



MD2 blockage prevents the migration and invasion of hepatocellular carcinoma cells via inhibition of the EGFR signaling pathway

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Background: The toll-like receptor (TLR) is an emerging signaling pathway in tumor invasion and metastasis. The activation of TLRs requires specific accessory proteins, such as the small secreted glycoprotein myeloid differentiation protein 2 (MD2), which contributes to ligand responsiveness. However, the role of MD2 in tumorigenesis and metastasis has rarely been reported. This study aimed to investigate the effects and underlying mechanisms of MD2 on the proliferation, migration, and invasion of hepatocellular carcinoma (HCC).

Methods: Cell counting kit 8 (CCK8), cell colony formation, wound healing, and transwell assays were conducted to determine cell viability, proliferation, migration, and invasion, respectively. Quantitative real-time PCR (qRT-PCR) was performed to assess the expression of MD2 in HCC cell lines and human normal liver cell lines as well as the silencing efficiency of MD2 blockage. Western blot and qRT-PCR assays were performed to detect the protein and mRNA expression levels of epithelial mesenchymal transformation (EMT) markers and epidermal growth factor receptor (EGFR) signaling molecules.

Results: MD2 was highly expressed in HCC tissues and cell lines. High expression of MD2 was associated with poor prognosis of HCC patients. In addition, MD2 silencing slightly inhibited the proliferation of HepG2 and HCCLM3, and significantly suppressed cell migration and invasion. Furthermore, MD2 blockage could distinctly prevent the EMT process by increasing the protein and mRNA levels of E-cadherin and Occludin, and decreasing the levels of Vimentin, N-cadherin, and Snail. Finally, the phosphorylation level of EGFR as well as its downstream molecular Src, Akt, I- κ B α , and p65 were downregulated in HCC cells with MD2 silencing.

Conclusions: Our findings suggest that high expression of MD2 may affect the EMT, migration, and invasion via modulation of the EGFR pathway in HCC cells.

Keywords: Hepatocellular carcinoma (HCC); myeloid differentiation protein 2 (MD2); epidermal growth factor receptor (EGFR); migration; invasion

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Introduction

Hepatocellular carcinoma (HCC) is the fourth most common cause of cancer-related mortality worldwide, and accounts for more than 80% of all liver cancer cases (1). More than 70% of HCC patients experienced tumor progression 1 year after diagnosis due to the extensive invasiveness of HCC (2,3), and the 5-year survival rate of advanced HCC is very poor (below 15%) (4,5). Thus, exploring the potential therapeutic targets for HCC is crucial.

Myeloid differentiation protein 2 (MD2), also known as lymphocyte antigen 96 (LY96), is a kind of small secreted protein (6). As the co-receptor for toll-like receptor (TLR) 4 and the main lipopolysaccharide (LPS)-binding module, MD2 is requisite for the localization of TLR4 on the cell surface and subsequently confers responsiveness to LPS (7,8). MD2 mediates the LPS-induced inflammatory and immune responses by forming a heterotetrameric complex with TLR4 (9,10). The TLR4 signaling pathway is an emerging pathway related to tumor cell invasion and migration (11-13). TLR4 signaling has been shown to have oncogenic effects both *in vitro* and *in vivo* in various cancer types, including colon (14), breast (15), prostate (16), skin (17), ovarian (18), cervical (19), and esophageal (20) cancers, as well as HCC (21).

Considering the essential role of MD2 in TLR4 signaling activation, targeting MD2 may be a potential pharmacological strategy for cancer treatment. However, the effect of MD2 on tumor cell invasion and migration has been rarely studied, and no studies addressing drugs targeting MD2 for cancer treatment specifically are currently available. Data from the TNMplot database (<https://tnmplot.com>) indicates that MD2 is overexpressed in human HCC tissues compared with normal liver tissues. Furthermore, the data of a 5-year analysis from the Kaplan-Meier plotter database (<https://kmplot.com/analysis/index.php>) shows that high expression of MD2 in tumor tissues is associated with poor prognosis of HCC. Although the role of MD2 in HCC is still unclear, these data point to the intriguing possibility that MD2 might play a role in HCC.

