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Original Research Article

STAT3 inhibitor enhances chemotherapy drug efficacy by modulating mucin 1 expression in non-small cell lung carcinoma

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Abstract

Purpose: *To evaluate the role of signal transducer and activator of transcription 3 (STAT3) and mucin 1(MUC1) in non-small cell lung carcinoma (NSCLC) and the use of their inhibitors to reduce chemoresistance.*
Methods: *Cisplatin or vinblastine was provided either with or without STAT3 inhibitor and evaluated for chemoresistance in NSCLC cells and a xenograft mice tumor model. Immunohistochemistry and Kaplan-Meier method of survival analysis were used to determine chemoresistance trends in patients. STAT3 inhibitor treatment, RNAi or ectopic overexpression of STAT3 or MUC1 in NSCLC cells were used to determine their inter-molecular relation and for modulating stemness-related genes.*
Results: *A major subset of chemoresistance patients exhibited a combined aberration of both STAT3 and MUC1 and exhibited a significantly reduced median overall survival ($p = 0.008$). Subsequent in vitro experiments in NSCLC cells showed that STAT3 levels modulate MUC1 expression ($p < 0.01$) and increase stemness gene expressions such as AKT (3-fold), OCT4 (4-fold), SOX2 (2-fold) and CXCR4 levels (2 -fold). In addition, co-treatment of STAT3 inhibitor with cisplatin or vinblastine enhanced drug efficiency in viability and invasion assays ($p < 0.01$) and in a xenograft mouse model ($p < 0.05$).*
Conclusion: *STAT3 inhibitor co-treatment with chemotherapy drugs increases drug efficacy and reduced tumor growth, and therefore, may improve outcomes in patients on NSCLC chemotherapies.*

Keywords: *STAT3, Non-small cell lung carcinoma, Mucin 1, Chemoresistance, Chemotherapy*

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INTRODUCTION

Non-small cell lung carcinoma (NSCLC) accounts for > 80 % of all lung cancers including lung adenocarcinoma and squamous cell carcinoma [1-4]. Treatment options such as surgical resection, radiotherapy, and chemotherapy are widely used for disease management [4-7]. However, tumor resistance to these options is a major problem and often leads to tumor recurrence and eventually failure of all treatment options. Stemness in cancer cell populations is widely implicated for facilitating the chemoresistance. Stemness in cancer cell population is widely implicated for facilitating the chemoresistance [7-10]. Developing tailor-made chemotherapeutic options based on cancer sub-types and the underlying facilitating mechanism for the chemoresistance has been proposed as an alternate strategy to improve outcomes [11]. In particular, chemotherapy options for lung cancers could be improved by synergistically treating with drugs that block tumor survival pathways.

Multiple studies have shown that STAT proteins particularly, STAT3 is abnormally activated in a wide variety of cancer type including NSCLC, and targeting them could provide therapeutic options [12-17]. Similarly, MUC1 protein, which is modulated by STAT3 levels, is also indicated to facilitate for tumor survival and progression [18,19]. Also, as both STAT3 and MUC1 are aberrantly associated with multiple cancer types including NSCLC, it is intriguing to hypothesize that both of them might play a critical role in lung cancer progression, and targeting this axis could aid in the chemotherapy efficiency.

In this study, we have investigated the relationship between STAT3 and MUC1 in the context of NSCLC cell survival and evaluated its potential if co-treated to improve chemotherapy response.

METHODS

Patients

Resected tissue samples were collected from a total of 58 patients with NSCLC. All patients samples were collected from the Binzhou City Central Hospital and selected based on the following criteria: (1) underwent complete resection of lung cancer with lymph node dissection followed by (2) chemotherapy as the only treatment used. The study group composed 28 men and 24 women, and all ranging from the age group of 42 - 68 years. The resected samples were evaluated for STAT3 and MUC1 overexpression. All samples were collected after prior ethical approval from the hospital review board at Binzhou City Central Hospital, Binzhou, Shandong (protocol approval no. BZLC2015)) and used as per the revised guidelines of International Ethical Guidelines for Biomedical Research involving Human Subjects, Council for International Organizations of Medical Sciences (CIOMS) and World Health Organisation (WHO), 1993 (update 2002) [20].

