Knockdown of MUC1 by RNA Interference (siRNA) Influenced Vascular Endothelial Growth Factor Receptor (VEGFR2) and Suppression of Growth of Pancreatic Cancer Cells (PANC1)

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Abstract. MUC1 is overexpressed approximately in most of pancreatic adenocarcinomas and has been revealed to be linked with a worse prognosis, beside effectiveness, MUC1 hold for regulating cellular and developmental pathways. The objective of the current study is to downregulate the overexpression of MUC1 by small interfering RNA (siRNA) on PANC1 and to investigate its effect on vascular endothelial growth factor (VEGFR-2) expression. Besides MUC1, the expression levels of VEGFR2 were investigated in PANC1. Then the expression level of MUC1 was downregulated by using MUC1 specific siRNA approach to identify whether MUC1 is involved in the regulation of VEGFR-2 mRNA and protein, which were determined by QRT-PCR and Western blot, respectively. Interestingly, siRNA is considered an emerging approach in cancer cell therapy. Furthermore, the impact of MUC1 silencing in pancreatic adenocarcinoma cell lines was investigated. Transwell and Matrigel assays were performed to study the functional significance of MUC1 activity on cell migration and invasion, respectively. The present study indicates that targeting MUC1 by siRNA in pancreatic adenocarcinoma cells is associated with silencing of VEGFR-2 expression in both mRNA and protein levels, demonstrating that MUC1 regulates the main driver of angiogenesis and metastasis. The current study showed that targeting MUC1 with siRNA decreases pancreatic cancer cell proliferation, migration, and invasion, by significantly decreasing the expression of the potent angiogenic receptor VEGFR-2. Therefore, targeting MUC1 with siRNA can be exploited as a therapeutic option for pancreatic cancer treatment.

1 Introduction

MUC has 12 types (MUC1-MUC12) [1], The transmembrane glycoprotein MUC1 is being expressed in most epithelial cells of various human tissue types. The MUC1 gene is located on the human chromosome 1q21-24, contains seven exons and its genomic structure has been almost entirely determined. The cell surface protein Mucin 1 (MUC1) or polymorphic epithelial Mucin (PEM) is Mucin encoded O-glycosylated protein via the MUC1 gene in

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humans. MUC1 is a single-pass type I transmembrane protein with a profoundly O-linked glycosylated extracellular domain that comprises twenty-amino acid core tandem repeats and stretches up to 200-500 nm from the cell shell [2].

MUC1 is ordinarily expressed and localized at the apical surface of glandular epithelial cells in several tissue kinds, involving breast, lung, esophagus, stomach, pancreatic prostate, ovary, and to a minor level, in hematopoietic cell [3]. Whereas, MUC1 is missing in other tissue types as the skin epithelium and mesenchymal cells [4].

In well tissues, the main function of Mucin is to keep the body from infection by pathogens that react to oligosaccharides in the extracellular domain. Therefore, it blocks the pathogen from getting the cell exterior by regulating cells' chemotaxis of the innate immune system and ultimately preventing pathogenic colonization [5]. MUC1 is expressed in a variety of cancers with a high ratio, which ranges from 43.6% in Colorectal cancer to 100% in Ovarian cancer [6]. MUC1 is a highly glycosylated protein with two subunits: the extracellular N-terminus, under normal state, has a protective role to cells from undesired effects and the intracellular C-terminus contributes to controlling tumor state as growth, survival, invasion, migration, and apoptosis [7].

MUC1 is overexpressed in more than 80% of pancreatic ductal adenocarcinoma persuaded a pro-angiogenic tumor microenvironment by elevation the ranks of NRP1 (neuropilin-1, a co-receptor of VEGF) and its ligand, VEGF. Production of tumor-associated MUC1 (tMUC1) linked with VEGF and NRP1 ranks in human PDA. Overexpression of tumor-associated MUC1 in cancer cells was linked with elevation levels of VEGF and VEGFR2.

The overexpression of tumor-association MUC1 in pancreatic cancer-induced signing by the neuropilin1-VEGF axis considerably increases tube creation, new vessel blood production, and metastasis by prompting epithelial to mesenchymal transition [8]. Therefore, the Reduction of tMUC1 in pancreatic cancer mice stops cancer evolution and metastasis and is associated with lesser levels of VEGF. Several studies demonstrated that MUC1 overexpression promotes VEGF creation by insulin-like growth factor-1 receptor/Akt cascades and enhances tumor development and angiogenesis in human breast carcinoma [9]. Moreover, Extracellular O-glycosylation of MUC1-N is responsible for weakening drug penetration, efficacy, and sensitivity [10].