This study aims to evaluate the precise mechanisms of MD2 on the invasion and migration of HCC. Our findings indicate that MD2 is involved in the migration and invasion of HCC cells, and its mechanism may be related to the regulation of the epidermal growth factor receptor (EGFR) signaling pathway. We present the following article in accordance with the MDAR reporting checklist (available at <https://dx.doi.org/10.21037/jgo-21-362>).

Methods

Cell culture

Human hepatoma carcinoma cell lines (Hep3B, HepG2, HCCLM3, and Huh7) and normal hepatocyte (HL7702) were obtained from the National Collection of Authenticated Cell Cultures (NSTI, Shanghai, China), and cultured in RPMI-1640 (Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Invitrogen, Beijing, China) at 37 °C in a 5% CO₂ cell incubator.

siRNA transfections

Cells were transfected with 4–5 ng/mL siRNA against MD2 (GenePharma, Shanghai, China) and a negative control (GenePharma, Shanghai, China) using Lipofectamine 3000 (Thermo Fisher Scientific, USA) for 24 h. Cells were then washed and cultured in fresh complete medium and the silencing efficiencies were detected by western blot and quantitative real-time PCR (qRT-PCR).

qRT-PCR analysis

RNA was isolated from each group as described in individual experiments using TRIzol (Invitrogen, cat. no. 15596026). cDNA was synthesized with 2–5 µg of RNA using a PrimeScript RT reagent kit (TaKaRa, Tokyo, Japan). Quantitative PCR was carried out using a SYBR[®] Premix Ex Taq[™] II kit (TaKaRa, cat. no. RR820A). The mRNA levels of *MD2* (forward primer: 5'-TGCCAGAAGACCGTGACC-3', reverse primer: 5'-TTTGCAGTAGAACTCCCCGTG-3'), *Vimentin* (forward primer: 5'-GACGCCATCAACACCGAGTT-3', reverse primer: 5'-CTTTGTCGTTGGTTAGCTGGT-3'), *N-cadherin* (forward primer: 5'-AGCTCCATTCCGACTTAGACA-3'; reverse primer: 5'-CAGCCTGAGCACGAAGAGTG-3'), *Snail* (forward primer: 5'-TGTGACAAGGAATATGTGAGCC-3', reverse primer: 5'-TGAGCCCTCAGATTTGACCTG-3'), *E-cadherin* (forward primer: 5'-ATTTTTCCCTCGACACCCGAT-3', reverse primer: 5'-TCCCAGGCGTAGACCAAGA-3'), *Ocludin* (forward primer: 5'-ACAAGCGGTTTTATCCAGAGTC-3', reverse primer: 5'-GTCATCCACAGGCGAAGTTAAT-3'), and *GAPDH* (forward primer: 5'-TGIGGGCATCAATGGATTTGG-3'; reverse primer: 5'-ACACCATGTATTCGGGTCAAT-3') were quantified using the LightCycle[®] 480 II (Roche Diagnostic, QC, Canada).

Western blot analysis

Lysates of total cells were extracted as described in individual experiments using lysis buffer with protease inhibitor, and the total protein concentration was analyzed using a BCA Protein Assay Kit (Beyotime, #P0012, Shanghai, China) according to the manufacturer's protocol. Samples were separated on 8–12% SDS-PAGE gels and transferred to PVDF membranes (Millipore, IPVH00010, USA), and subsequently blocked with 5% non-fat dry milk for 1.5 h at room temperature and incubated overnight at 4 °C with appropriate primary antibodies: anti-MD2 (1:3,000, #ab24182, Abcam), E-cadherin (1:3,000, #20874-1-AP, Proteintech), Occludin (1:3,000, #27260-1-AP, Proteintech), Vimentin (1:3,000, #10366-1-AP, Proteintech), Snail (1:3,000, #13099-1-AP, Proteintech), p-EGFR (F-3) (1:3,000, #sc377547, Santa Cruz), EGFR (1:3,000, #sc373746, Santa Cruz), p-Src (Y419) (1:1,000, #6943S, Huabio), Src (1:3,000, #2109, Cell Signaling), p-IκB-α (B-9) (1:1,000, #sc8404, Santa Cruz), p-p65 (1:3,000, #3033, Cell Signaling), p-AKT (Ser473) (1:3,000, #4060, Cell Signaling), AKT (1:3,000, #4685, Cell Signaling), and GAPDH (1:3,000, #6004-1-Ig, Proteintech). Horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibodies were used as secondary antibodies. Protein signals were visualized using a BeyoECL Plus kit (Beyotime, #P0018, Shanghai, China) and quantified by ChemiDoc XRS+ software (Bio-Rad, USA).

