

Myeloid cell leukemia-1 promotes epithelial-mesenchymal transition of human gastric cancer cells

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Abstract. Epithelial-mesenchymal transition (EMT) is a critical process that occurs during cancer progression, and cancer stem cells have been shown to acquire the EMT phenotype. Myeloid cell leukemia-1 (Mcl-1) has been implicated in cancer progression and is overexpressed in a variety of human cancers. However, the interaction between Mcl-1 and EMT in human gastric cancer (GC) is unclear. We investigated the impact of Mcl-1 expression levels on EMT and the underlying signaling pathways in human GC cells. We used the human GC cell lines, AGS and SNU638, and small interfering RNAs (siRNAs) to evaluate the effects of Mcl-1 knockdown on cell adhesion, migration and invasion. Expression of Mcl-1 and other target genes was determined using reverse transcription-polymerase chain reaction assays and western blotting. The results revealed that expression levels of Mcl-1 mRNA and protein in the AGS and SNU638 cells were reduced following transfection with Mcl-1 siRNAs. Knockdown of Mcl-1 led to increased cellular adhesion to fibronectin and collagen. Expression levels of vimentin, MMP-2, MMP-9 and Snail protein were decreased following knockdown of Mcl-1. However, expression of E-cadherin was increased in the AGS cells following knockdown of Mcl-1. The expression of cancer stemness markers, such as CD44 and CD133, was not altered by knockdown of Mcl-1. Knockdown of Mcl-1 suppressed tumor cell migration and invasion in both human GC cell lines. Signaling cascades, including the β -catenin, MEK1/2, ERK1/2 and p38 pathways, were significantly blocked by knockdown of Mcl-1. Our results indicate that Mcl-1 expression induces EMT via β -catenin, MEK1/2 and MAPK signaling pathways, which subsequently stimulates the invasive and migratory capacity of human GC cells.

Introduction

Gastric cancer (GC) remains one of the most common causes of cancer-related death worldwide, although its incidence is decreasing. Despite recent improvements in the early diagnosis and effective treatment of GC, its progression and metastasis are major contributors to GC-related death (1-3). Therefore, an understanding of the molecular and biological changes underlying the progression and metastasis of GC is required to predict outcomes, personalize treatment and improve the survival rates of GC patients.

The B-cell leukemia/lymphoma-2 (Bcl-2) protein family regulates the integrity of the outer mitochondrial membrane and intrinsic pathways of apoptosis. The Bcl-2 family comprises pro- and anti-apoptotic members. The pro-apoptotic members control the release of cytochrome *c*, and subsequent activation of caspases. In contrast, anti-apoptotic members such as Bcl-2, Bcl-xL, Bcl-w, A1 and myeloid cell leukemia-1 (Mcl-1) promote cell survival by inhibiting pro-apoptotic proteins, including Bim, Bax, and Bak (4-7).

Mcl-1 is a rapidly inducible, anti-apoptotic Bcl-2 protein with a very short half-life. Cells with increased Mcl-1 expression levels exhibit inhibition of apoptosis and cell cycle progression, and chemoresistance (8-11). Increased expression of Mcl-1 occurs in a variety of human cancers and is strongly associated with resistance to therapies, tumor progression, and poor prognosis in most cancers, including GC (12-17). Therefore, Mcl-1 could be a promising molecular target with respect to improving treatment strategies and outcomes for cancer patients.

Epithelial-mesenchymal transition (EMT) is a complex process that has been observed in embryonic development, differentiation of normal tissues and organs, wound healing, and cancer progression. During EMT, cells lose their epithelial characteristics and gain mesenchymal phenotypes, which are correlated with increased motility and invasion (18-22). Mesenchymal cells tend to dedifferentiate and acquire stem cell or tumorigenic phenotypes, such as invasion, metastasis, resistance to apoptosis and drug resistance during EMT progression (18-22).

