Knockdown of c-Met inhibits cell proliferation and invasion and increases chemosensitivity to doxorubicin in human multiple myeloma U266 cells in vitro

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Abstract. c-Met, a receptor tyrosine kinase and its ligand, hepatocyte growth factor, are critical in cellular proliferation, motility and invasion and confer resistance to specific chemotherapy drugs. However, little is known about the impact of c-Met knockdown on the biological functions of human multiple myeloma U266 cells. The present study was designed to determine the role of c-Met in the proliferation and invasion of U266 cells, using RNA interference technology in vitro. In our study, the c-Met short hairpin RNA (shRNA) was successfully transfected into U266 cells, which resulted in the significant inhibition of transcription and expression of c-Met. The down-regulation of c-Met inhibited the proliferation potential, adherence and invasiveness of U266 cells, and also increased chemosensitivity to doxorubicin. The c-Met shRNA in U266 cells induced apoptosis and increased the accumulation of cleavage PARP and cleavage caspase-3. However, the expression of Bcl-2 and Bax did not change following the c-Met knockdown. Taken together, our data reveal that the down-regulation of c-Met inhibits proliferation and invasion and increases the chemosensitivity of U266 cells. Thus, the targeting of c-Met could be an effective therapeutic approach against multiple myeloma.

Introduction

Multiple myeloma (MM) is the second most common hematological malignancy, characterized by the clonal proliferation of neoplastic plasma cells in the bone marrow (BM). At present, MM is an incurable disease, in spite of the fact that most patients to a certain extent respond to chemotherapy. High-dose chemotherapy with stem cell support has achieved higher response rates than conventional therapies, but few patients remain in long-term remission. New effective anticancer targets for both early and advanced MM is a dynamic research area (1-5).

The interaction of MM cells with extracellular matrix (ECM) proteins and BM cells, as well as factors in the BM milieu (cytokines, chemokines and angiogenesis), play a crucial role in MM pathogenesis (6-9). The interaction of MM cells with the BM microenvironment activates MM cell proliferation and the anti-apoptotic signaling cascades (10,11). These molecular events are triggered directly, via cell adhesion molecule-mediated interactions, or indirectly by growth factors released by BM stromal cells (BMSCs), MM cells or both (12,13).

Hepatocyte growth factor (HGF) is a multifunctional protein. c-Met is the receptor for HGF, a protein product of a proto-oncogene (14). It is a transmembrane tyrosine kinase with structural and functional features of a growth factor receptor (15). On binding with its ligand, autophosphorylation of the receptor stimulates its intrinsic tyrosine kinase activity with resultant changes in cellular motility, growth and invasion. c-Met overexpression has been found in numerous human tumors (16,17). Previous studies have revealed that c-Met and its ligand HGF are overexpressed in human myeloma cell lines (JJN-3, U-266, OH-2 and JW) and primary human MM cells (18). In addition, HGF has been reported to promote MM cell growth and migration in vitro (19). In the present study, we investigated the role of c-Met in the proliferation and invasion of human MM U266 cells using RNA interference (RNAi) technology in vitro.

RNAi is a sequence-specific, post-transcriptional gene silencing method initiated by double-stranded RNAs, which are homologous to the target gene (20). In the present study, the highly expressed c-Met U266 cell line was used as a cellular model of MM for gene studying.

Materials and methods

Cell culture. ECV304 and U266 cells (Wuhan University, P.R. China) were suspended in RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Gibco) and cultured at 37°C in a humidified atmosphere containing 5% CO₂.
Table I. The sequences of the designed c-Met-specific shRNAs.

