغر 3-222- بي، في خطوط خلايا سرطان الثدي في الانسان.

مواد وطرق البحث: تم زرع خطوط خلايا سرطان الثدي للإنسان (MCF-7 وMDA-MB-231) مع الخلايا الحاكمة، و الرنا-قصير التداخل تعطيل السنديكان (1) عن العمل، وتم تحليل ظهورالرنا-متناهي الصغر 3-222- بي، بواسطة جهازبيسي آرالكمي.وكذالك، تم تقييم ظهوربروتين البيتا-الكاتينين بعد عملية تعطيل السنديكان (1) عن العمل، بواسطة الويسترن بلوت.

النتائج: لم يلاحظ أي تغير ملحوظ لمستويات ظهورالرنا-متناهي الصغر 3-222- بي و بروتين البيتا-الكاتينين بعد عملية تعطيل السنديكان (1) عن العمل في خطوط الخلايا MCF-7 و MDA-MB-231.

الإستنتاج: لا يُعد السنديكان (1) منظمًا لظهورالرنا-متناهي الصغر 3-222- بيولا لتغير السلوك الخلويفي خلايا سرطان الثدي وعلى ذلك تعتبر عدوانية خلايا MDA-MB-231 وخلايا MCF-7 مستقلة عن الرنا-متناهي الصغر 3-222- بي.