Inhibition of Hes1 enhances lapatinib sensitivity in gastric cancer sphere-forming cells

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Abstract. It has been considered that the neurogenic locus notch homolog protein (Notch) signaling pathway serves an essential role in cellular differentiation, proliferation and apoptosis. However, the function of the Notch signaling pathway in gastric cancer stem cells (GCSCs) and epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) sensitivity remains unclear. The present study aimed to delineate the role of the Notch1 signaling pathway in GCSCs and lapatinib sensitivity. Sphere-forming cells were separated from human gastric cancer MKN45 parental cells. The sphere-forming cells exhibited characteristics of CSCs and higher Notch1 expression compared with that of parental cells. To investigate the role of the Notch1 signaling pathway in GCSCs, the expression of transcription factor Hes1 (Hes1) was knocked down using small interfering RNA against Hes1. It was observed that Hes1 expression was significantly downregulated in knocked down cells. The inhibition of Hes1 suppressed the properties of CSCs, as indicated by significant decreases in the expression of the transcription factor sex determining region Y-box 2, epithelial cell adhesion molecule and the homeobox protein Nanog and reduced spheroid colony formation. In addition, epithelial-mesenchymal transition was significantly impaired in sphere-forming cells following Hes1 knockdown. Furthermore, the inhibition of Hes1 effectively enhanced lapatinib sensitivity in sphere-forming cells. These results suggest that sphere-forming gastric cancer cells possess the characteristics of CSCs, and that the Notch1 signaling pathway serves an essential role in the maintenance of CSCs and lapatinib sensitivity.

Introduction

Epidermal growth factor receptor (EGFR) and human EGFR2 (HER2) are frequently upregulated or mutated in various types of cancer, and serve essential roles in cancer cell proliferation, survival, migration and differentiation (1,2). EGFR is frequently mutated or overexpressed in lung, brain, colon, pancreatic and breast cancer (3-6). Furthermore, HER2 is often overexpressed in breast, gastric, esophageal, pancreatic and ovarian cancer (7,8). EGFR-tyrosine kinase inhibitors (TKIs), including gefitinib and erlotinib, are used either alone or in combination with radiation or chemotherapy in cancer therapy (9). The EGFR-TKI lapatinib, and the monoclonal antibody trastuzumab have been approved for the treatment of HER2-overexpressing breast cancer (10). Lapatinib is a potent adenosine triphosphate (ATP)-competitive dual kinase inhibitor that inhibits EGFR and HER2, and has demonstrated antiproliferative activity against human HER2-amplified breast cancer cell lines (11). Wainberg et al (12) reported that lapatinib selectively inhibits the proliferation of HER2-amplified human gastric cancer cells. However, lapatinib is currently unsatisfactory for the treatment of patients with gastric cancer (13). A previous clinical trial suggested that HER2-targeted therapy is associated with drug resistance in cancer cells (14). However, it is unknown whether such resistance mechanisms are associated with gastric cancer stem cells (GCSCs).

The CSC hypothesis was introduced to explain the pathogenesis of numerous cancer types. CSCs have the ability to self-renew and proliferate indefinitely, which can initiate tumor formation and cause tumor recurrence. CSCs are distinguished from the bulk of the tumor cell population by their ability to successfully seed new tumors when implanted in low numbers into experimental animals (15). Furthermore, CSCs are more resistant to chemotherapy and radiotherapy than their corresponding differentiated cancer cells (16-18). The neurogenic locus notch homolog protein (Notch) signaling pathway serves an important role in the determination of cell fate in various organ systems (19). The Notch pathway encompasses four types of receptors (Notch1, -2, -3 and -4) and five membrane proteins ligands that include Delta-like ligands (DLL1, -2 and -3) and Serrate/Jagged (JAG1 and -2). The Notch1 signaling pathway is essential in maintaining the characteristics of CSCs and is associated...
with the self-renewal of various types of CSCs, including breast and pancreatic CSCs (20). Hes1 is the downstream target gene of Notch1 pathway (21). Hes1 may have an important function in the maintenance of cancer stem cells self-renewal and epithelial-mesenchymal transition (EMT) process (22).

In the present study, the role of the Notch1 signaling pathway in GCSCs and lapatinib sensitivity was examined. Furthermore, the current study aimed to elucidate the molecular mechanism underlying resistance to lapatinib in GCSCs.