<table>
<thead>
<tr>
<th>shRNA</th>
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<tr>
<td>shRNA-Met</td>
<td>5'-GATCCGCAAGCCAGATTCTGC&lt;br&gt;CGAACCATCAAGAGTGGTTCG&lt;br&gt;GCAGAATCTGGCTTGCTTATA-3'&lt;br&gt;5'-AGCTTAAAAGCAAGCCGAT&lt;br&gt;TCTGCGAACCACCTTTGTAGT&lt;br&gt;GGTTCCAGAATCTGGCTTGCTTATA-3'&lt;br&gt;5'-GATCCGTCACACTCTCGAGAT&lt;br&gt;CGATGCTCAAAGGGCATCTG&lt;br&gt;ATTGCGAGTCTGACTTTTTTA-3'&lt;br&gt;5'-AGCTTAAAGATCTGACAC&lt;br&gt;CGATAGATCCGCTCTTAGACATC&lt;br&gt;2TTCTAGTGAGTGCAGAC-3'</td>
</tr>
<tr>
<td>shRNA-control</td>
<td>5'-GATCCGCAAGCCAGATTCTGC&lt;br&gt;CGAACCATCAAGAGTGGTTCG&lt;br&gt;GCAGAATCTGGCTTGCTTATA-3'&lt;br&gt;5'-AGCTTAAAAGCAAGCCGAT&lt;br&gt;TCTGCGAACCACCTTTGTAGT&lt;br&gt;GGTTCCAGAATCTGGCTTGCTTATA-3'&lt;br&gt;5'-GATCCGTCACACTCTCGAGAT&lt;br&gt;CGATGCTCAAAGGGCATCTG&lt;br&gt;ATTGCGAGTCTGACTTTTTTA-3'&lt;br&gt;5'-AGCTTAAAGATCTGACAC&lt;br&gt;CGATAGATCCGCTCTTAGACATC&lt;br&gt;2TTCTAGTGAGTGCAGAC-3'</td>
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Construction of short hairpin RNA (shRNA) expression vectors. The vector pSilencer 3.1 H1 neo (Ambion Inc., Austin, TX, USA) was used to generate shRNA specific for c-Met. The region of c-Met mRNA (GenBank: NM_000245) ranging from 1,045 to 1,067 bp was selected as the RNAi target site, designated as shRNA-Met (21). The template oligonucleotides encoding the target sequence were designed and synthesized. shRNA-control encoding a non-specific shRNA was also synthesized and used as the negative control (Table I). These oligonucleotides were annealed and subcloned into the HindIII and BamHI sites of the vector according to the manufacturer’s instructions. These recombinant vectors were designated as pSilencer-shRNA-Met and pSilencer-shRNA-control, respectively.

Transfection of cells. Transfection of U266 cells was performed using the Amaxa cell optimization kit V (Amaxa, Köln, Germany) according to manufacturer’s instructions, using pSilencer-shRNA-Met and pSilencer-shRNA-control as mentioned above. These cells were cultured in the presence of 400 µg/ml G418 (Gibco) for 4 weeks. The positive clones were picked and expanded to establish cell lines. RT-PCR and Western blot analysis were used to identify the successful transfection. The stable transfection cell clones were designated as U266/shRNA-Met and U266/shRNA-control, respectively.

RT-PCR analysis. Total RNA was isolated from cells, using TRIzol reagent (Invitrogen). RNA (4 µg) was reverse transcribed to cDNA by the Thermoscript RT-PCR System reagent (Gibco). Primers were designed for the PCR reaction. The sequences for human c-Met were 5'-CCGAGGTTGTATGTATGAGTG-3' (sense) and 3'-GGAGAGTGGTCTAGAGGAGG-5' (antisense). Primers for the control GAPDH were 5'-GAGGTCTACTGATATTCCTC-3' (sense) and 5'-GTCTGAGCATCTAGAGTTC-3' (antisense). The PCR amplification consisted of 35 cycles: 15 sec at 94°C for denaturing, 30 sec at 58°C for annealing and 45 sec at 72°C for elongation. Bands obtained on 1.5% agarose gel electrophoresis were analyzed and quantified with an image analyzer. The transcript levels were normalized according to the GAPDH transcripts. Results were expressed in relation to the control value.

Western blot analysis. The cultured cells (1x10⁶) were collected and lysed. Protein concentration was determined by the Lowry method using BSA as the standard. Equal amounts of protein were separated on 6-15% SDS/PAGE gels. The protein was then electrophoretically blotted onto polyvinylidene fluoride (PVDF) membranes, which were blocked with TBST containing 5% non-fat milk at 4°C overnight and then incubated with anti-cleaved caspase-3 (Asp175) and anti-cleaved PARP (Asp214; Cell Signaling Technology, Beverly, MA, USA), anti-c-Met, anti-bax, anti-bcl-2 and GAPDH (Santa Cruz, USA) for 2 h. After washing with TBST, the blots were incubated with HRP-labeled secondary antibodies for 2 h at room temperature. The blots were visualized by the enhanced chemiluminescence (ECL) reagent kit (Beyotime, P.R. China).