Wound healing assay

Cells were seeded and transfected with siRNA against MD2 and a negative control. Cells were allowed to reach 70–80% confluence, and were then starved for 24 h. The cells were subsequently wounded with a sterile plastic tip and cultured in serum-free medium. Cell migration was monitored for 24 h using microscopy (Nikon, Tokyo, Japan).

Transwell assay

Cells were transfected with siRNA against MD2 and a negative control and plated on top of a thick layer of Matrigel in transwell chambers (BD Biosciences, USA). After culturing for 18 hours, non-invasive cells on the upper surface of filters were removed completely. Invasive cells that adhered to the lower surface of the filter were rinsed with phosphate buffered saline (PBS), fixed with methanol, stained with 0.05% crystal violet, and counted.

Cell viability assay

Cell counting kit 8 (CCK8) assay was used to determine cell viability. Cells (5×10^3 cells/well) were transfected with siRNA against MD2 and a negative control, laid out in 96-well plates, and incubated for 24, 48, and 72 h. Next, the cell medium was removed and CCK8 reagent was added (diluted with the medium at 1:10), and incubated at 37 °C for 30 min. The absorbance was measured at 595 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader.

Colony formation assay

Cells (5×10^4 cells/well) were transfected with siRNA against MD2 or a negative control, and seeded in six-well plates for 5 days. During this period, the medium was renewed every 3 days. Colonies were fixed with 4% paraformaldehyde for 15 min and stained with 0.05% crystal violet at room temperature for 15 min. Images were obtained using a Nikon camera (Nikon, Tokyo, Japan).

Statistical analysis

All experiments were performed at least in triplicate. Data were analysed using GraphPad Prism Version 9.0.0 (GraphPad Software Inc., La Jolla, CA, USA). Values were presented as means ± standard deviation (SD). Statistical significance was evaluated using one-way analysis of variance (ANOVA) followed by Tukey's test. A P value of <0.05 was considered to be statistically significant.

Results

MD2 was highly expressed in HCC and was associated with poor prognosis

To investigate the relationship between MD2 and HCC, we first analyzed the expression of MD2 in hepatoma carcinoma using the TNMplot database, and found that the MD2 gene (*LY96*) was significantly higher in HCC tumor tissues than in normal liver tissues (*Figure 1A*). Next, we detected the mRNA level of MD2 in several HCC cell lines, and obtained the same results as above; that is, that MD2 was higher in HCC cell lines (Hep3B, HepG2, HCCLM3, and Huh7) than in normal liver cell line (HL7702) (*Figure 1B*). Furthermore, we analyzed the prognosis via the Kaplan-Meier plotter database, and found that the 5-year survival rate of patients with lower MD2 expression was higher than those with