2 Materials and Methods

2.1 Cell culture and conditions

PANC-1 is an epithelioid carcinoma cell line originating from human pancreatic cells. The cell lines were cultured in a growth medium: DMEM (Gibco/Invitrogen) containing 10% fetal bovine serum (FBS) (Hyclone), 100 U/ml penicillin, and streptomycin (100µg/ml). Cells were incubated at 37˚C in humidified air with 5% CO2. For cell freezing, the growth medium was supplemented with 5% (v/v) DMSO in 1 ml aliquots of approximately 5 x 10^6 cells and stored at -80º C. For reviving the cells, one ml aliquot of cells was thawed directly in a water bath at 37ºC. The cells were transferred to 9 ml warm media in a 15-ml conical tube, centrifuged at 1,200 rpm for 5 minutes to pellet cells. The media was discharged and the cells were re-suspended in 10 ml warm medium and cultured into two T25 flasks, each of which contained 5 ml warm media. The cells were incubated for two days and then the old medium was removed and changed with new media. When the cells were 70-90% confluent the cells were collected, in order to increase the cells number. Then 2 ml of trypsin (Gibco/Invitrogen) was supplemented to the flask, incubated for 2 to 5 minutes, and then the cells were examined under a microscope. After the cells separated from the flasks, 5 ml whole growth medium

was added and the cells were centrifuged, collected, and re-cultured in a new flask after removing the old media and re-suspended in a new one. This study has been granted Ethical Permission from Huazhong University of Science and Technology, China.

2.2 RNA isolation

Complete cellular RNA was produced from cells by Trizol reagent (Invitrogen™), following the procedure of total RNA isolation. Briefly, the media was aspirated and the cells were washed with ice-cold PBS (1-2 ml). Then the PBS was aspirated and 1 ml TRIzol was added per $5x10^6$ cell after scraping the plate gently. The TRIzol was removed by a pipette and the TRIzol/cell lysate was dropped into a 1.5 ml Eppendorf tube and left at room temperature for 5 minutes. After the addition of 0.2 ml chloroform, the tube was forcefully shaken for 15 seconds after that left at room temperature for 5 minutes then centrifuged at 12,000 rpm for 5 Minutes at 4°C. After removing the liquid phase, another Eppendorf tube of 1.5 ml was used. Then 0.5ml isopropanol was supplemented to the liquid phase and mixed gently. The sample was left at room temperature for 5 min and centrifuged at 12,000 rpm for 10 minutes at 4°C. Then a precipitant was detectible at the bottom of each tube. One ml of 75% ethanol was added to DEPC-treated H_2O. To clean RNA precipitation the tube reversed several times and re-centrifuged 10000 rpm at 4℃ for 5 minutes. The ethanol was poured out and the pellets were air-dried. Then 40μl RNase-free water was added; fully dissolving the RNA. Finally, the RNA concentration was calculated by determining the absorbance at 260 nm. Prepared RNAs were stored at -80°C for further application.

2.3 Transfection of cancer cells

PANC1 cancer cells were grown (50-75% confluence) in a 6-well plate before they were transfected with MUC1 siRNA (RiboBio, Guangzhou, China). After 24 hours, the cells were transfected using 30 μl of transfection reagent (RNA Transfection Reagent, RiboBio) according to the manufacturer's instructions. The siRNA sequences (Silencer, RiboBio) were as in [Table 1].

siRNAs and transfection reagents were preincubated in CP-buffer (RiboBio) (30 μl containing 20 nM MUC1 siRNA and 20 nM control siRNA) at room temperature for 15 min prior to addition of the mixture to each well. Then, the medium was aspirated and replaced with an antibiotic-free complete medium after 24 hours in culture. The efficacy of mRNA knockdown was evaluted 48 hours after transfection by QRT-PCR. The mRNA levels were normalized to the GAPDH and the results were expressed as a ratio of target mRNA expression and GAPDH (relative units).

2.4 Primers

The primers that used in current research are presented in (Table 1) the primers were synthesized by Wuhan Kingari Bioengineering Co., Ltd.