Cell proliferation assay. Before the cell proliferation assay, trypsin blue exclusion test of cell viability was performed and the viability of the three groups of cells (untreated U266, U266/shRNA-control and U266/shRNA-Met) was >98%. Cell proliferation in vitro was analyzed with 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma, USA). Briefly, cells were seeded in quadruplicate at a density of 2x10⁵ cells/well in 96-well plates in 200 µl of medium and cultured for 24, 48 and 72 h, respectively. Medium without any cells (200 µl) was provided as the blank control. Subsequently, 20 µl of MTT substrate (5 mg/µl in PBS) were added to each well and the plates were returned to standard cell incubator conditions for 4 h. After a brief centrifugation, supernatants were carefully removed and 200 µl DMSO were added to each well. After the insoluble crystals were completely dissolved, colorimetric analysis was performed (wavelength, 490 nm). The inhibition rate was calculated as follows: 100%-(OD value of U266 cells/OD value of blank control)%.

Flow cytometry analysis of apoptosis. Cells were washed twice with ice-cold PBS and fixed with 70% ethanol at 4°C overnight. After washing with PBS, cells were incubated in 0.5 ml PBS containing 50 µg/ml RNase A for 30 min at 37°C and then PI was added to achieve the final concentration of 50 µg/ml for 30 min on ice in the dark. The resultant cell suspension was then subjected to flow cytometry analysis (Coulter Epics XL). The percentage of apoptotic cells (sub-G₁) and cells in the G₀/G₁, S and G₂/M phases was calculated.

Adhesion assay. Cells were seeded in quadruplicate at a density of 1x10⁴ cells/well in 96-well plates coated with 10 g/l BSA, 50 mg/l Matrigel or 10 mg/l fibronectin (Fn). After the cells were cultured at 37°C for 60 min, MTT assay was performed as described above (22-25). BSA-coated wells were provided as the negative control. The percentage of adhesion was calculated as follows: 100%-(OD value of treated/OD
value of negative control)%. Each experiment was carried out in triplicate.

**U266 cell adherence to ECV304 cells.** ECV304 cells were plated onto 96-well plates at a density of 5x10^4 cells/well. After 48-h incubation, the supernatant was aspirated and cells were resuspended at a density of 5x10^4 cells/well. After 30 min, the wells were gently washed twice with PBS to remove unattached cells. Rose bengal (25%; 100 µl) was added to each well and left standing for 5 min. The supernatant was then aspirated, the wells were gently washed twice with PBS and finally 200 µl of 95% ethanol/PBS (1:1) were added to each well. After 20-min incubation, the absorbance was recorded at 570 nm.

**Invasion assay.** The invasion assay was performed, using Transwell polycarbonate membrane (Corning, Lowell, MA, USA) following the manufacturer's instructions. Briefly, the underside of each polycarbonate microporous membrane was coated with Matrigel (1:100) at 37°C for 5 min and left standing overnight. The membrane was then inserted into 24-well plates, forming upper and lower compartments, and 50 µl Matrigel (1:30) and 200 µl sterile water were added to the upper compartment and left standing at 37°C. After 2 days, 200 µl of the invasion buffer [2 ml BSA (2%) + 38 ml RPMI-1640] were added into the upper compartment. After 1 h, the upper compartment fluid was aspirated. Cells at a density of 5x10^6 cells/well were added into the upper compartment and 800 µl of the Fn solution (10 µg/ml) were added into the lower compartment. The cells were allowed to migrate for 48 h. The inserted membranes were then fixed in 10% formalin, stained with hematoxylin and eosin and rinsed by dipping in water. The cells on the upper surface of the membrane were removed with a cotton bud. The membranes were air-dried overnight, excised from the insert and mounted onto glass slides for microscopic analysis. The migrated cells were counted at high-power magnification (x40) from four randomly selected fields. Each experiment was repeated three times.