EMT has been implicated in cancer progression and metastasis, and is associated with poor clinical prognosis in a variety

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of human cancers (18-22). However, the interaction between Mcl-1 and EMT in human GC is unclear. We investigated the impact of Mcl-1 expression levels on EMT and the underlying signaling pathways in human GC cells.

Materials and methods

Cell culture and transfection with small interfering RNAs (siRNAs). Human GC cell lines, AGS and SNU638, were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and the Korean Cell Line Bank (Seoul, Korea), respectively. Cells were cultured in RPMI-1640 medium containing 25 mM HEPES and supplemented with 10% fetal bovine serum (FBS) (both from Hyclone, Logan, UT, USA), 50 U/ml penicillin, and 50 μ g/ml streptomycin (Gibco, Grand Island, NY, USA). Cultures were incubated at 37°C in 5% CO₂ in a humidified environment. Cells were seeded on plates at a density such that they would be 40-50% confluent at the time of transfection. The Mcl-1-specific and control-scrambled siRNA duplexes were purchased from Bioneer (Daeheon, Korea) and Qiagen (Germantown, MD, USA), respectively. The siRNAs were transfected into cells using Lipofectamine[®] RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR) assays. Total RNA was isolated from the cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For each sample, 1 μ g of total RNA was used to generate complementary DNA in a reaction containing 50 ng/ μ l oligo-dT (Promega, Madison, WI, USA) that was incubated at 72°C for 10 min. We then added MMLV transcription reagents (Promega) and RNasin (Takara, Otsu, Shiga, Japan) to each reaction and incubated the samples at 42°C for 1 h and 72°C for 15 min. PCR amplification was performed using gene-specific primers and GoTaq[®] DNA polymerase (Promega). The primers we used were specific for Mcl-1 (5'-TCC TCT TGC CAC TTG CTT TT-3' and 5'-TGC TGG AGT AGG AGC TGG TT-3'); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 5'-ACC ACA GTC CAT GCC ATC AC-3' and 5'-TCC ACC ACC CTG TTG CTG TA-3'). Amplicons were separated by electrophoresis on 1% (w/v) agarose gels containing ethidium bromide.

Western blotting. Proteins were extracted from the cells using RIPA buffer (1 M Tris-HCl, 150 mM NaCl, 1% Triton X-100 and 2 mM EDTA) supplemented with 1 mM PMSF, Halt[™] Phosphatase Inhibitor Cocktail and Halt[™] Protease Inhibitor Cocktail (both from Thermo, Rockford, IL, USA). Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% bovine serum albumin (BSA) buffer, PVDF membranes were probed with the appropriate primary antibody. We used antibodies against human Mcl-1, Snail, vimentin, E-cadherin, phosphorylated β -catenin, β -catenin, MEK1/2, phosphorylated ERK1/2, ERK1/2, p38 and phosphorylated p38 (all from Cell Signaling Technology, Danvers, MA, USA). Antibodies against human MMP-2, MMP-9, GAPDH and β -tubulin were purchased from Santa Cruz Biotechnology

(Santa Cruz, CA, USA). Antibodies against human CD44 and CD133 were purchased from R&D Systems (Minneapolis, MN, USA) and eBioscience (San Diego, CA, USA), respectively. Protein bands were detected using a chemiluminescent horseradish peroxidase substrate (Millipore) and an ImageQuant[™] LAS 4000 Luminescence imager (Fujifilm, Tokyo, Japan). The density ratio (%) of protein bands was quantified using MultiGauge V3.2 image analyzer software (Fujifilm).

Cell adhesion assays. Cell adhesion assay was conducted by coating fibronectin (2 μ g/ml; Calbiochem, La Jolla, CA, USA) and collagen type I and IV (40 μ g/ml; Corning Inc., Corning, NY, USA), respectively. The coated wells were washed with PBS, and blocked with 0.2% BSA medium for 30 min. Then the transfected cell suspension was added into the coated wells and incubated for 1 h at 37°C. Non-adherent cells were removed by washing with PBS. The attached cells were reacted with WST-1 solution (Daeil Lab Inc., Seoul, Korea) medium at 37°C for 1 h. The optical density was measured at 450 nm. All experiments were carried out in triplicate.