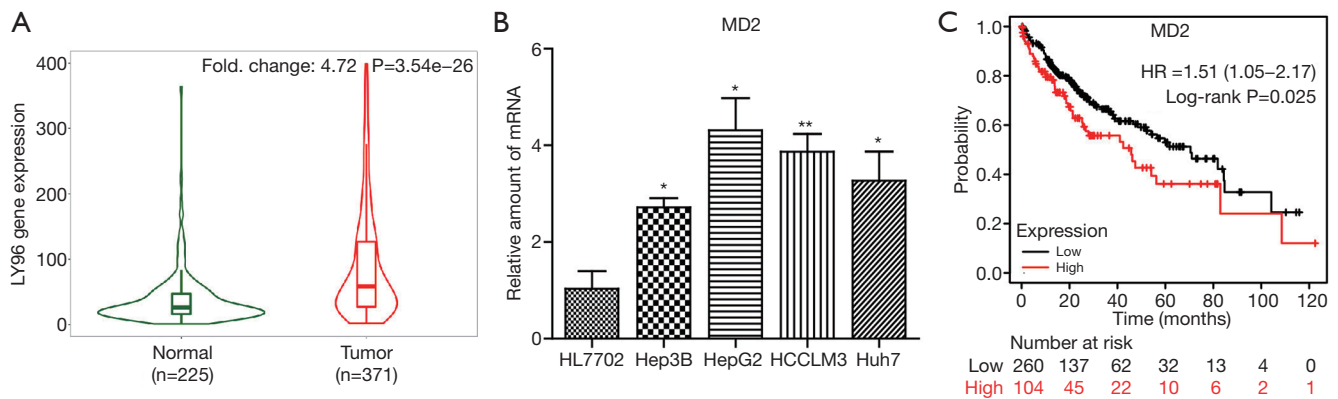


Figure 1 MD2 was highly expressed in hepatoma carcinoma and was associated with prognosis. (A) The TNMplot database was used to analyze the MD2 levels in HCC and normal liver. (B) qRT-PCR analysis of MD2 mRNA levels in HL7702, Hep3B, HepG2, HCCLM3, and Huh7. (C) The Human Protein Atlas database was used to analyze the relationship between MD2 expression and prognosis. *, $P < 0.05$ compared with HL7702 group; **, $P < 0.01$ compared with HL7702 group. MD2, myeloid differentiation protein 2; HCC, hepatocellular carcinoma; qRT-PCR, quantitative real-time PCR.

higher MD2 expression (Figure 1C). These results indicated that MD2 was highly expressed in hepatoma carcinoma and was associated with poor prognosis.

Silencing of MD2 inhibited migration and invasion of HCC cells

To explore the role and function of MD2 in HCC, we examined migration and invasion, which are important causes of tumor recurrence and metastasis, by silencing MD2 (Figure 2). First, we inhibited the expression of MD2 in HepG2 and HCCLM3 using three siRNAs, and investigated its efficiency to select the best one (Figure 2A, 2B, 2E, 2F). Wound healing and transwell assays showed that MD2 silencing significantly inhibited cell migration and invasion of HepG2 cells (Figure 2C, 2D) and HCCLM3 cells (Figure 2G, 2H). These results indicated that silencing of MD2 prevented the progress of HCC by decreasing the cell migration and invasion ability.

Silencing of MD2 slightly inhibited the proliferation of HCC cells

We investigated the effect of MD2 on cell proliferation of HCC cells (Figure 3). CCK8 assay of HepG2 and HCCLM3 cells indicated that silencing of MD2 had no significant inhibitory effect on cell viability at 24 h and a slight inhibitory effect at 48 or 72 h (Figure 3A, 3C). Colony formation assay showed that silencing of MD2 had little

inhibitory effect on the number and size of cell colonies (Figure 3B, 3D). These results indicated that MD2 silencing had little inhibitory effect on HCC cell proliferation.

Silencing of MD2 inhibited epithelial mesenchymal transformation (EMT) of HCC cells

EMT is an important process in tumor cell metastasis and invasion. To address the connection between MD2 and EMT, we detected the expression levels of EMT markers. As expected, silencing of MD2 prevented the procession of EMT by increasing the protein and mRNA levels of *E-cadherin* and *Occludin*, but decreasing the levels of *Vimentin*, *N-cadherin*, and *Snail* in HepG2 cells (Figure 4A, 4B). Similarly, MD2 silencing in HCCLM3 cells also increased the protein and mRNA levels expression of *E-cadherin* and *Occludin*, and decreased the levels of *Vimentin*, *N-cadherin*, and *Snail* (Figure 4C, 4D). These results showed that MD2 silencing had a significant inhibitory effect on EMT in HCC cells.

Silencing of MD2 inhibited the activation of EGFR signaling

EGFR signaling plays a key role in regulating EMT transformation of tumor cells and promoting tumor metastasis. To investigate the influence of MD2 on the EGFR signaling pathway, we detected the phosphorylation changes of EGFR and its downstream molecules, Src and