Cell culture

H441 human NSCLC cells were purchased from American Type Culture Collections (ATTC, MD) and maintained in high glucose DMEM medium supplemented with 2 mM L-glutamine and 10 % fetal bovine serum. Cells were maintained in 37 ºC humidified conditions with 5 % CO_2.

Drugs

C188-9 (STAT3 inhibitor) (Sigma, USA) were prepared as 20 mM stock in DMSO and stored at -20 ºC. Cells were treated with C188-9 at a final concentration of 3 µM and evaluated for changes as indicated. Cisplatin and vinblastine (Sigma, USA) were prepared as 100 mM stocks in DMSO and treated as indicated.

RNAi and overexpression experiments

siRNA targeting for human STAT3 or MUC1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were transfected with respective siRNA and evaluated for changes in mRNA and protein after 72 h. For overexpression experiments, pCMV6–STAT3 or MUC1 (Origene, MD) constructs were transfected and analyzed for STAT3 and MUC1 expression at 72 h post transfection.

RT-PCR

Total RNA was isolated from 72 h post-transfected cultures and reverse transcribed as described previously [21]. PCR was performed with a 7500 Applied Biosystem instrument using TaqMan probes with universal PCR Master Mix (Life Technologies, USA). The following prob-es were used: TaqMan: MUC1: Hs00904314_g1; STAT3: Hs01047580_mL; glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*): Hs02758991_g1; (Applied Biosystems, USA). Untreated samples were used as references to determine the change in gene expression.

Immunoblotting

The cell lysates were separated on a 4-20 % gel under reducing conditions, transferred to a PVDF membrane (Bio-Rad, USA), and blotted using specific antibodies. The membranes were blocked with 3 % milk and probed with primary antibodies. The primary antibodies and the dilutions were used as follows: rabbit STAT3 (79D7, Cell Signaling Technology, USA, 1:1000), rabbit MUC1 (D908K, Cell Signaling Technology, USA, 1:1000), rabbit OCT4 (P0056, Sigma, USA 1:500), rabbit CXCR (UMB2, Abcam USA, 1:500), rabbit SOX2 (D6D9, Cell Signaling Technology, USA, 1:1000), rabbit AKT (C67E7, Cell Signaling Technology, USA, 1:1000) and mouse Actin (8H10D10 Cell Signaling Technology, USA, 1:2000). Following the primary antibody incubation, the membranes were then a washed and incubated with respective secondary antibodies conjugated with horseradish peroxidase (HRP) and developed using a chemiluminescence substrate (Super Signal West Dura, Pierce Biotechnology, USA). Densitometry analysis was used to determine the relative fold change in protein levels.

Immunohistochemistry

Resected tissue samples were paraffin embedded and processed as previously described [21]. Also, the sections were de-paraffinized, antigen-retrieved and probed for STAT3 or MUC1 proteins. Expression levels from the non-cancerous region sections were compared as controls. Two pathologists who were blinded to the clinical outcome independently evaluated immunostaining scores and samples were grouped either or and negative for overexpression of STAT3 or MUC1 based on staining intensity on different samples.

Cell viability and invasion assay

Cell viability after treatments was measured using calorimetrically 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma, USA). Furthermore, control or STAT3 knockdown (post 72 h siRNA transfected) cells (1×10^4 per well) were seeded in a 96-well plate and treated with 10 µM cisplatin or vinblastine. After 16 h, 50 µL of MTT reagent (1 mg/mL) was added and incubated for 1 h. Absorbance values were measured at 570 nm using a microplate reader and percent changes in viability for different groups were calculated. These time incubations were used to allow probing for changes in drug response within the time-span of siRNA induced STAT3 reduction is retained. For STAT3 inhibitor based experiments, STAT3 inhibitor and drugs at 10 µM or 30 µM were co-treated.

For the migration assay, cells were seeded to upper Transwell chambers (Costar) at 1×10^4 cells/well and plated with or without drugs (10 µM). For invasion assays, cells were plated on the matrigel-coated surface (BD biosciences) in a transwell chamber and plated with or without drugs (10 µM). After overnight incubation, cells that have invaded and migrated to the other side of the matrix were fixed and stained with a solution of 0.5 % crystal violet prepared in 20 % ethanol. Cells were counted in 5 fields under an inverted microscope at ×200 magnification.