Cell invasion assays. We conducted cell invasion assays using Transwell chambers with 8- μ m pores (Corning Inc.). Transwell chambers were coated with 1% gelatin in RPMI-1640 overnight and then allowed to dry at room temperature. Cells transfected with the Mcl-1 and scrambled siRNAs were resuspended in 120 μ l of 0.2% (w/v) BSA solution and seeded into the upper chambers. The lower chambers contained 400 μ l of 0.2% (w/v) BSA solution supplemented with 10 μ g/ml human plasma fibronectin (Calbiochem) as a chemoattractant. After incubation for 24 h, the cells that had migrated to the bottom surface of the Transwell were fixed with 70% ethanol and stained with Diff-Quik solution (Sysmex, Kobe, Japan). The cells in the upper chambers were removed using a cotton tip. Stained cells in the lower chambers were counted using light microscopy, from five randomly selected fields of view (0.5x0.5 mm²).

Cell migration assays. Cell migration was measured using Ibidi Culture-Inserts (Ibidi, Regensburg, Germany). The cells transfected with the Mcl-1 and scrambled siRNAs were seeded on the culture-inserts and incubated at 37°C in a humidified environment. After 24 h, the culture-inserts were gently removed using sterile tweezers to create a cell-free gap. Cell migration into the cell-free gap was followed for 24 h, and photographed using an inverted microscope. The distance between gaps was normalized to 1 cm after images were captured at three random sites.

Statistical analysis. The associations between experimental groups were analyzed using a Student's t-test. A value of P<0.05 was considered to indicate a statistically significant result.

Results

Impact of Mcl-1 knockdown on EMT in the human GC cells. To study the biological role of Mcl-1 in GC progression, we used siRNAs to knock down endogenous Mcl-1 expression in AGS and SNU638 cells. Expression levels of Mcl-1 mRNA and protein in all tested cells were reduced following transfection with the Mcl-1 siRNAs (Fig. 1). To investigate the relationship

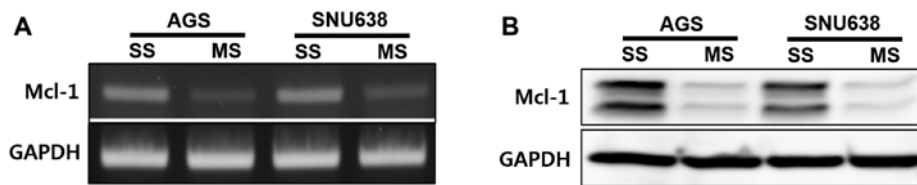


Figure 1. Effects of Mcl-1 knockdown on human GC cells. Expression level of (A) Mcl-1 mRNA and (B) Mcl-1 protein were decreased following transfection of human GC cell lines (AGS and SNU638) with Mcl-1 siRNAs. SS, scrambled siRNA; MS, Mcl-1 siRNA; Mcl-1, myeloid cell leukemia-1; GC, gastric cancer.