2.5 Western blot analysis

After 48 hours, PANC1 cells were harvested by trypsin/EDTA washed two times with ice-cold PBS, and moved to Eppendorf tubes implanted in a frozen container. Then, the cells were, destroyed by means of lysis buffer RIPA, 1%PMSF, and 2% protease/phosphatase inhibitors cocktail. Cell lysates were centrifuged at 12,000 rpm for 10 min at 4°C. The top supernatant was then transferred to new Eppendorf tubes for discarding the bottom pellets.

The protein extract was studies by the BCA™ Protein Assay Kit. Thereafter, identical quantities of protein (20μg) were miscellaneous by identical sizes of protein loading buffer. Then, samples were immersed in boiling water for 10 minutes and then overloaded on 10% SDS-PAGE gel. Electrophoresis was then directed in Tris-Glycine SDS running buffer for 2 hours. Proteins were then moved into motivated PVDF membrane by Bolt® Transfer Buffer holding methanol at 80 Voltage for two hours. The membrane was incubated for 24 hours at 4 °C with a blocker buffer of BSA solution containing 0.1% TWEEN® 20 (TBST), which was also used to thoroughly rinse the membrane. To investigate protein, the membranes were incubated with anti-MUC1mAb and anti-VEGFR2mAb (1:500). The membrane was then washed with TBST 3 times. Then, a secondary antibody conjugated to HRP, anti-rabbit IgG (1:20000), was put into the membranes in a blocking buffer and kept at room temperature for 2 hours shaking. Then, the membrane was washed 3 times and investigated in a dark room by means of the Amersham ECL Prime Western Blotting. After that the membranes were then exposed, washed, blocked, and probed with rabbit anti-beta actin antibody (1:2000) and developed as previously described. Finally, the band was examined via ImageJ software.

2.6 Data analysis method

Calculation of RNA levels was carried out by using the ΔΔCT Method, as described below.
ΔΔCT Method:

$$\Delta CT = CT\ (\text{target gene}) - CT\ (\text{internal standard gene, GAPDH})$$

$$\Delta\Delta CT = \Delta CT\ (\text{target gene}) - \Delta CT\ (\text{internal standard gene})$$

$$\text{Gene quantification (relative unit)} = 2^{-\Delta\Delta CT}$$

The protein relative levels were calculated according to the following formulas

$$\Delta 1 = \text{Test Protein} / \beta\text{.actin}$$

$$\Delta 2 = \text{Control protein} / \beta\text{.actin}$$

$$\text{Protein quantification (in relative unit)} = \Delta 1 / \Delta 2$$

3 Results

3.1 Expression of MUC1 and VEGFR2 mRNA levels on PANC1

In humans, the expression of MUC1 is specific for both cells and tissues and is changed during cancer development. In the current search, the production levels of MUC1 and VEGFR-2 were investigated in a pancreatic adenocarcinoma cell line PANC1 to identify their relative expressions. Moreover, this study aims to (i) determine the relationship between MUC1 downregulation and VEGFR2 expression and (ii) investigate the role of MUC1 in PANC1 cell line proliferation and migration. To address these objectives, the mRNA levels of MUC1 and VEGFR2 were assessed via QRT-PCR compared with MUC1 mRNA levels. The results demonstrated relatively high expression levels of VEGFR2 in these cell lines as shown in Figure 1A.

3.2 Effect of different MUC1siRNA on MUC1 RNA levels

MUC1 mRNA expression level, following treatment with MUC1 siRNAs, was reduced gradually compared with non-treated PANC1 and non-target siRNA-treated cells (negative control). These results indicate that the siRNA MUC1 (2) led to high downregulation of MUC1 followed by siRNA MUC1 (3) and then siRNA MUC1 (1) as shown in Figure 2, which is then used for further investigations in the rest of the experiments and denoted as MUC1 siRNA.

To confirm the previous results, the cells were treated with MUC1 siRNA and their mean was compared with the non-treated cells. The current study results for the second time identified that the MUC1 siRNA significantly downregulates MUC1 in the PANC1. These results are represented in Figure 3A.

In order to investigate the effect of MUC1 downregulation on VEGFR2, the expression levels of VEGFR-2 mRNA levels were examined on PANC1 cancer cells after being treated with MUC1 siRNA.

This study revealed that the VEGFR2 mRNA expression levels were significantly reduced by MUC1siRNA as demonstrated in Figure 3B and the results were clearly compared in Figure 1B. Therefore, VEGFR2 may be developmentally regulated by a mechanism dependent on MUC1 expression.