Xenograft animal model

All animal experiments were performed with prior approval from Institute's Animal Care Committee at Binzhou City Central Hospital, Binzhou, Shandong. Housing and followed the guidelines in compliance with the Animals (Scientific Procedures) Act, 1986 (UK) (amended 2013) and reported as per the ARRIVE Guidelines for reporting animal research [22].

To induce tumors in mice, 1×10^5 H441 cells were mixed with matrigel and subcutaneously inoculated into the flanks of six weeks old BALB/c/nude mice (n = 5 per group). Drugs were administered as i.v injection at a concentration of 3 mg/kg, once a week. The STAT3 inhibitor was provided along with the drug injections at a concentration of 3 µM/kg. The tumor size was measured using calipers in five-day intervals. Five weeks after the inoculation, the endpoint analysis for MUC1 mRNA analysis was made.

Data analysis

Kaplan-Meier method of analysis was used to calculate the overall survival times in patients. Patients (n = 3) with the lack of survival time info or mortality due to other causes were censored. Of them, 2 were STAT3 positive patients and 1 was MUC1 positive patient. Chi-square test was used to find the correlation between the gene expression profile and tumor outcome. For other statistical analysis, one-way analysis of variance (ANOVA) was used. Statistical significance was set at $p < 0.05$. All calculations were made using GraphPad Prism Version 6.

RESULTS

STAT3 and MUC1 levels in chemoresistant NSCLC

STAT3 and MUC1 expressions were evaluated by separating the patient groups based on their tumor types (Table 1). Our results showed that both squamous cell carcinoma and adenocarcinoma types exhibited high levels of STAT3 or MUC1 expression (Figure 1B), which indicate a role for these proteins in tumor progression [13,23]. In addition, we noted a considerable population of samples showing a combined overexpression of both STAT3 and MUC1 (Table 1 and Figure 1B). Statistical analysis through Chi-square test yielded a statistically significant association for the over-expression of STAT3 and MUC1 with tumor characteristics ($p = 0.027$). Further analysis using Kaplan-Meier method was made to determine if there are differences in the overall survival (OS) of these patients (Figure 1B). Our results showed that median OS for STAT3 and MUC1 over-expression patients were 78 and 75 months respectively, while it is reduced to 46 months ($p < 0.008$) in patients with the combined

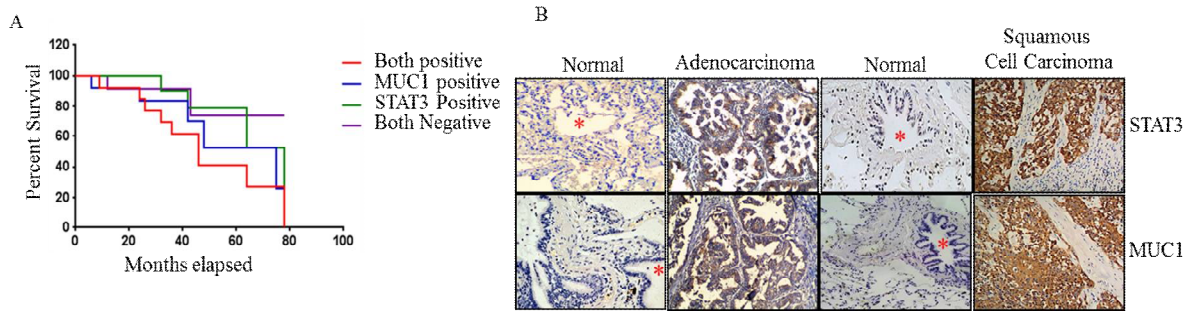

Figure 1: STAT3 and MUC1 levels with NSCLC prognosis. (A) Kaplan-Meier survival analysis in different patient groups. Patients with both STAT3 and MUC1 positive expression displayed significantly worse outcomes compared with other patients (P = 0.008). (B) Representative immunohistochemical staining for STAT3 and MUC1 in NSCLC patient samples. Staining from the non-tumor region in patients were shown as controls. * represents the bronchial/alveolar regions of the lung. Magnification at 20X.

