Targeting MUC1 and JNK by RNA interference and inhibitor inhibit the development of hepatocellular carcinoma

Juan Wang,¹ Wei-hua Ni,¹ Ke-bang Hu,² Xiao-yu Zhai,¹ Fei Xie,¹ Jing Jie,¹ Nan-nan Zhang,¹ Li-na Jiang,¹ Hong-yan Yuan¹ and Gui-xiang Tai¹

¹Department of Immunology, College of Basic Medical Sciences, Jilin University, Changchun; ²Department of Urology, The First Hospital of Jilin University, Changchun, China

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Correspondence
Gui-xiang Tai, Department of Immunology, College of Basic Medical Sciences, Jilin University, 126 Xinmin Street, Jilin, Changchun 130021, China.
Tel: +86-431-8561-9476; Fax: +86-431-8561-9105; E-mail: taiguixiang@163.com

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Mucin 1 (MUC1), as an oncogene, is overexpressed in hepatocellular carcinoma (HCC) tissues and promotes the progression and tumorigenesis of HCC through JNK/TGF-β signaling pathway. In the present study, RNA interference (RNAi) and JNK inhibitor SP600125, which target MUC1 and/or JNK, were used to treat HCC cells in vitro, and the results showed that both silencing the expression of MUC1 and blocking the activity of JNK inhibited the proliferation of HCC cells. In addition, MUC1-stable-knockdown and SP600125 significantly inhibited the growth of tumors in the subcutaneous transplant tumor models that established in BALB/c nude mice rather than MUC1 or JNK siRNAs transiently transfection. Furthermore, the results from immunohistochemical staining assays showed that the inhibitory effects of MUC1 gene silencing and SP600125 on the proliferation of HCC cells in vivo were through the JNK/TGF-β signaling pathway. These results indicate that MUC1 and JNK are attractive targets for HCC therapy and may provide new therapeutic strategies for the treatment of HCC.
steatosis, and insulin resistance.\textsuperscript{23} Recently, studies have confirmed that JNK plays a key role in the progression and tumorigenesis of HCC by regulating various cellular biological functions.\textsuperscript{24} Early findings showed that the transforming actions of several oncoproteins, such as Ras, c-fos, Met, Bcr-Abl, and mutant HER2, were JNK-dependent in various cancers.\textsuperscript{25\textendash}29} Collectively, all of these data indicate that JNK activity is necessary for the efficient transformation and tumorigenesis of these oncoproteins, therefore making JNK a dominant target for cancer therapy.

RNA interference (RNAi) can silence target gene expression and thereby block the production of disease-causing proteins.\textsuperscript{30,31} The effectiveness of RNAi in cancer therapy has been characterized by high efficiency and specificity.\textsuperscript{32} The major advantage of RNAi in cancer therapy is targeting multiple genes of various cellular pathways involved in tumor progression. Significant effort and capital have been invested in bringing siRNA therapeutics to the market. At least 22 RNAi-based drugs have entered clinical trials, and many more are in the development pipeline, indicating that siRNA has potential clinical use for cancer therapeutics.\textsuperscript{33}

In this study, we inhibited the biological functions of MUC1 and JNK by RNAi and/or the use of an inhibitor in MUC1-expressing HCC cells to determine whether such treatments are effective against HCC, and the results revealed that both MUC1 and JNK are attractive targets for the pathogenesis and gene therapy of HCC.

Materials and Methods

Cell lines and culture. SMMC-7721 was purchased from the Cell Bank of the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Cells were cultured in IMDM medium (Gibco, Grand Island, NY, USA) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (Gibco, Grand Island, NY, USA) in an incubator at 37°C under 5% CO\textsubscript{2}. The stable MUC1-knockdown cells (MR1-D4) and SMMC-7721 negative control cells (NC) were generated as previously described\textsuperscript{16} and maintained with 600 μg/mL G418 (Sigma-Aldrich, St. Louis, MO, USA).

Cell viability assay. Cell viability was determined at different time points based on WST-1 cell viability assays according to the manufacturer’s protocol (Roche, Basel, Switzerland). The absorbance was measured using a microplate reader at a wavelength of 450 nm.

Enzyme-linked immunosorbent assay (ELISA). Cells were grown in complete medium and treated with or without JNK inhibitor (30 μM) for 16 h and then incubated for 24 h in serum-free medium. Cell culture supernatants were assayed using a TGF-β ELISA kit (eBioscience, San Diego, CA, USA) according to the manufacturer’s instructions. The absorbance was measured at a wavelength of 450 nm.