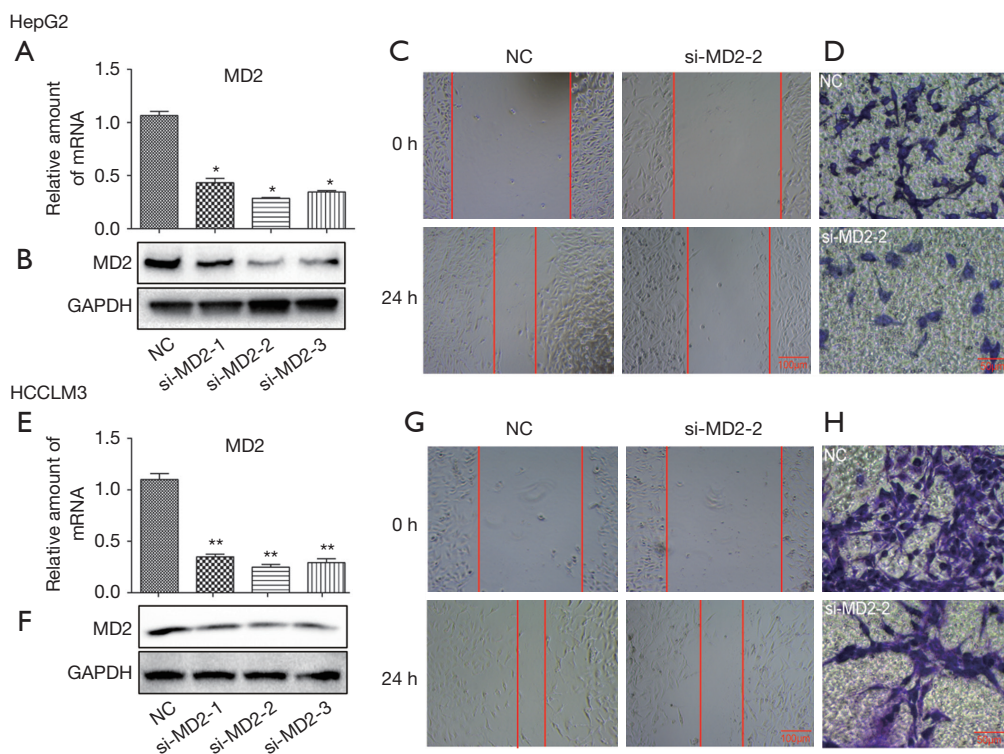


Figure 2 Silencing of MD2 inhibited cell migration and invasion. (A) qRT-PCR was used to detect the silencing efficiency of siRNA in HepG2 cells. (B) Western blot was used to detect the silencing efficiency of siRNA in HepG2 cells. (C) Wound healing assay was used for migration ability detection in HepG2 cells (100 \times). (D) Transwell assay and crystal violet staining were used for invasion abilities detection in HepG2 cells (200 \times). (E) qRT-PCR was used to detect the silencing efficiency of siRNA in HCCLM3 cells. (F) Western blot was used to detect the silencing efficiency of siRNA in HCCLM3 cells. (G) Wound healing assay was used for migration ability detection in HCCLM3 cells (100 \times). (H) Transwell assay and crystal violet staining were used for invasion abilities detection in HCCLM3 cells (200 \times). *, $P < 0.05$ compared with NC group; **, $P < 0.05$ compared with NC group. MD2, myeloid differentiation protein 2; qRT-PCR, quantitative real-time PCR; NC, normal control.

AKT. The results demonstrated that the phosphorylation levels of EGFR, Src, and AKT were decreased in MD2-silenced HepG2 and HCCLM3 cells (Figure 5A,5B). We then also inspected the changes of NF- κ B that were reported as downstream of both EGFR and MD2 signals. In the same way, MD2 silencing inhibited the phosphorylation of I κ B- α and p65 in HepG2 and HCCLM3 cells (Figure 5A,5B). These results showed that silencing of MD2 significantly inhibited the activation of EGFR signaling in HCC cells.

Discussion

HCC is one of the main causes of cancer-related mortality worldwide, and exhibits characteristics of rapid growth and high invasiveness (1,22). Therefore, it is important

to investigate the mechanisms involved in HCC invasion and migration as well as explore novel therapeutic targets. Our study documented, for the first time, the effects and mechanisms of MD2 on the invasion and migration of HCC via regulation of the EGFR pathway.

Previous studies have found that the MD2 and TLR4 signaling pathways play an important role in acute inflammation (23,24). Our previous research also identified a crucial role of these pathways in some chronic diseases, such as diabetes (25,26), obesity (27,28), etc. Studies in recent years have highlighted the critical function of TLR4 signaling in cancer progression, invasion, migration, angiogenesis, and immune escape (12,29,30). The MD2/TLR4 complex plays a carcinogenic effect by activating the TIRAP, PI3K/AKT, IKK- α , and NF- κ B signals (31,32).