Table 1: Patient's count split up based on histology of NSCLC and MUC1 and STAT3 staining

Histology	**STAT3 positive**	**MUC1 positive**	**Both** STAT3 and MUC1**positive**	**Both** STAT3 and MUC1 **negative**
Squamous Cell Carcinoma	4	5	16	4
Adeno Carcinoma	5	6	15	2
Others	0	0	1	0
Total	***9***	***11***	***32***	***6***

overexpression of STAT3 and MUC1. The median OS for patients with no expressions of both proteins was undefined.

Among the 58 NSCLC patients, ~55 % had both STAT3 and MUC1 high expression, while 15% had STAT3 and 18 % had MUCl high expression levels respectively. Chi-square test was performed and statistically, significant correlation was identified between STAT3 and MUC1 expressions and tumor pathology type (p = 0.023).

STAT3 levels regulate MUC1 expression in cells

To understand the relationship between STAT3 and MUC1 in cells, NSCLC cells (H441) that are known to express both the proteins were evaluated for changes in either of their gene knockdown conditions. We observed a significant decrease in the MUC1 mRNA (75 %) and protein levels (> 3 fold) (Figure 2), upon STAT3 knockdown. Treatments with STAT3 inhibitors were also able to recapitulate similar reduction in MUC1 mRNA (65 % reduction) and protein (> 3 fold), indicating that STAT3 activity could regulate the MUC1 expression in cells. In contrast, MUC1 knockdown showed a very slight reduction in STAT3 levels (25 % mRNA reduction), suggesting that STAT3 might act upstream and regulates MUC1 expression I cells (Figure 2).

STAT3 and MUC1 regulate stemness in cells

The stemness and survival related signaling pathways were investigated upon STAT3 or MUC1 knockdown in cells by evaluating for PI3K/AKT, OCT4, c-Src, CXCR4, and SOX2. The results showed a decrease in AKT (~ 3 fold), OCT4 (> 4 fold), SOX2 (> 2 fold) and CXCR4 levels (2 fold) in STAT3 knockdown cells, while Src expression remains unchanged (Src data not shown). In contrast, MUC1 knockdown showed minimal changes in OCT4, SOX2, and CXCR4, and unchanged for Src (Figure 3). Over-expression of STAT3 data also showed a reversal in the expression patterns of these proteins while it remained unchanged with MUC1 overexpression.

STAT3 inhibition sensitizes cells to chemotherapeutic agents

It was observed that STAT3 knockdown and drug treatments significantly decreased the number of viable cells (20 % change in drug alone and 50 % change in STAT3 knockdown and drug group; $p < 0.05$). Similar results were observed upon STAT3 inhibitor treatments as well (Figure 4).

Subsequent analysis with STAT3 inhibitor in migration and invasion assays also showed that STAT3 inhibitor significantly enhanced drug response in inhibiting both the migration and invasion capacity of the cells. (40 % change in drug alone groups and 70 % change in drug and inhibitor groups; $p < 0.01$) (Figure 4).