Western blotting. Western blotting was performed as previously described.\textsuperscript{34} Primary antibodies: MUC1 (GP1.4) was purchased from NeoMarkers (Fremont, CA, USA); c-Myc and GAPDH were purchased from Epitomics ( Burlingame, CA, USA); p21\textsuperscript{WAF1}, JNK, p-JNK, pSmad2C (Ser-465/467), pSmad2L (Ser-245/250/255) and MMP-9 were purchased from Cell Signaling Technology (Danvers, MA, USA); pSmad3L (Ser-213) and pSmad3C (Ser-423/425) were purchased from Abcam (Cambridge, MA, USA).

RNAi. SiRNA oligonucleotides corresponding to the target sequence for human MUC1: GCAGCCCTCTCGATATATACC and JNK1/2: AGAAGGUGGCAUUCUUT\textsuperscript{35} for both in vitro and in vivo experiments were designed and synthesized by Ribobio Co. Ltd (Guangzhou, China). In vitro, a negative-control siRNA (NC-siRNA) (Ribobio, Guangzhou, China) was used as a medium control and a negative-control siRNA-Cy3 (Ribobio, Guangzhou, China) was used to detect the transfection efficiency. The siRNAs were transfected into SMMC-7721 cells using x-fect Transfection Reagent (Takara Biotechnology Co. Ltd, Dalian, China). In vivo, MUC1-siRNA, JNK-siRNA and NC-siRNA were 5’-cholesterol-conjugated and 2’-O-methyl-modified, and 5 nmol siRNA in 0.05 mL saline buffer was locally injected.

Results

The inhibition effects of silencing the expression of MUC1 and blocking the activity of JNK on HCC progression and tumorigenesis are through the JNK/TGF-β signaling pathway. Our previous studies found that MUC1 promotes HCC progression and tumorigenesis by activating the JNK/AP-1/TGF-β pathway in HCC cells.\textsuperscript{12,13,37} Previously, a MUC1-knockdown cell clone designated as MR1-D4 and a negative control clone designated as NC were established by transfecting SMMC-7721 cells with MUC1-targeted or nonspecific siRNAs in the expression vector pGCSilencer U6.Neo.GFP\textsuperscript{16} and the results from Western blotting showed that the expression of MUC1 in MR1-D4 cells was obviously decreased compared to SMMC-7721 or NC cells (\textit{P} < 0.01; Fig. 1a). In this study, to further verify the molecular mechanism leading to the inhibition effects of
MUC1 gene silencing and SP600125 on HCC cells, the phosphorylation of JNK, Smad3L/C and Smad2L/C and the expression levels of the corresponding target genes, such as c-Myc, p21 and MMP-9, in these cells were detected by Western blotting. The results showed that the phosphorylation of JNK, Smad3L/C and Smad2L/C and the expression of c-Myc, p21 and MMP-9, in these cells were detected by Western blotting. The results showed that the phosphorylation of JNK, Smad3L/C, and Smad2L/C and the expression of c-Myc and...
MMP-9 were downregulated, while the phosphorylation of Smad3C and p21 were upregulated in these cells when the expression of MUC1 and the activity of JNK were inhibited, respectively (Fig. 1b–d). Furthermore, the TGF-β1 levels in the culture supernatants of MR1-D4 and SP600125-treated SMMC-7721 cells and the respective controls were measured by ELISA, and the results showed that both silenced the expression of MUC1 and inhibited the activity of JNK markedly decreasing the autocrine TGF-β1 (P < 0.01; Fig. 1e). All of these results demonstrate that the inhibition effects of silencing the expression of MUC1 and blocking the activity of JNK in HCC progression and tumorigenesis are through the JNK/TGF-β signaling pathway, making MUC1 and JNK as attractive targets for HCC therapy.

MUC1 gene silencing inhibits the proliferation of HCC cells in vitro. To discover a new method for the treatment of HCC by targeting MUC1, in the present study, the expression of MUC1 was silenced by different methods. The results from WST-1 cell viability assay showed that the viability of MR1-D4 cells was reduced in a time-dependent manner compared to the SMMC-7721 or NC cells (Fig. 2a). Furthermore, SMMC-7721 cells were transient transfected with MUC1-targeted or unspecific siRNAs using x-fect transfection reagent. The transfection efficiency was above 95% and MUC1-siRNA could significantly inhibit the expression of MUC1 compared to the negative control or NC-siRNA (P < 0.01; Fig. 2b,c). The viability of these MUC1-knockdown cells was reduced compared to the controls at 24 h, but the inhibitory effect of MUC1-siRNA decreased as time progressed when measured by WST-1 cell viability assay (Fig. 2d). These results indicate that MUC1 gene silencing by MUC1-stable-knockdown is much more effective than MUC1-transient-knockdown in inhibiting the proliferation of HCC cells in vitro.