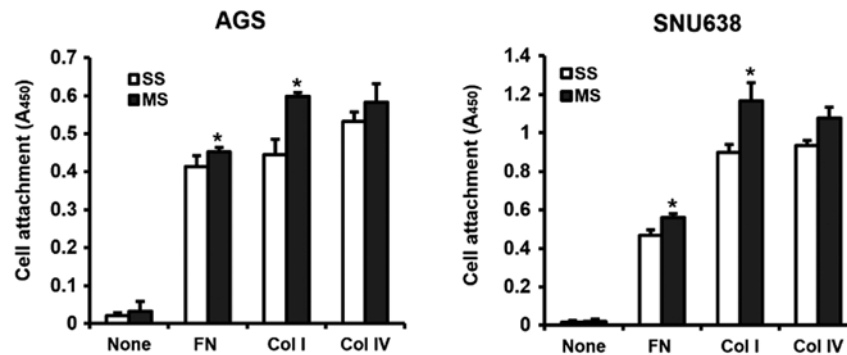


Figure 2. Mcl-1 knockdown leads to increased cellular adhesion to fibronectin and collagen I in human GC cells. The cell adhesion ability was measured after transfection of the siRNAs using three cell adhesion substrates including fibronectin and collagen I and IV. The adherent cells were stained with crystal violet, dissolved with sodium dodecyl sulfate, and then quantified by reading the absorbance at 540 nm using a plate reader. The cell adhesion to fibronectin and collagen I was significantly increased in the Mcl-1 siRNA-transfected AGS ($P=0.023$ and 0.034 , respectively) and SNU638 cells ($P=0.045$ and 0.025 , respectively) compared to the ability of the scrambled siRNA-transfected cells. Each bar represents the mean \pm SE of 3 experiments. * $P<0.05$ vs. scrambled siRNA-transfected cells. SS, scrambled siRNA; MS, Mcl-1 siRNA; FN, fibronectin; Col I, collagen I; Col IV, collagen IV; Mcl-1, myeloid cell leukemia-1; GC, gastric cancer.

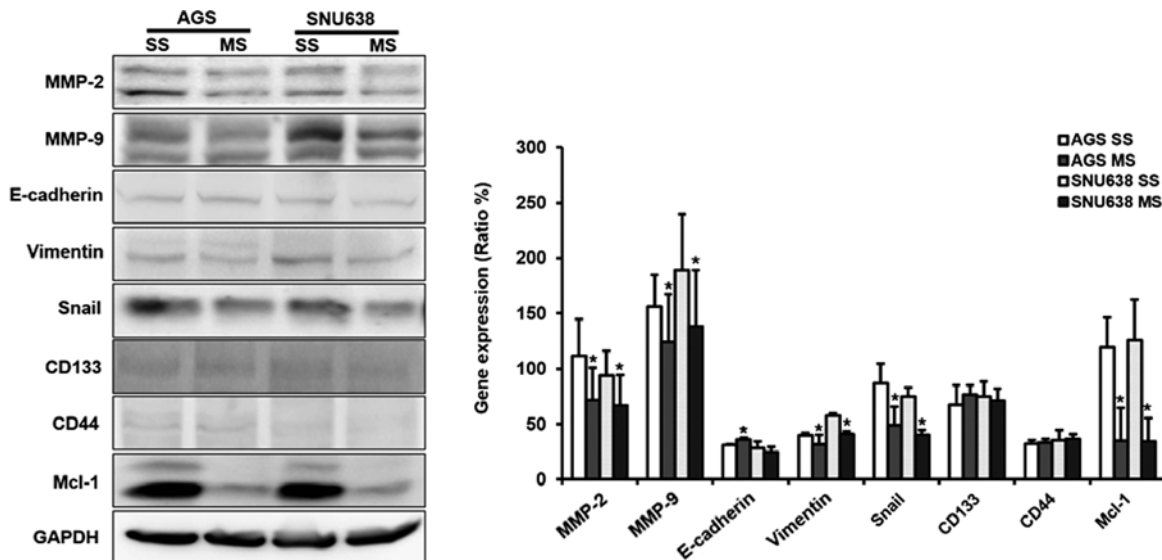


Figure 3. Mcl-1 knockdown affects the expression of EMT-associated genes in human GC cells. Protein expression levels of vimentin, MMP-2, MMP-9 and Snail were decreased following Mcl-1 knockdown. The protein expression level of E-cadherin was increased when Mcl-1 expression was knocked down in the AGS cells, but was not significantly different in the SNU638 cells. Expression of the CD44 and CD133 proteins was unaltered by Mcl-1 knockdown of Mcl-1. Data are presented as the mean \pm SE from three experiments. * $P<0.05$ vs. scrambled siRNA-transfected cells. SS, scrambled siRNA; MS, Mcl-1 siRNA; EMT, epithelial-mesenchymal transition; Mcl-1, myeloid cell leukemia-1; GC, gastric cancer.