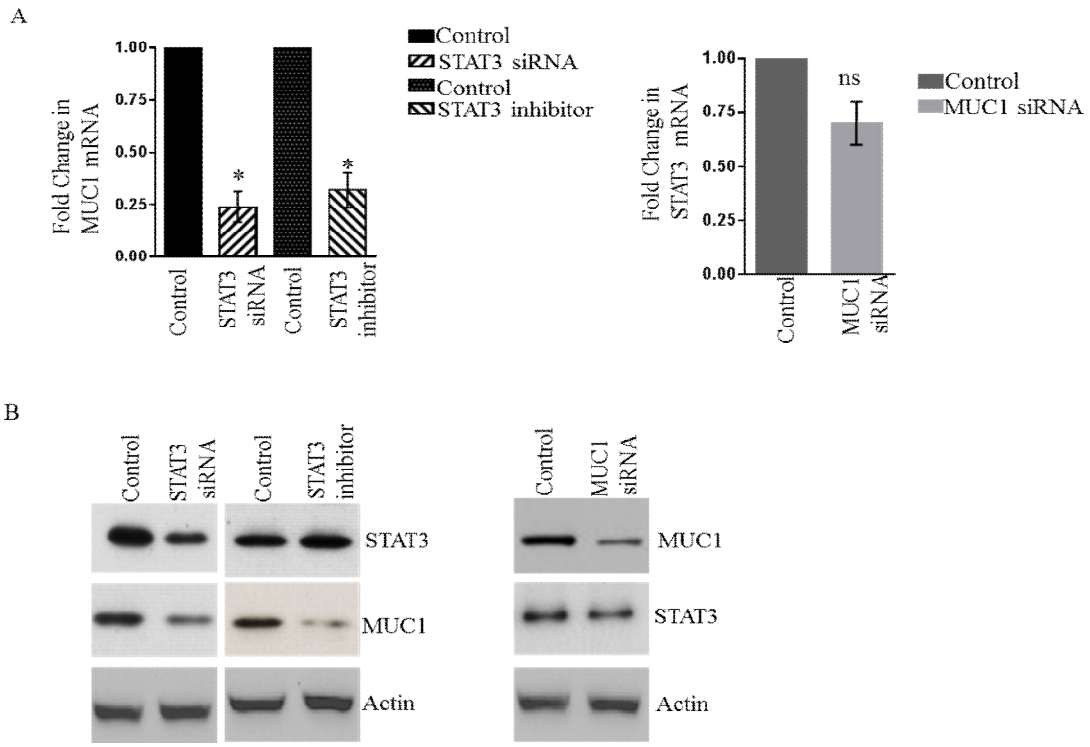

Figure 2: STAT3 modulates MUC1 expression in cells. (A) MUC1 mRNA changes upon STAT3 siRNA or inhibitor treatment and STAT3 mRNA changes upon MUC1 siRNA treatments. (B) Protein changes in treatments as described in (A). STAT3 siRNA or inhibitor treatments showed a significant reduction in MUC1 mRNA and protein, while a less apparent reduction was seen in STAT3 mRNA and protein due to MUC1 siRNA treatments. (* $p < 0.01$)