**Increased chemosensitivity to doxorubicin.** The cells were seeded in triplicate on 96-well plates at 1x10^4 cells/well and incubated for 24 h. Then the medium was gently removed and replaced with fresh medium, containing doxorubicin (Sigma) in varying concentrations (doxorubicin±), that is, 0.1X peak plasma concentration (PPC), 1X PPC and 10X PPC, and with the medium without doxorubicin as the control. After 48-h incubation, the cells were treated with MTT as described above. The inhibition rate was calculated as follows: 100%-OD490(absorption) / OD490 (absorption)%. The assay was repeated three times.

**Statistical analysis.** The data were expressed as the means ± SD, and the Student's t-test was used to determine the significance of differences in multiple comparisons. A value of p<0.05 was considered to be statistically significant.

**Results**

**Knockdown of c-Met efficiently suppresses c-Met expression.** The silencing effects of c-Met-specific shRNA in U266 cells was first evaluated using RT-PCR and Western blot analyses. The results revealed that the c-Met mRNA expression in the U266/shRNA-Met cells was 45.4±1.9%, significantly lower than that in the U266/shRNA-control cells (91.7±3.1%) or the untreated U266 cells (93.7±2.7%; p<0.05; Fig. 1A). Western blot analysis revealed similar results. The density of the c-Met protein in the U266/shRNA-Met cells was 40.9±2.4%, significantly lower than that in the U266/shRNA-control cells (99.8±2.9%) or the untreated U266 cells (99.4±3.8%; p<0.05; Fig. 1B). There was no significant difference between the U266/shRNA-control and the untreated U266 cells (p>0.05).

**Knockdown of c-Met inhibits cell proliferation, induces cell cycle arrest and promotes apoptosis in U266 cells.** We then investigated whether c-Met shRNA decreases the proliferation of U266 cells. As shown in Fig. 2, compared to the untreated U266 cells, the proliferation capacity of U266/shRNA-Met was inhibited by 72.51±1.49% (p<0.01), 66.08±1.91% (p<0.01) and 68.27±1.87% (p<0.01) at 24, 48 and 72 h, respectively. There was no significant difference in cell cycle distribution between the U266/shRNA and the untreated U266 cells (p>0.05). The percentage of cells in G0/G1, S and G2/M phase was 90.4±3.2%, 6.8±2.1% and 2.8±1.2%, respectively, in the untreated U266 cells. Knockdown of c-Met significantly increased the percentage of cells in G0/G1 phase (94.8±2.1%; 0.05<p<0.01) and the percentage of apoptosis (26.2±3.1%; 0.01<p<0.05), but did not change the percentage of cells in S and G2/M phase (p>0.05).

**Conclusion.** The c-Met silencing effects in U266 cells were confirmed by RT-PCR and Western blot analyses. Knockdown of c-Met efficiently decreases c-Met expression and inhibits cell proliferation, induces cell cycle arrest and promotes apoptosis in U266 cells. This effect might be associated with the regulation of cell cycle and apoptosis. Future studies are needed to further investigate the molecular mechanisms of U266 cell proliferation and apoptosis in the context of c-Met silencing.
was no significant difference in the proliferation capacity between the U266/shRNA-control and untreated U266 cells (p>0.05). To determine the apoptosis-inducing potential of c-Met/shRNA in U266 cells, flow cytometric analysis of PI-stained cells was performed. As indicated in Fig. 3A, the percentage of cells in the G0/G1 phase in the U266/shRNA-Met group (50.1%) was much higher than that observed in the U266 shRNA-control group (30.3%). The percentages of cells in the S and G2/M phase in the U266/shRNA-Met group were 3.98 and 45.9%, respectively, lower than those in control group, which were 12.1 and 57.6%, respectively. The apoptotic rate in U266/shRNA-Met cells was 17.9%, much higher than that (4.39%) in the control group (Fig. 3B).