between Mcl-1 and EMT in the human GC cells, cell adhesion assays were performed. The cell adhesion ability was measured after transfection of the siRNAs using three cell adhesion substrates including fibronectin and collagen I and IV. The cell adhesion to fibronectin and collagen I was significantly

increased in the Mcl-1 siRNA-transfected AGS ($P=0.023$ and 0.034 , respectively) and SNU638 cells ($P=0.045$ and 0.025 , respectively) compared to that of the scrambled siRNA-transfected cells (Fig. 2). To investigate phenotypic changes induced by EMT, the expression levels of EMT-associated

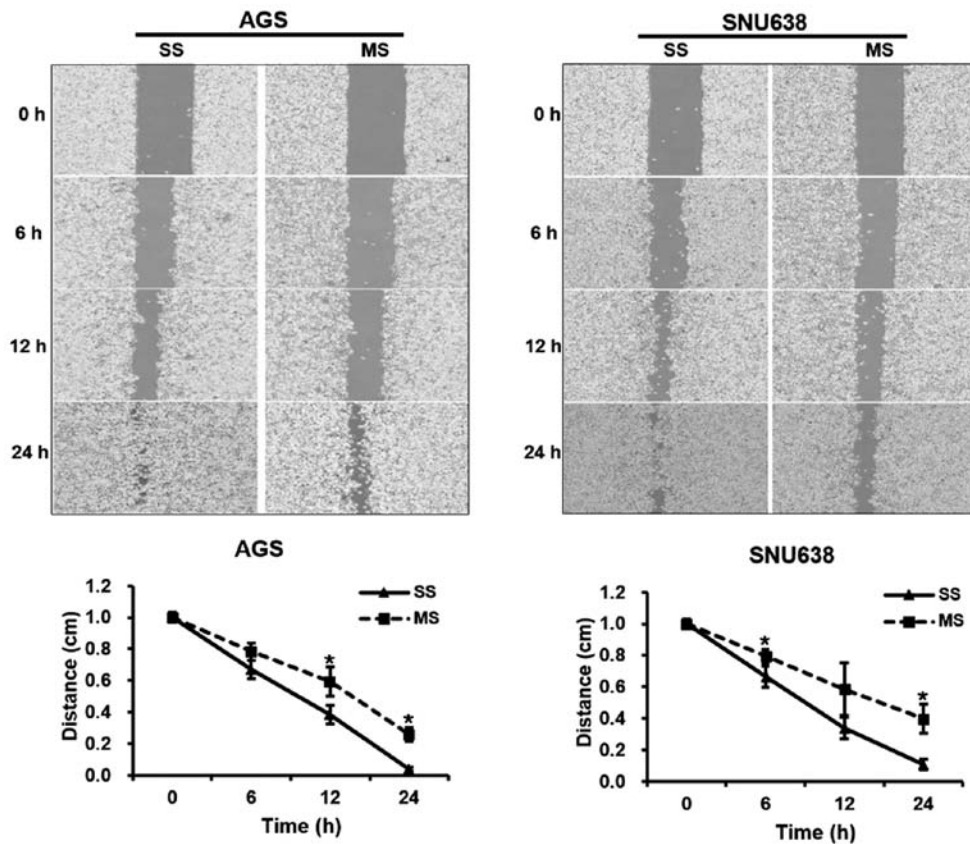


Figure 4. Mcl-1 knockdown affects the migration capacity of human GC cells. Wound-healing assays using siRNA-transfected cells were performed. Cell migration is expressed as the relative healing distance (mean \pm SE, $n=3$, * $P<0.05$). Cell migration was significantly affected in the cells transfected with the Mcl-1 siRNAs. SS, scrambled siRNA; MS, Mcl-1 siRNA; Mcl-1, myeloid cell leukemia-1; GC, gastric cancer.