3.3 Effects of MUC1 siRNA transfection on MUC1 protein levels

In order to investigate the role of MUC1 in human PANC1, the cells were transfected with MUC1-siRNA. After 48 hours of cell transfection, the effect of siRNA on MUC1protein expression levels was examined by western blot analysis and compared with control cells. The results verified that the protein expression of MUC1 was effectively reduced after MUC1 silencing (Figure 4) as detected by Western blotting. These results demonstrated that MUC1 silencing significantly decreased MUC1 expression in pancreatic adenocarcinoma cell line PANC1.

3.4 Effect of MUC1 siRNA transfection on VEGFR2 protein levels

The binding of VEGF to the VEGFR-2 receptors at the cell surface triggers several signaling events, including MUC1 expression. Therefore, it was of interest to analyze the expression levels of VEGFR2 in PANC1 cells following MUC1 silencing. The results demonstrated that VEGFR2 proteins (Figure 5) were efficiently reduced after MUC1 silencing. Moreover, we found that MUC1 silencing significantly blocked the shedding of VEGFR-2 mRNA [Figure 1B], indicating that MUC1 is crucially involved in this process in PANC1 cell lines. These results together confirmed that there is a relation between MUC1 and VEGFR2 expressions. Furthermore, since both receptors are involved in cancer cell development, metastasis, and angiogenesis, targeting these receptors may effectively participate in cancer treatment.

Table 1. Primers and their sequences

siRNA	Sequence
siRNA1	GCACCCAGUCUCCUUUCUUdTdT
siRNA2	GCCUCUCCAAUAUUAAGUUdTdT
siRNA3	GCUAUCCCAGCACCGACUAdTdT
negative control	GCAACUGUCCCUUUCCCUUdTdT
GAPDH Forward	5`-CATCATCCCTGCCTCTACTGG-3`
GAPDH Reverse	5`-GTGGGTGTCGCTGTTGAAGTC-3`
MUC1 Forward	5`-CAGCCACTTCTGCCAACTTG-3`
MUC1 Reverse	5`-AGCTCACCAGCCCAAACAG-3`
VEGFR2 Forward	5`-GGGCATGTACTGACGATTATGG-3`
VEGFR2 Reverse	5`- GGAGGAATGGCATAGACCGTA-3`

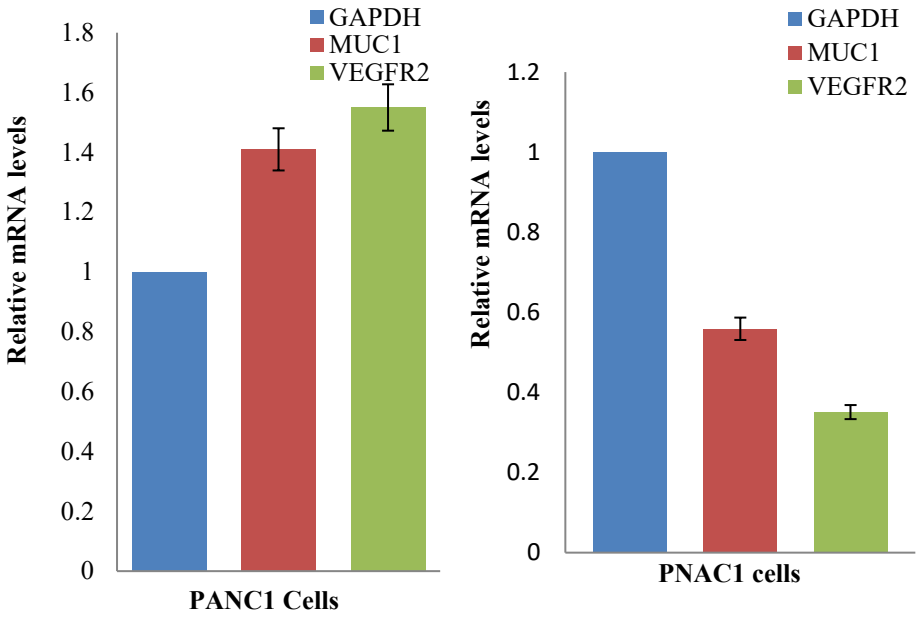

Fig. 1. Expression of MUC1 and VEGFR2 mRNA levels on PANC1 cells. A: Normal Expression of Non-treated (Without silencing) PANC1 cells. B: Reduced Expression of treated (With silencing) PANC1cell. The results of mRNA levels were analyzed by qRT-PCR, and quantification of three independent QRT-PCR results is shown. PANC1 cancer cells expressed high VEGFR2 mRNA compared with MUC1 mRNA. Data are presented as the mean ± SEM.