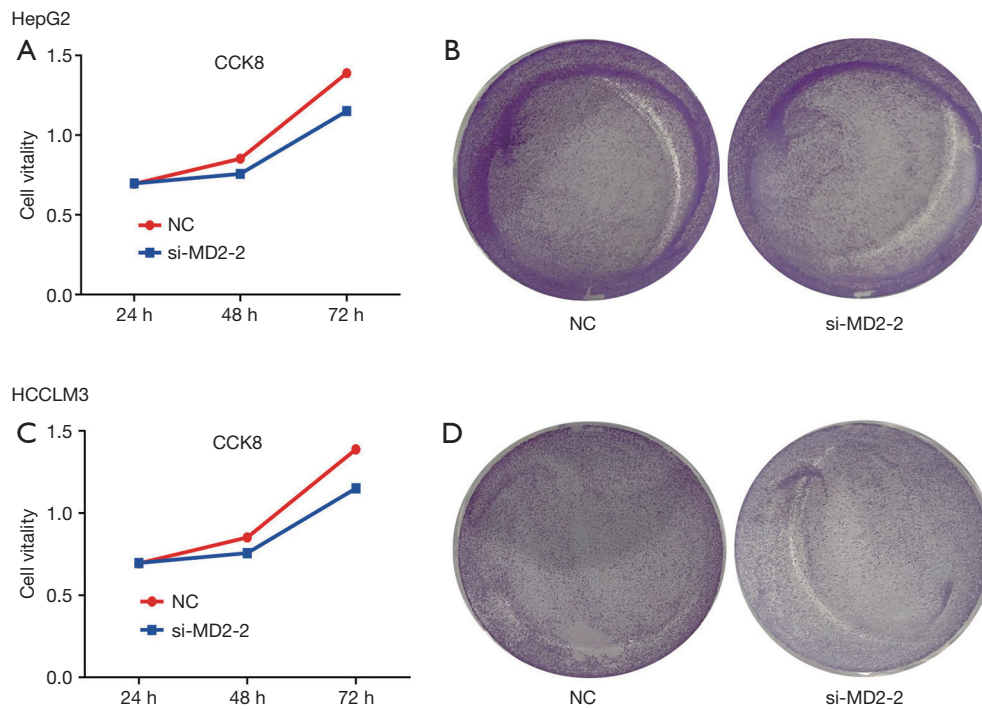


Figure 3 Silencing of MD2 slightly inhibited the proliferation of hepatoma carcinoma cells. (A) CCK8 was used to detect the cell vitality of HepG2 cells. (B) Colony formation assay and crystal violet staining were used to detect the cell proliferation of HepG2 cells. (C) CCK8 was used to detect the cell vitality of HCCLM3 cells. (D) Colony formation assay and crystal violet staining were used to detect the cell proliferation of HCCLM3 cells. MD2, myeloid differentiation protein 2; CCK8, cell counting kit 8.

The expression of TLR4 is significantly increased in a variety of cancers (33). High expression of TLR4 in tumor tissues is associated with poor survival in patients with various cancers (34,35). Although previous studies have demonstrated the potential therapeutic implications of inhibiting TLR4 pathway in cancer treatment, few studies have focused on the specific contribution of MD2, which has been known as an essential cofactor in TLR4 signaling for many years. As far as we know, the function of MD2 on liver cancer has never been studied.

In this study, we explored the expression levels of MD2 using the TNMplot database, and found that the expression level of MD2 was higher in various tumor tissues as compared with paired normal tissues, especially in HCC. In addition, MD2 expression was upregulated in HCC cell lines, such as Hep3B, HepG2, HCCLM3, and Huh7, as compared with the human normal hepatocyte, HL7702. Survival analysis using The Human Protein Atlas database showed that higher expression of MD2 predicted poorer survival compared to lower expression. Furthermore, MD2

silencing had a weak inhibitory effect on the proliferation of HCC cells, however exhibited an obvious inhibitory effect on cell migration and invasion. Therefore, the first finding of this study was the validation of the effect of MD2 on suppressing the invasion and migration of HCC cells.