genes (MMP-2, MMP-9, Snail, E-cadherin, and vimentin) were also assessed. We observed lower expression levels of vimentin, MMP-2, MMP-9 and Snail in the Mcl-1 siRNA-transfected AGS and SNU638 cells, compared to these levels in the scrambled siRNA-transfected cells. The E-cadherin expression level was increased in the Mcl-1 siRNA-transfected AGS cells, but this level was not significantly different in the SNU638 cells (Fig. 3). We investigated the possible effect of Mcl-1 on the expression of cancer stemness markers such as CD44 and CD133. CD44 and CD133 expression levels were unaltered by knockdown of Mcl-1 (Fig. 3). Our results indicate that Mcl-1 expression is associated with the induction of molecular and cellular alterations consistent with EMT.

Mcl-1 knockdown affects migration and invasion of human GC cells. For the cell migration assays, the artificial wound gap became significantly narrower for cells transfected with the control-scrambled siRNAs in comparison with that for the Mcl-1 siRNA-transfected cells at 12 and 24 h in the AGS cell line ($P=0.001$ and 0.019 , respectively). Similar results were noted at 6 and 24 h for the SNU638 cell cultures ($P=0.033$ and 0.023 , respectively, Fig. 4). For the cell invasion assays, 160.3 ± 93.8 and 117.7 ± 70.7 invading Mcl-1 siRNA-transfected AGS and SNU638 cells, respectively, were observed. In contrast, for cultures transfected with the scrambled siRNAs, 424.0 ± 146.2 and 382.7 ± 109.4 invading AGS and SNU638 cells, respectively were observed. These differences in invading cell numbers were significantly different ($P=0.018$ for AGS cells

and $P=0.009$ for SNU638 cells, Fig. 5). Our findings indicate that Mcl-1 expression is required for GC cell migration and invasion, subsequently leading to tumor metastasis.

Mcl-1 knockdown affects β -catenin, MEK1/2 and MAPK signaling pathways in human GC cells. We assessed phosphorylation levels of proteins in the β -catenin, MEK1/2 and MAPK signaling cascades using western blotting to determine their involvement in EMT regulation. The phosphorylation level of β -catenin was increased in the AGS and SNU638 cells when Mcl-1 was knocked down. Phosphorylation levels of MEK1/2 were decreased in the AGS and SNU638 cells when Mcl-1 expression was knocked down. Phosphorylation of ERK1/2 and p38 was decreased in the Mcl-1 siRNA-transfected SNU638 cells (Fig. 6).

Discussion

Metastatic gastric cancer (GC) is incurable and ultimately claims the life of the majority of these patients (1-3). Tumor metastasis is a complex process involving tumor cells migrating from the primary tumor mass to distant organs or tissues. The tumor microenvironment is thought to drive tumor initiation and progression, with anti-apoptotic effects stimulated, cell proliferation, angiogenesis, invasion, metastasis and EMT of tumor cells observed (23,24).