Effects of c-Met on cell apoptosis-associated protein expression. We have demonstrated that the knockdown of c-Met induces U266 cell apoptosis. To further elucidate how the c-Met regulation affects apoptosis, caspase-3, PARP, bcl-2 and bax which are associated with apoptosis, were detected...
respectively (Fig. 5 c). Thus, the adhesion of shRNA-Met u266, u266/shRNA-control and u266/shRNA-Met cells, 0.751±0.007, 0.742±0.004 and 0.312±0.005 for the untreated adherence capacities to eCV304. The absorbance values were (Fig. 5B). In addition, the U266 cell line revealed various percentages of adhesion to ECM (Matrigel) were 83.1±6.2, 82.6±9.1 and 34.1±2.5% for the untreated U266, the U266/shRNA-control cells or the U266/shRNA-Met cells. However, in our present study, no significant differences were found in the expression of both Bcl-2 and Bax proteins in the U266/shRNA-control and the untreated U266 cells or the U266/shRNA-Met cells.

Effects of c-Met on cell adhesion. Suppressing c-Met expression had a clear inhibitory effect on the adhesion of transfected U266 cells to ECM (Fn and Matrigel) and to eCV304 cells. The percentages of adhesion to ECM (Fn) were 45.9±2.4, 41.7±4.7 and 11.2±1.7% for the untreated U266, the U266/shRNA-control and U266/shRNA-Met cells (Fig. 5A). The percentages of adhesion to ECM (Matrigel) were 83.1±6.2, 82.6±9.1 and 34.1±2.5% for the untreated U266, U266/shRNA-control and U266/shRNA-Met cells, respectively (Fig. 5B). In addition, the U266 cell line revealed various adherence capacities to eCV304. The absorbance values were 0.751±0.007, 0.742±0.004 and 0.312±0.005 for the untreated U266, U266/shRNA-control and U266/shRNA-Met cells, respectively (Fig. 5C). Thus, the adhesion of shRNA-Met to ECM and to eCV304 cells was significantly suppressed (P<0.01).

Effects of c-Met on cell invasion. As depicted in Fig. 6, for each x400 field under the microscope, the number of migrated U266/shRNA-Met cells on the membrane was 211±14, significantly lower than that of the U266/shRNA-control (451±11; p<0.01) or untreated U266 cells (463±10; p<0.01).

Increased chemosensitivity to doxorubicin. We investigated whether the inhibition of c-Met by shRNA affected the sensitivity of U266 cells to the anti-tumor drug, doxorubicin. At all the indicated concentrations of doxorubicin, the inhibition rate in the U266/shRNA-Met cells was markedly higher than that in the untreated U266 or U266/shRNA-control cells (p<0.01). There was no significant difference between the U266/shRNA-control and the untreated U266 cells (p>0.05; Fig. 7).

Discussion

HGF/c-Met has been proved to be involved in the progression of many malignancies and the spread of disease (26-29). In MM patients, elevated levels of HGF have been found in serum and BM, compared to healthy controls (30-33), indicating that a high serum level of HGF at the time of diagnosis has a strong negative prognostic impact (30,33). Previous basic studies have also revealed that c-Met and its ligand HGF are overexpressed in human myeloma cell lines (JJN-3, U-266, OH-2 and JW) and primary human MM cells (18,34,35).

The silencing of c-Met by shRNA has been reported in various tumor cells (36,37). This suggests that shRNA is an effective technique for tumor study and treatment. However, there have been no reports of silencing c-Met in myeloma cells until recently. The nucleofector technique is a new non-viral transfection method, especially designed for hard-to-transfect cell lines (38). In the present study, we successfully transfected shRNAs targeting the c-Met gene into the human MM cell line, U266, using the nucleofector technique, and the c-Met expression was effectively inhibited at both the mRNA and protein levels.

A recent study has also indicated that c-Met signaling could be important in myeloma cell proliferation (39). We examined the effect of c-Met silencing on the proliferation capacity of U266 cells. The proliferation capacity of shRNA-Met cells was suppressed compared to the untreated U266 or U266/shRNA-control cells. This is in agreement with results from other studies, in that the down-regulation of c-Met leads to myeloma cell death, thus indicating that the level of c-Met expression is important for the survival of myeloma cells (40,41).