We also demonstrated that MD2 inhibited the invasion and migration ability of HCC cells via regulation of the EMT process. EMT, which is characterized by the loss of apical basal cell polarity of epithelial and the mesenchymal cells, is the key link of tumor invasion, migration, and metastasis (36-38). Inhibition of EMT regulators is an important approach in anticancer research. The multi-targeted tyrosine kinase inhibitors like sorafenib and regorafenib have an established place in the systemic treatment for advanced HCC. It was reported that sorafenib (39) and regorafenib (40) could suppress the EMT of HCC cells. The expression levels of various molecules also change during EMT. The expression of epithelial molecular markers E-cadherin and the occlusal protein (Occludin) were downregulated, while the expression of the E-cadherin transcription factor (Snail)

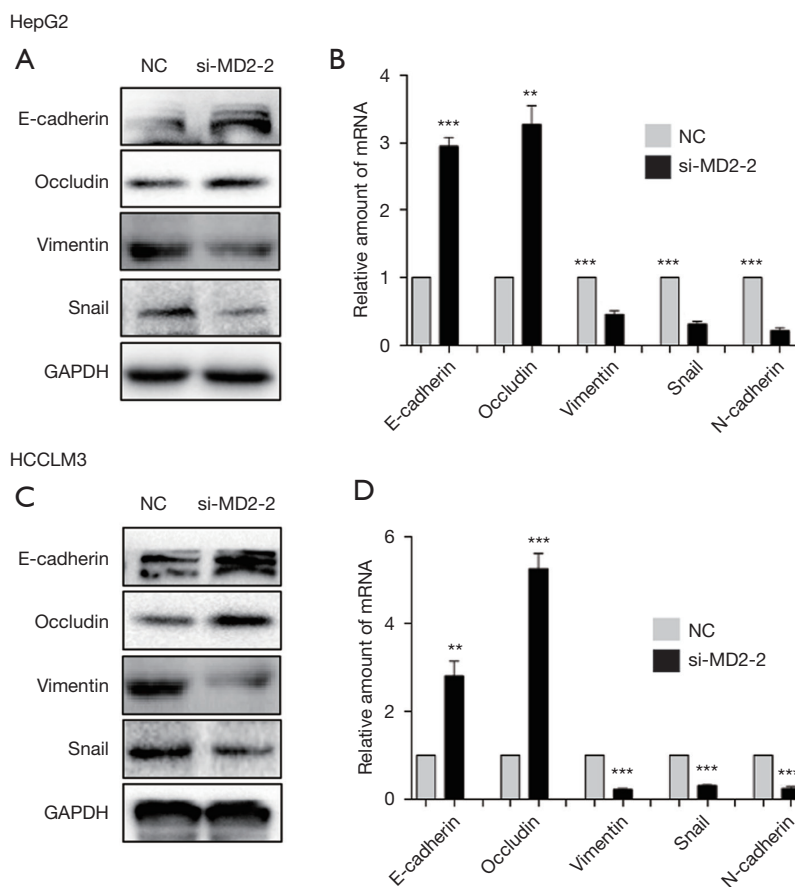


Figure 4 Silencing of MD2 inhibited cell EMT. (A) Western blot detected the expression of EMT markers in HepG2 cells. (B) qRT-PCR detected the expression of EMT markers in HepG2 cells. (C) Western blot detected the expression of EMT markers in HCCLM3 cells. (D) qRT-PCR detected the expression of EMT markers in HCCLM3 cells. **, P<0.01 compared with NC group; ***, P<0.001 compared with NC group. MD2, myeloid differentiation protein 2; EMT, epithelial mesenchymal transformation; qRT-PCR, quantitative real-time PCR; NC, normal control.