Blocking the activity of JNK inhibits the proliferation of HCC cells in vitro. To investigate the influence of blocking the activity of JNK on HCC cell proliferation, the activity of JNK in the SMMC-7721 cells was inhibited using SP600125 and RNAi, respectively. As SP600125 is a commonly used JNK inhibitor, it could significantly inhibit the phosphorylation of JNK and the proliferation of SMMC-7721 cells in both dose- and time-dependent manners (Fig. 3a,b). When the activity of JNK in SMMC-7721 cells was inhibited by transfection with JNK-targeted or nonspecific siRNAs, the transfection efficiency was above 95% and the expression of JNK was significantly reduced (P < 0.01; Fig. 3c,d). The result from the WST-1 cell viability assay showed that the viability of the JNK-knockdown cells was significantly reduced compared to the controls at 24 h (P < 0.01), but the inhibitory effect of JNK-siRNA decreased as time progressed (Fig. 3e). All of these results demonstrate that blocking the activity of JNK by SP600125 is more effective than JNK RNAi in inhibiting the proliferation of HCC cells in vitro.

Silencing the expression of MUC1 and blocking the activity of JNK suppress the growth of tumors in mice. To evaluate
whether targeting MUC1 and JNK could suppress the growth of tumors in vivo, subcutaneous transplant tumor models were established in BALB/c nude mice. First, SMMC-7721, NC and MR1-D4 cells were inoculated subcutaneously into BALB/c nude mice. As shown in Figure 4(a, b), the tumors in the MR1-D4 group were much smaller. In the following studies, SMMC-7721 cells were inoculated subcutaneously into BALB/c nude mice, and these mice were intratumorally injected with SP600125, JNK-siRNA, or MUC1-siRNA, and the respective controls when the tumors were formed, and the results showed that the tumors in the mice treated with SP600125 were much smaller than the control group (P < 0.01; Fig. 4c–e), while there was no significant difference in the tumors when treated with JNK-siRNA (Fig. 4f–h) or MUC1-siRNA (data no shown). As JNK-siRNA had no effect on the growth of these tumors, the expression of JNK in these tumors was detected by immunohistochemical staining to confirm the silencing effect of JNK-siRNA in vivo, and the result showed that JNK-siRNA did not downregulate the expression of JNK in vivo compared to NC-siRNA, suggesting that new drug delivery methods should be developed in order to achieve ideal effectiveness (Fig. 4i, j). Taken together, these results further indicate that both MUC1 and JNK are potential targets for HCC therapy, providing new methods for HCC therapy.

The inhibition effects of silencing the expression of MUC1 and blocking the activity of JNK on HCC growth are through the JNK/TGF-β signaling pathway in vivo. To verify the molecular mechanism by which inhibiting the expression of MUC1 and the activity of JNK reduced the growth of HCC cells in vivo,
immunohistochemical staining was performed, and the results showed that p-JNK, TGF-β, pSmad3L, pSmad2L/C, c-Myc and MMP-9 expression were highly positive in tumor tissues of the SMMC-7721 group but were weakly positive or even negative in tumor tissues from the SP600125-treated group and MR1-D4 groups (Fig. 5a–d). In addition, in contrast to the above results, the expression of pSmad3C and p21 in the tumor tissues of these groups exhibited opposite patterns (Fig. 5a–d). These results demonstrate that inhibiting the expression of MUC1 and the activity of JNK reduces the growth of HCC cells through the JNK/TGF-β signaling pathway in vivo.