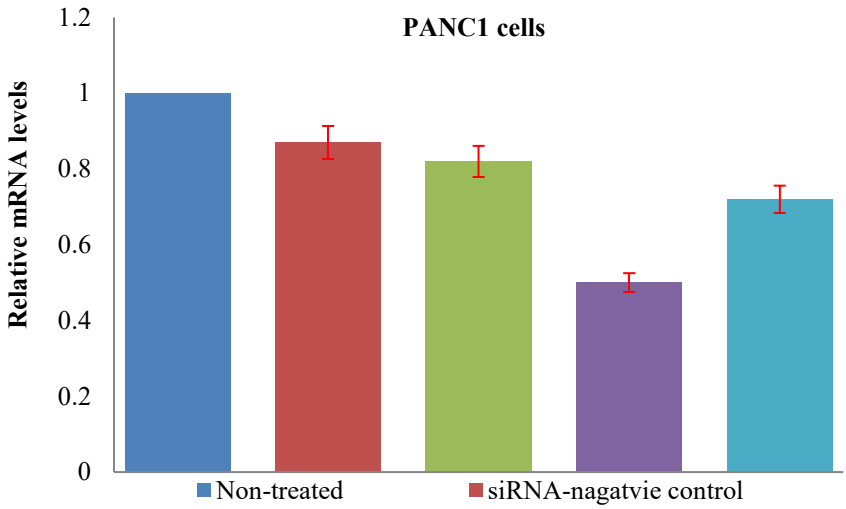

Fig. 2. Expression levels of MUC1 mRNA in different experimental groups determined by QRT-PCR. The PANC1 cancer cells were categorized as no-treated cells and treated cells with negative control of siRNA and different MUC1 siRNAs (from 1 to 3), respectively. The siRNA reduced MUC1 and mRNA expression levels. Data are presented as the mean ± SEM of three independent experiments.

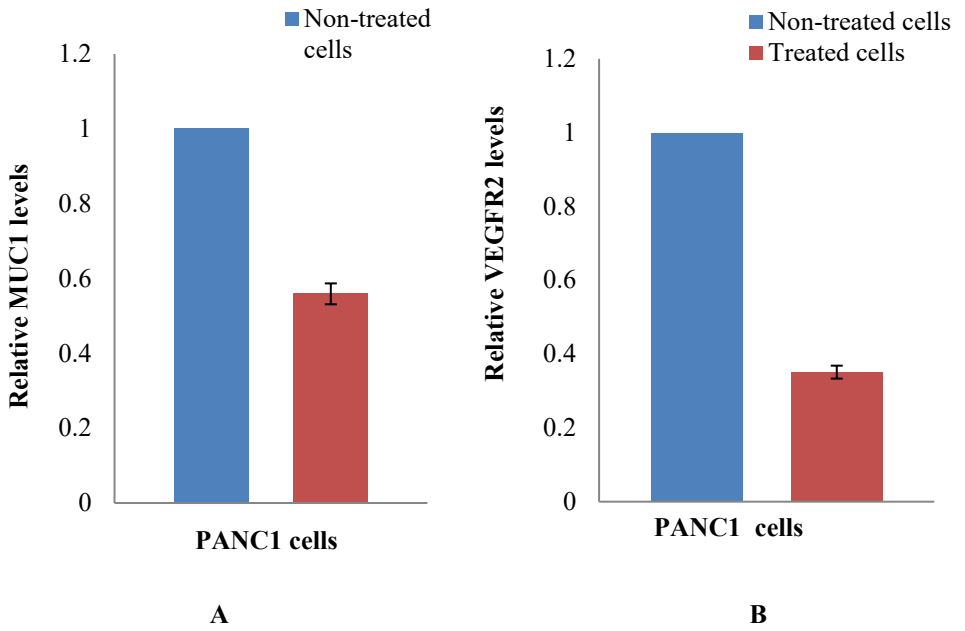

Fig. 3. Comparison between non-treated and MUC1 siRNA-treated PANC1 cancer cells A: based on relative MUC1 mRNA levels. B: based on relative VEGFR-2 mRNA levels. The results of mRNA were analyzed by qRT-PCR. Graph bars represent the mean of three independent experiments ± SEM.