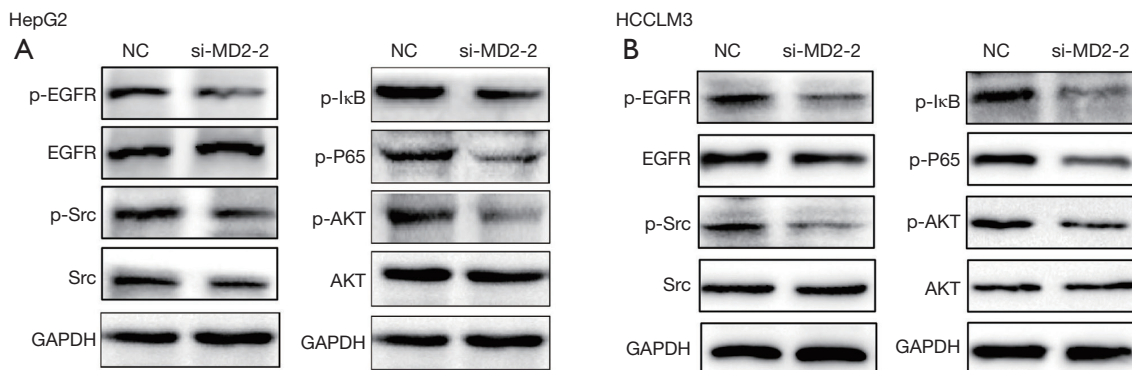


Figure 5 Silencing MD2 inhibited the activation of EGFR signaling. (A) Western blot detected the phosphorylation changes of EGFR, Src, AKT, I-κBα, and p65 in HepG2 cells. (B) Western blot detected the phosphorylation changes of EGFR, Src, AKT, I-κBα, and p65 in HCCLM3 cells. MD2, myeloid differentiation protein 2; EGFR, epidermal growth factor receptor; NC, normal control.

was upregulated in the EMT process, which simultaneously acquired mesenchymal markers such as Vimentin and N-cadherin (41,42). E-cadherin mainly mediates cell-to-cell adhesion and participates widely in cell-to-cell connection, and is crucial for maintaining the integrity of normal epithelial cells. Snail can recognize and bind the promoter sequence of the E-cadherin gene, inhibit the transcription of E-cadherin, affect the adhesion between epithelial cells, and promote the occurrence of EMT (43). Occludin, one of the tight junction proteins between cells, can increase the transmembrane resistance of epithelial cells and prevent cell diffusion (44). Vimentin is abundantly expressed in numerous tumor types and is known as a canonical marker of EMT reprogramming, which is related to the acquisition of invasive and migratory tumor cell phenotypes (45). Serving as an indicator of ongoing EMT, N-cadherin is expressed in almost all interstitial cells and is associated with the development of various cancer types (46). As expected, the silencing of MD2 could block the EMT process of hepatoma cells, accompanied by the upregulation of E-cadherin and Occludin expression, and the downregulation of Snail, Vimentin, and N-cadherin expression.

As the expression product of the proto-oncogene *CerbB-1*, EGFR plays an important role in a variety of cancer processes, such as regulating the cell cycle, inducing angiogenesis, accelerating tumor invasion and metastasis, promoting tumor drug resistance, and so on (47). Also, studies have indicated that the EGFR signaling pathway is closely related to the occurrence of EMT in lung, ovarian, uterine, liver, and other cancers. EGFR can mediate the occurrence of tumor EMT through Src, AKT, TGF- β , NF- κ B, MAPK, and other signals (48,49). Src, NF- κ B, and AKT are vital downstream molecules in the EGFR signaling pathway. Studies have demonstrated that Src inhibitors can significantly inhibit the EMT process of tumor cells, while Src activation can destroy cadherin-dependent intercellular adhesion and promote the interstitial phenotype of tumor cells (50). Increased expression and activity of NF- κ B leads to the loss of E-cadherin through direct regulation of the target gene of Snail (51). Moreover, the activation of NF- κ B can upregulate the expression of Vimentin, thereby indirectly affecting the level of Occludin (52). AKT signal activation can directly upregulate the expression of EMT-related transcription factors such as Snail, Slug, Twist, and ZEB, as well as inhibit the expression and promote the degradation of intracellular E-cadherin (53,54). In this study, we investigated these EMT-related signaling pathways to clarify the regulatory mechanism of MD2.

We found that MD2 silencing could inhibit the activation of the EGFR signaling pathway via suppression of the phosphorylation of EGFR and its downstream molecules Src, NF- κ B, and AKT.

Conclusions

In summary, our data indicated that silencing of MD2 could block the occurrence of EMT by inhibiting the EGFR signaling pathway, thereby inhibiting the migration and invasion of HCC cells. These findings reveal that MD2 blockage may be a novel method in regulating the invasion and migration of HCC.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are

appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Institutional ethical approval and informed consent were waived.

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