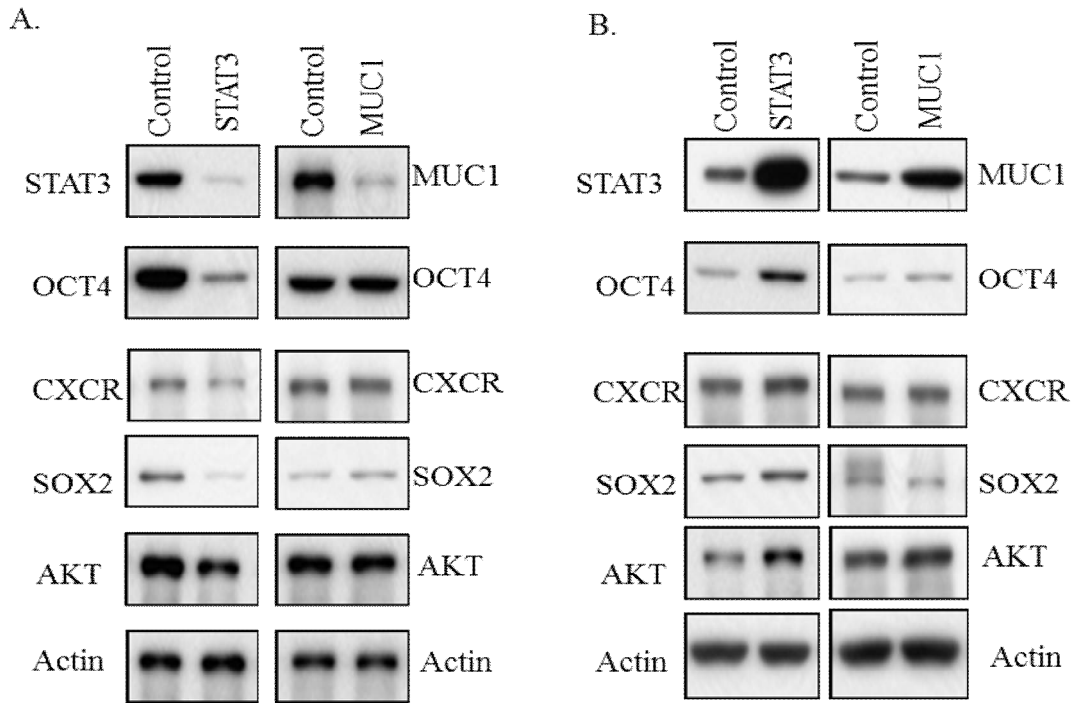

Figure 3: STAT3 and MUC1 regulate stemness in cells (A & B) Immunoblot analysis of stemness-related genes OCT4, CXCR SOX2, AKT upon knockdown or overexpression of STAT3 or MUC1. (A) Knockdown of STAT3 and MUC1. STAT3 knockdown showed the reduction in OCT4, CXCR, SOX2. (B) Overexpression of STAT3 and MUC1. STAT3 overexpression increased OCT4, CXCR, SOX2 levels