It has also been reported that c-Met signaling results in the inhibition of proliferation and induction of apoptosis in a wide variety of tumors. For example, exposure to ARQ 197, a novel and selective inhibitor of the human c-Met receptor tyrosine kinase, resulted in the inhibition of proliferation of c-Met-expressing cancer cell lines, as well as the induction of caspase-dependent apoptosis in cell lines (42). In vitro pharmacological c-Met inhibition and siRNA targeted against c-Met triggered caspase-dependent apoptosis in DBLCL cells (43) and HGF has strong proliferative and anti-apoptotic effects on...
both MM cell lines and primary MM cells (44). In agreement with these results, our study reveals that the decreased proliferation capacity of the U266 cells in the shRNA-Met group is associated with increased rates of apoptosis and that cell cycle arrest occurs at the G1/S phase, as measured by flow cytometric analysis of PI-stained cells.

Active caspases play an important role in the induction of apoptosis. After caspase-3 was activated, PARP was cleaved (45). The cleavage of PARP has often been used as an indicator of apoptosis. In the present study, U266/shRNA-Met cells possessed a higher level of cleaved caspase-3 (p17) fragments and cleaved PARP. These results reveal that the c-Met knockdown by shRNA is sufficient to trigger caspase-dependent apoptosis, which could partially be the reason for the decrease of cell viability.

The Bcl-2 family proteins have been reported to regulate apoptosis by controlling the mitochondrial membrane permeability (46). It has been found that some anti-MM agents, such as dexamethasone, thalidomide and proteasome inhibitors (PS-341), induce apoptosis in myeloma cell lines or patient cells associated with the down-regulation of Bcl-2 or/and the up-regulation of Bax (47). However, in this study, we found that although c-Met was knocked down in U266 cells and apoptosis occurred, the expression of Bcl-2 and Bax did not change. This is in agreement with the results from the study by Kitamura et al., in that the gene expression of bcl-2 or bcl-x(L) was not affected by treatment with c-met-antisense oligonucleotides in LoVo cells (48). This suggests that apoptosis in U266 cells, induced by the knockdown of c-Met, is independent of the modulation of Bcl-2 and Bax. Future study is required in order to identify the exact mechanism.

MM had a disseminated growth pattern, with cancer cells usually spread throughout the skeleton. This dissemination is dependent on the migration of cells through endothelial barriers and on the adhesion to other cells, as well as to the matrix components. The involvement of HGF in transendothelial migration of myeloma cells has recently been documented (49). Our study also reveals that the down-regulation of c-Met expression inhibits the adhesion and invasion ability of myeloma cells in vitro, which is consistent with the results of Hov et al., in that PHA-665752, a small-molecule inhibitor of c-Met, prevents the HGF-induced migration and adhesion of myeloma cells (50). These findings suggest that c-Met could be an important target for the migration and adhesion of MM cells.

The importance of myeloma cell adhesion for survival and resistance to chemotherapeutic drugs has been documented in several studies (51,52). Drug resistance is an important cause of treatment failure and mortality in MM patients. Lou et al. reported that the inhibition of the c-Met receptor tyrosine kinase signaling enhances the chemosensitivity of glioma cell lines to CDDP (53). A recent study has also indicated that c-Met antisense oligodeoxynucleotides increases the sensitivity of human glioma cells to paclitaxel (54). In our study, the down-regulation of c-Met expression increased the chemosensitivity to doxorubicin in the human MM cell line, U266, suggesting that c-Met is an adjuvant chemotherapeutic target for MM.

We considered that more MM cell lines should be included in this study besides U266, to confirm the availability of suppression of proliferation and invasion mediated by the knockdown of c-Met in MM cells. Furthermore, animal experiments should also be performed to further confirm the inhibitory effects of proliferation and metastasis mediated by c-Met knockdown on MM in vivo. Therefore, the anti-cancer effect induced by c-Met knockdown requires further investigation. In conclusion, our findings indicate that the c-Met expression is inhibited by c-Met shRNA and that the shRNA-mediated silencing of c-Met not only results in the inhibition of cell proliferation, adherence and invasive potential, but also increases the chemosensitivity to doxorubicin in U266 cells, independent of the Bcl-2 pathway. Our results provide further evidence that c-Met is an important target for MM treatment and that the c-Met shRNA could serve as an effective tool.

Acknowledgements

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