EMT is a physiological process that is activated during wound healing, inflammation or embryogenesis. Recently,

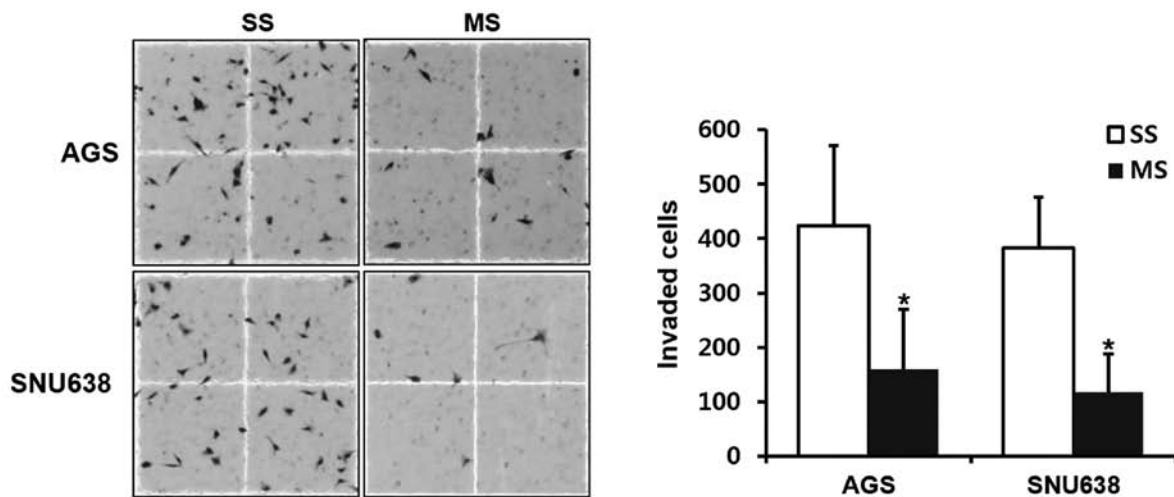


Figure 5. Mcl-1 knockdown affects the invasive abilities of human GC cells. Less invading cells were noted in the group transfected with the Mcl-1 siRNAs compared with this number in the control group (mean \pm SE, $n=3$, $P<0.05$). SS, scrambled siRNA; MS, Mcl-1 siRNA; Mcl-1, myeloid cell leukemia-1; GC, gastric cancer.

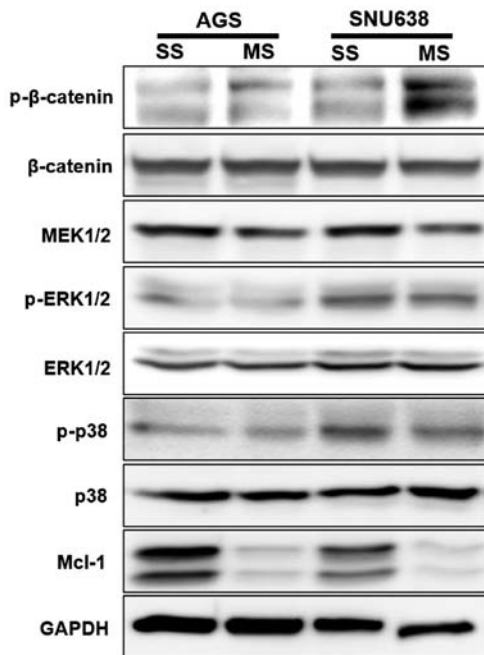


Figure 6. Mcl-1 knockdown affects the β -catenin, MEK1/2 and MAPK signaling pathways in human GC cells. Phosphorylation levels of β -catenin were increased in the AGS and SNU638 cells when Mcl-1 expression was knocked down. Phosphorylation levels of MEK1/2 were decreased in the AGS and SNU638 cells when Mcl-1 expression was knocked down. Phosphorylation levels of ERK1/2 and p38 were decreased in the SNU638 cells when Mcl-1 expression was knocked down. SS, scrambled siRNA; MS, Mcl-1 siRNA; Mcl-1, myeloid cell leukemia-1; GC, gastric cancer.

EMT has also been described for cancer cells, allowing them to acquire motility and invasiveness. EMT is considered an essential step in driving the early phases of tumor metastasis (18-22). EMT induces phenotypic changes with respect to the shape and polarity of epithelial cells. These phenotypic changes in epithelial cells include a remodeled cytoskeleton, loss of cell-cell adhesion, the ability to overcome *anoikis* and apoptosis, and the acquisition of mobile and invasive characteristics, which are all typical of mesenchymal cells (18-22).