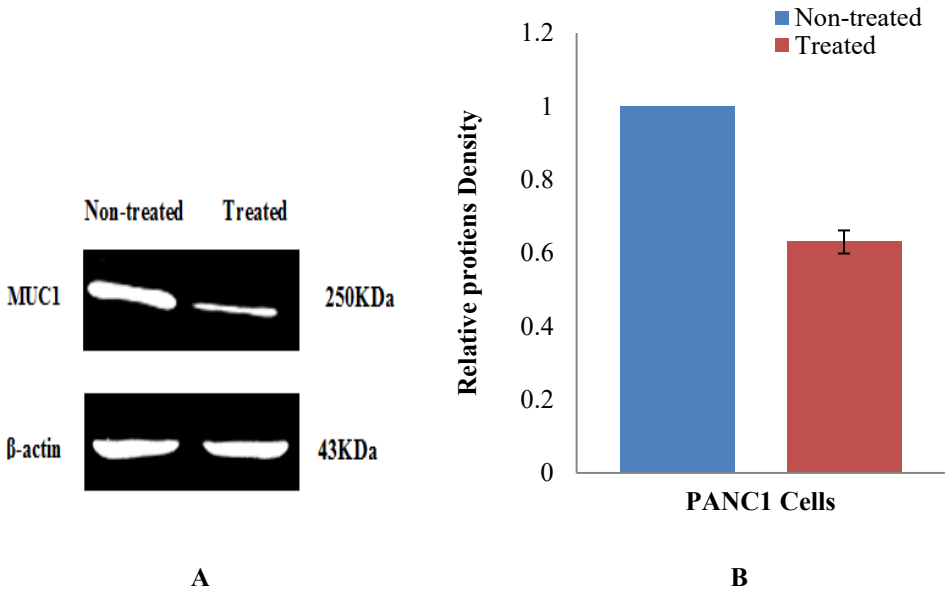

Fig. 4. MUC1 knockdown decreases the expression of MUC1 protein in PANC1 adenocarcinoma cell line, A: Effect of MUC1siRNA transfection on MUC1 protein expressions level B: The relative protein levels from the semi-quantification of three Western blots are shown. The siRNA reduced MUC1 expression at the protein level.

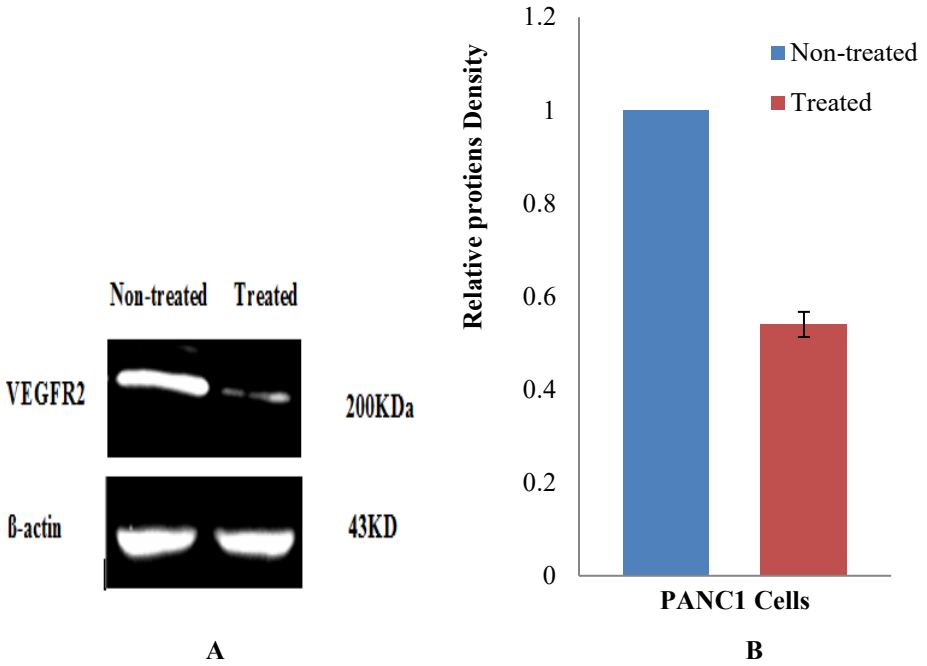

Fig. 5. MUC1 knockdown decreases the expression of VEGFR2 protein in PANC1 adenocarcinoma cell line. A: Effect of MUC1siRNA transfection on VEGFR2 protein expressions level, B: The relative protein levels from the semi-quantification of three Western blots are shown. The siRNA reduced MUC1 expression at the protein level.