Discussion

Our previous studies revealed that MUC1 promotes HCC progression and tumorigenesis and mediates autocrine TGF-β signaling by activating the JNK/AP-1 pathway in HCC cells, and makes MUC1 and JNK as attractive targets for HCC therapy.
Mucin 1 is a tumor-associated antigen that is aberrantly overexpressed on most epithelial malignant tumors, including HCC and breast, lung, ovarian, prostate and pancreatic cancers. In recent years, numerous preclinical and clinical trials of immune-based therapies that target MUC1 have been reported, including anti-MUC1 monoclonal antibodies, vaccines (peptides, carbohydrate, glycopeptides, DNA, and dendritic cells), aptamers (peptides that resemble DNA and RNA), and adoptive T-cell transfer with MUC1-specific cytotoxic T lymphocytes (CTLs). JNK is a member of a larger group of serine/threonine (Ser/Thr) protein kinases known as the MAPK family. The contributions of JNK in HCC pathogenesis strongly suggest that JNK signaling could be a promising target for developing novel chemoprevention and targeted therapies for HCC. To date, many small molecule inhibitors that might modulate specific components of JNK signaling have been developed. These inhibitors can be broadly classified into two categories: ATP-competitive and ATP-non-competitive inhibitors. The ATP-competitive inhibitors include SP600125, CC-401, CC-930, AS601245, JNK-IN-8 and so on; among these inhibitors, CC-401 and CC-930 have entered phase I and II clinical trials, but both of these trials have been terminated. The most representative ATP-non-competitive inhibitor is D-JNKI-1 (also named as XG-102/AM-111), for which phase III clinical trials evaluating the efficacy and safety of the drug in reducing post-cataract surgery intraocular inflammation and pain have been completed, although D-JNKI-1 has not been applied to the treatment of cancers. Regarding gene therapy, no RNAi therapy targeting either MUC1 or JNK has been reported in clinical trials (Fig. 6).

During recent decades, there have been remarkable advances and profound changes in cancer therapy. One of the most rapidly growing fields of research in cancer therapeutics was the discovery of RNAi, which is able to effectively and specifically downregulate the expression of genes. In the present study, the expression of MUC1 and JNK in HCC cells was silenced using RNAi. Figure 2 shows that the cell viability of MUC1-siRNA transiently transfected cells was increased as time progressed, while the viability of MR1-D4 cells was significantly reduced in a time-dependent manner. In addition, Figure 3 shows almost the same results when SMMC-7721 cells were treated with JNK-siRNA or SP600125. These results indicate that siRNA transient transfection in vitro is less efficient, possibly because the number of siRNAs in single cells decreased as the cells divided and due to rapid enzymatic degradation.

Among the 63 clinical trials on RNAi are currently listed in the NIH clinical studies database, 18 studies have primary results (completed, terminated, or withdrawn), indicating that RNAi has potential clinical uses for cancer therapeutics. However, some limitations make their clinical application difficult, including delivery problems, side-effects due to off-target actions, the disturbance of physiological functions of the cellular machinery involved in gene silencing, and the induction of innate immune responses. Unmodified siRNA is unstable in the bloodstream, can be immunogenic, and does not readily cross membranes to enter cells. Therefore, chemical modifications and/or delivery materials are required to bring siRNA to the site of action without causing adverse effects. A broad range of materials is under exploration to address the challenges of in vivo delivery, including polymers, lipids, peptides, antibodies, aptamers and small molecules. In the present study, we designed 5‘-cholesterol-conjugated and 2‘-O-methyl-modified MUC1- and JNK-siRNA that enhanced the delivery efficiency and abolished the immunostimulatory activity of unmodified/native RNA duplexes when administered in a delivery vehicle. Unfortunately, there was no significant difference of the tumors in these mice when treated with JNK-siRNA (Fig. 4f–h) or MUC1-siRNA (data not shown) compared to that treated with NC-siRNA, while the tumors in the MR1-D4 group were much smaller than that in the SMMC-7721 or NC groups (Fig. 4a,b). The key challenge to realizing the broad potential of siRNA-based therapeutics is the need for safe and effective delivery methods, our results suggest that a more effective delivery method for siRNAs by targeting MUC1 or JNK is needed to be investigated for HCC therapy. Beside MUC1 and JNK, TGF-β signaling inhibition is an emerging strategy for cancer therapy (Fig. 6). To date, many inhibitors by targeting TGF-β signaling inhibition are in clinical development. The future of TGF-β inhibitors in cancer therapy as tumor micro-environment-targeting agents is promising and opens new challenges in terms of biomarkers and patient selection.

In summary, our study reveals that MUC1 and JNK are attractive molecular targets for HCC therapy. It is anticipated that several MUC1- and JNK-targeted therapies, such as via the use of RNAi or inhibitors, will be successfully developed and used in the clinic in the near future. We believe that
promising new avenues for the treatment of HCC are on the horizon, which will undoubtedly lead to better, more effective and faster therapies in the future.

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Disclosure Statement

The authors have no conflict of interest.

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