Therefore, markers involved in EMT activation may be associated with the modulation of pro- and anti-apoptotic genes.

Mcl-1 is an anti-apoptotic Bcl-2 protein that is highly expressed in a variety of human cancers. Expression of Mcl-1 has been shown to contribute to tumorigenesis, and is associated with the acquisition of invasive and metastatic capabilities by tumor cells through the inhibition of apoptosis, cell cycle progression, promotion of cancer cell replication, invasion and metastasis (8-11). Furthermore, expression of Mcl-1 is associated with advanced stages and poor clinical outcome of many human cancers including GC (12-17).

During EMT, expression of epithelial markers such as E-cadherin, γ -catenin, cytokeratin and occludin are down-regulated in cancer cells. Simultaneously, expression levels of mesenchymal markers such as vimentin, fibronectin, N-cadherin, Twist and Snail are increased. In addition, proteolytic enzymes such as MMPs, which are required for the degradation of the extracellular matrix (ECM) in normal tissue surrounding tumors, are activated (18-22). These morphological and cellular alterations are critical steps in EMT, and common steps in tumor metastasis.

First, to further explore the role of Mcl-1 in cell-cell adhesion of human GC cells, we used three common ECM proteins, including fibronectin and collagen I and IV, to examine whether knockdown of Mcl-1 could affect the adhesive capacity of human GC cells. Our study showed that knockdown of Mcl-1 led to increased adhesion of human GC cells to fibronectin and collagen I, but not collagen IV. This result indicates that altered expression of Mcl-1 may be associated with altered adhesion to specific components of the ECM such as fibronectin and collagen I in human GC cells.

Next, we evaluated the expression of EMT-associated genes and their corresponding proteins in human GC cells. Expression levels of vimentin, MMP-2, MMP-9 and Snail were decreased in cells where Mcl-1 expression was knocked down. E-cadherin expression was increased in AGS cells following knockdown of Mcl-1. Our results indicate a positive relationship between Mcl-1 expression and induction of EMT in human GC cells.

Cancer stem cells are a small subset of tumor cells that possess extensive proliferative potential; therefore they can initiate and propagate tumors. During EMT, epithelial cells acquire stem cell phenotypes. There is a link between EMT and cancer stem cells, with a correlation observed for EMT occurrence, GC progression and resistance to treatment (25-27). However, the expression of CD44 and CD133 was not altered by Mcl-1 knockdown in our study.

Molecular signaling pathways involved in the induction of EMT have been identified during development, differentiation, and carcinogenesis. Signaling pathways, including β -catenin and MAPK, phosphatidylinositol-3 kinase/Akt and NF- κ B have been implicated in the induction of EMT in cancer cells. These pathways are responsible for increased cell proliferation, apoptosis, EMT, invasion, metastasis and chemoresistance in a number of human cancers (28,29). We evaluated the impact of Mcl-1 expression on oncogenic signaling pathways. Our study showed that the β -catenin, MEK1/2, ERK1/2 and p38 pathways were significantly blocked when Mcl-1 expression was knocked down.

In summary, knockdown of Mcl-1 led to increased adhesion of human GC cells to fibronectin and collagen I. Knockdown of Mcl-1 inhibited EMT induction, as the expression levels of vimentin, MMP-2, MMP-9 and Snail in human GC cells were decreased. Additionally, knockdown of Mcl-1 suppressed tumor cell migration and invasion. The β -catenin, MEK1/2, ERK1/2 and p38 pathways were significantly blocked by knockdown of Mcl-1. These results revealed that Mcl-1 expression induces EMT via the β -catenin, MEK1/2 and MAPK signaling pathways, thereby stimulating the invasive and migratory capacities of human GC cells.

Acknowledgements

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