4 Discussion

The heterodimeric protein MUC1 is abnormally overexpressed in pancreatic, non-small cell lung (NSCL), breast, and other types of cancers. Remarkably, MUC1 is made of 2-subunits: the component of the heterodimer, MUC1 N-terminal subunit (MUC1-N), which is extracellularly complexed with the C-terminal subunit (MUC1-C), the transmembrane part [11]. The resultant complex developed to preserve epithelia from tension by using a MUC1-N-associated as a physical barrier and MUC1-C-activated signaling for self-renewal, repair, and persistence [12]. MUC1 can trigger the TAK1→IKK→NF-κB inflammatory pathway [13]. Based on this capability and its overexpression in the tumor, MUC1 acts as an oncogenic protein that interacts with (i) certain nuclear transcription factors, including NF-κB, MYC, β-catenin/TCF4 and p65, (ii) the cell surface receptor such as tyrosine kinases (RTKs) and VEGFR-2[14], [15] .Interestingly, there are numerous reports suggesting that VEGFR2 is the main if not exclusive VEGF signaling receptor for endothelial cell functions including permeability, proliferation, migration, and angiogenesis. Therefore, inhibiting VEGFR2 is a useful approach for blocking VEGF signaling, the key inducer of angiogenesis and metastasis in cancer cells. These findings reflect the importance of this protein in cellular signaling, proliferation, angiogenesis, and invasion. Therefore, recently, VEGF has been used as a tumor diagnostic biomarker and as a therapeutic target [16]. Through, for example, designing VEGF and VEGFR inhibitors [17]. In the present experiments, the influence of the transfection of three diverse sequences of MUC1 siRNAs was examined on PANC1 cancer cells. RNAi is a powerful tool that accomplishes silencing genes in an effective mode. The understanding of the RNAi method of effect has soon revealed a varied range of promising uses, both *in vitro* or *in vivo*. In the past period, several papers have exposed that small

siRNAs have the ability to downregulate MUC1 genes with sequence homology and that the knockdown interceded by RNAi apparatus is a global phenomenon in eukaryotic cells [18], [19] [20]. Briefly, siRNAs are widely used to block the expression of several mark genes, for the reason of their elevated specificity and their apparent non-toxicity. Therefore, this study aimed to knock down MUC1 expression by a siRNA-based method and to test its influence on VEGFR-2 expression in PANC1 cancer cell lines. Furthermore, the effect of MUC1 silencing on PANC1 cancer cell proliferation, migration, and invasion was also considered. In this study, an RNAi-based method was used to achieve an effective knockdown of MUC1 gene expression. The overexpression of MUC1 looks like to performs a critical part in several pathological processes, especially in cancer. MUC1 in pancreatic cancer cells looks to stimulate tumor cell proliferation and tumor metastasis. The present study indicates that affecting MUC1 in pancreatic adenocarcinoma cells is linked with silencing of VEGFR-2 expression in both mRNA and protein levels, demonstrating that MUC1 regulates the major driver of angiogenesis and metastasis.

In brief, our study confirmed the predominant role of siRNA in the field of gene silencing due to its elevated efficacy. One successful method in cancer therapies is developed a selective downregulation of those genes overexpressed in tumor tissues. These results together reported affective siRNA-based mode for MUC1 gene knockdown *in vitro* in a PANC1.

5 Conclusion

MUC1 overexpression might contribute to angiogenesis and metastasis of pancreatic cancer and might related to elevated expression of VEGFR2, stimulating angiogenesis and formation of the new blood vessel for the tumor microenvironment in pancreatic cancer. In current study, MUC1 expression was downregulated in pancreatic cancer cells by using siRNA. Knockdown of MUC1 expression successfully led to downregulation of VEGFR2 expression and inhibition of PANC1 cancer cell proliferation. Thus, these results together indicate that the downregulation of MUC1 may be used to target angiogenesis, proliferation, migration, and invasion of pancreatic cancer cells. RNA interference is a quite new way of inhibiting gene expression and has remained regarded as a favorable tool for cancer healing. RNA interference is a unique molecular healing method because it employs the cell's natural RNA interference pathway to silence specific and selective genes.

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