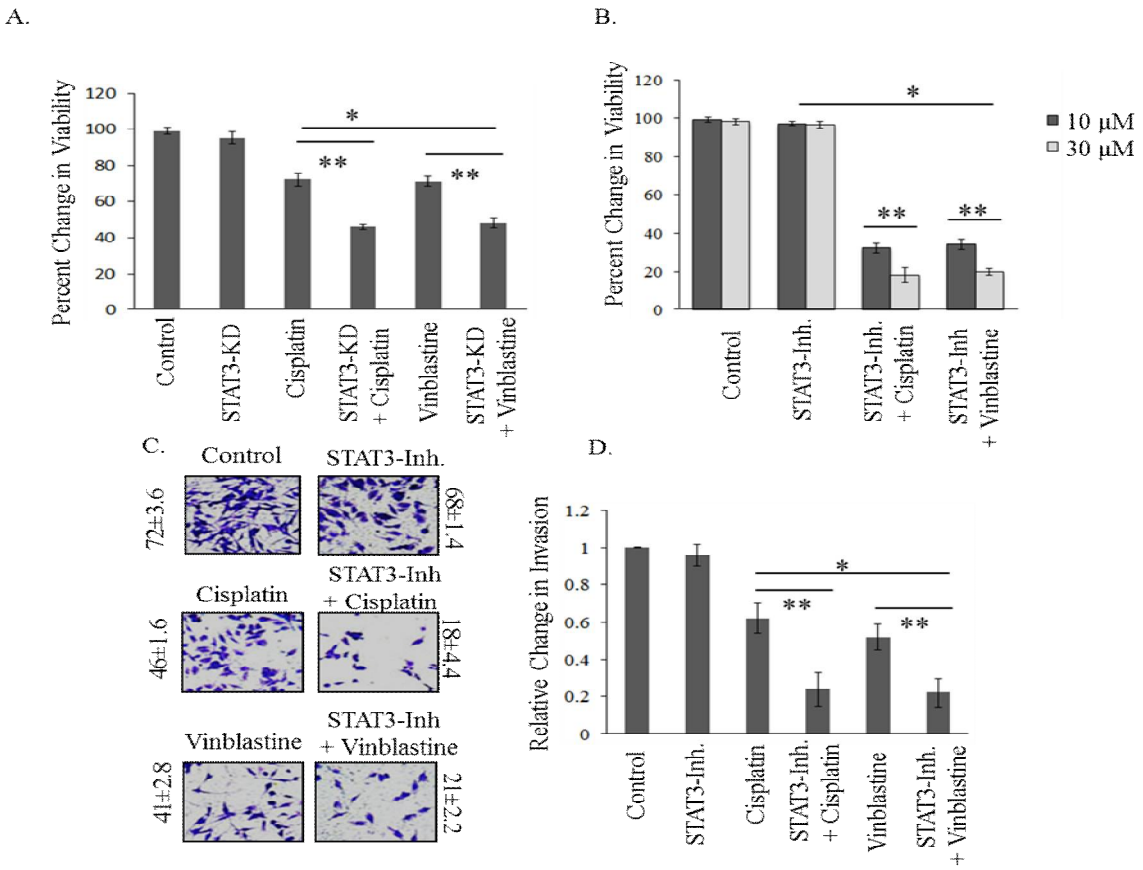

Figure 4: STAT3 inhibition sensitizes cells to chemotherapeutic drugs. (A & B) Cell viability evaluation upon STAT3 knockdown/inhibitor treatments and with drug treatments. Reduced STAT3 levels by knockdown or STAT3 inhibitor treatment alone had no effect on cell viability. Chemotherapeutic drugs cisplatin and vinblastine reduced the cell viability, which is enhanced manifold with STAT3 knockdown or STAT3 inhibitor co-treatment (* $p < 0.01$; ** $p < 0.05$). (C&D) Cell Migration and Invasion changes in STAT3 inhibitor co-treatment with drugs . (C) Cell Migration changes. Numerical value represents the average number of migrated cells. (D) Invasion Assay. Chemotherapeutic drugs reduce the cell migration and invasion, which is further enhanced manifold with STAT3 inhibitor co-treatment. (* $p < 0.05$; ** $p < 0.01$). STAT3 inhibitor treatment alone did not have any effect on cell migration and invasion

STAT3 inhibitor reduces tumor growth in mice by modulating MUC1 expression.

We evaluated the efficacy of STAT3 inhibition and chemotherapy drug treatments in xenograft mouse models. As shown in figure 5A, we observed an enhanced reduction (> 2 fold change

STAT3 inhibitor co-treatment with drugs enhances tumor growth reduction in mice

The results showed that STAT3 inhibitor co-treated mice exhibited an enhanced reduction in tumor volume compared to drug alone treated mice (> 2 fold change from day 25; drug alone vs STAT3 inhibitor and drugs, $p < 0.05$) (Figure 5A). In addition, a significant decrease in MUC1 mRNA in STAT3 inhibitor and drug-treated tumor tissues (> 30 % reduction in all STAT3 inhibitor-treated groups; $p < 0.05$) (Figure 5B) were observed, while no changes in MUC1 mRNA were observed in drug alone treated mice. Together, these data indicate that STAT3 inhibition modulates MUC1 expression but not sufficient to induce tumor reduction (Figure 5), however, with the drugs it chemo-sensitizes the cells to respond and inhibits tumor growth.

DISCUSSION

A wide range of mechanisms is proposed for inappropriate STAT3 and MUC1 activation in cancer [24-26]. This increased knowledge of signaling pathways about tumor mechanisms could be translated into therapeutic strategies with less toxicity than current treatments. In this study, we investigated whether STAT3 and MUC1 combined overexpression are correlating with NSCLC prognosis. Our results showed an association of combined elevated expression of STAT3 and MUC1 in cancer patient samples with poor chemotherapy response (median overall

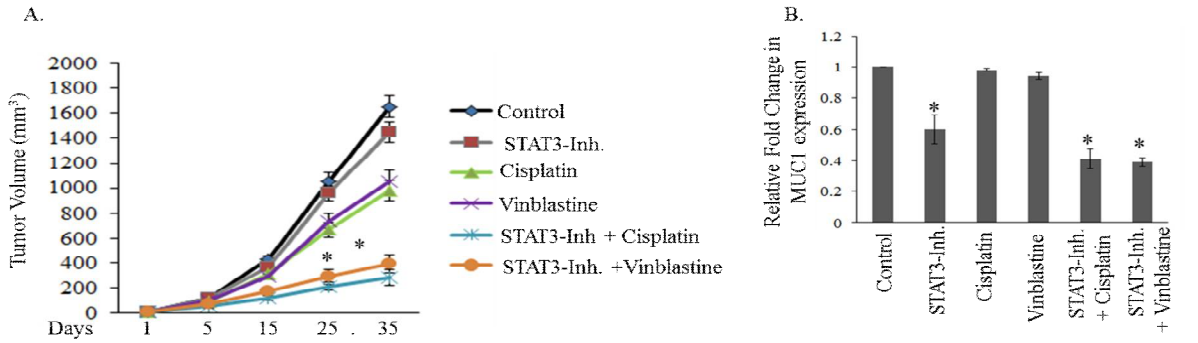

Figure 5: STAT3 inhibitor reduces tumor growth in mice by modulating MUC1 expression. (A) Change in tumor volume with different treatments and at different time points. STAT3 inhibitor treatments combined with cisplatin or vinblastine showed the maximum reduction in tumor growth (* $p < 0.05$) from Day 25. (B) MUC1 expression in tumor samples upon different treatments for Day 25. STAT3 inhibitor treatment and STAT3 inhibitor combined with cisplatin or vinblastine treatments showed a significant reduction from control (*$p < 0.05$), while no significant difference was seen between them

survival). Furthermore, the relationship between STAT3 and MUC1 were investigated in NSCLC cells and explored the potential to aid in chemotherapy drug treatments. Following *in vitro* and *in vivo* analyses, we concluded that STAT3 levels regulate MUC1 expression and reducing their activity could sensitize the cancer cells to chemotherapy drugs.

Our results demonstrated that STAT3 protein is constitutively over-expressed in NSCLC, and could contribute to the regulation of MUC1 mRNA and protein levels. Earlier reports have also indicated the cross-talk of these proteins and have implicated for their functions to promote tumor cell survival [17]. However, the results from - STAT3 and MUC1 knockdown experiments it is evident that - that STAT3 acts at the upstream signaling level and regulate the MUC1 expression. Although other signals such a PI3K/AKT are thought to affect the MUC1 protein, given that STAT response element is present in the promoter region of MUC1 gene, it is likely that STAT3 contribution would be critical to MUC1 expression.

Multiple remarkable studies have shown that stemness in cancer cells contribute to the poor response of chemotherapy [27-29]. Recent reports have shown that MUC1 plays a critical role in regulating de-differentiation, and stemness in NSCLC, and in accordance with those observations, we have also observed that the MUC1 overexpression could increase the expression levels of OCT4, SOX2, and CXCR4 to some extent. However, the results also revealed that STAT3 could have a more prominent effect on these proteins; thereby suggesting that STAT3 mediated regulation of stemness could be beyond MUC1 involvement. Given that STAT3 mediates MUC1 regulation in NSCLC, these changes in the stemness-related protein expressions suggests that MUC1 could possibly act as a mediator of STAT3 either independently or in coordination with other proteins in this pathway and confer stemness to NSCLC.

Previous studies have shown that sensitizing cancer cells to chemotherapeutic agents can reduce the side effects as well as balance the efficacy and toxicity of chemotherapy [30]. Molecular pathways driving cancer progression or essential for the cancer cell survival could be targeted to sensitize the cells for chemotherapy. Multiple studies have highlighted the significance of MUC1 in cancer progression and are particularly associated with aggressiveness of tumors. In addition, approaches targeting MUC1 in lung cancer cells have also shown to sensitize the cancer cells to chemotherapy drugs and inhibit their growth [18]. Similarly, STAT3 inhibitors have also been used in the same context, to promote the cancer cell death [31]. Given that both STAT3 and MUC1 proteins have been shown to be critical for tumor cell survival and in the context of our data showing that STAT3 to regulate MUC1 levels, we evaluated the efficacy of chemotherapy drug treatments with additional STAT3 inhibition. Our results demonstrated that STAT3 inhibition sensitizes the NSCLC cells for cisplatin and vinblastine treatments and showed decreased viability and invasion potency. Following *in vivo* experiments also showed that these synergistic treatments significantly decreased tumor growth in mice, compared with cisplatin or vinblastine treatments alone. More significantly, we have observed that this tumor reduction is associated with an MUC1 reduction, thereby suggesting that strategic reduction of STAT3 could significantly enhance chemotherapy response.

CONCLUSION

Co-overexpression of STAT3 and MUC1 is seen in a significant proportion of NSCLC patients with poor prognosis. STAT3 regulates MUC1 and confers stemness, and targeting them could significantly enhance chemotherapy response in cells and mice. Overall, the results suggest that STAT3 inhibitor co-treatment with chemotherapy drugs could cause a synergistic effect in cancer patients and provide improved outcomes in chemotherapy.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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