Materials and methods

Culture of sphere-forming cells. MKN45 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), which were seeded at a density of 2x10^4 cells/ml in 100 mm ultralow attachment plates (Costar; Corning Incorporated, Corning, NY, USA). Cells were grown in serum-free RPMI-1640 and Nutrient Mixture F-12 medium (both Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with B27 (1:50; Hyclone; GE Healthcare Life Sciences), 20 ng/ml EGF, 20 ng/ml basic fibroblast growth factor (bFGF) (both R&D Systems, Minneapolis, MN, USA), 5 µg/ml bovine insulin (Cell Applications, Inc., San Diego, CA, USA), 0.5 µg/ml hydrocortisone (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and penicillin/streptomycin (Hyclone; GE Healthcare Life Sciences). MKN45 cells were grown for 3 days in the above sphere culture maintained at 37˚C in a 5% CO_2 atmosphere and produced spheres, which were dissociated through incubation with 0.05% trypsin/EDTA (Sigma-Aldrich; Merck KGaA) on day 4. Dissociated MKN45 cells were cultured in the aforementioned sphere conditions maintained at 37˚C in a 5% CO_2 atmosphere for another 3 days and then harvested.

Tumorigenicity assay. A total of 40 4-week-old female nude mice (weight 18-20 g) were obtained from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China). The mice, were given SPF grade feed and purified water and were kept in cages (5 mice/cage) in a room with a constant temperature (22±1˚C) and a 12 h dark/light cycle. For in vivo experiments, sphere-forming and parental cells were suspended in PBS (Hyclone; GE Healthcare Life Sciences), and injected subcutaneously into the limbs of mice. The protocol used in the present study was approved by the Ethics Committee of Chongqing Cancer Institute of Biotechnology (Chongqing, China). Groups of mice were inoculated with sphere-forming or parental cells at 5x10^3, 5x10^4, 5x10^5 or 5x10^6 (five mice/group). Tumor growth was monitored every 2 days following the second week of inoculation.

Lentivirus transfection. Inhibition of transcription factor Hes1 (Hes1) was achieved by infecting cells with the Hes1-small interfering (si)RNA lentivirus (Shanghai GenePharma Co., Ltd., Shanghai, China). Transduction was performed using a GenePharma Lentivirus Transduction kit (Shanghai GenePharma Co., Ltd.). The target sequence for the Hes1 siRNA was 5’-AGATCAATGCCATGAATTCA-3’. Cells were cultured in six-well plates at 20-30% confluence and incubated for 12 h at 37˚C with 5% CO_2. Cells were then infected with Hes1-siRNA- or scrambled control-siRNA-expressing lentivirus (5x10^8 TU/ml, 40 µl, Shanghai GenePharma Co., Ltd.). Following the infections, the culture medium was replaced with supernatant fluid that contained an appropriate viral titer (1 ml/well). After incubating at 37˚C for 12 h, the viral supernatant was replaced with fresh medium. The infected cells were selected using 2 mg/ml puromycin (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) following incubation for 48 h. Successful infection was confirmed by expression of green fluorescent protein using an inverted fluorescence microscope (Leica DMI4000 B; Leica Microsystems GmbH, Wetzlar, Germany). The knockdown efficiency was determined using western blotting as described below.

Spheroid colony formation assay. Cells were inoculated into each well (20 cells/well) of ultralow attachment 48-well plates (Costar; Corning Incorporated) and supplemented with 300 µl of RPMI-1640 containing 40 ng/ml bFGF and 20 ng/ml EGF. After incubation for 4 weeks at 37˚C with 5% CO_2, the total number of spheroid colonies/well was counted.

Cell chemosensitivity examination. Cells cultured in medium were incubated at 37˚C with 5% CO_2 and treated with 6 mM 5-fluorouracil (5-Fu) or 5 µmol/l lapatinib (both Sigma-Aldrich; Merck KGaA). After 48 h of exposure, 20 ml of 0.5 mg/ml MTT solution (Sigma-Aldrich; Merck KGaA) was added for an additional 4 h and then 100 ml dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) was added for 15 min. The plates were agitated at a low speed for 5 min and the absorbance was measured at a wavelength of 570 nm using a spectrophotometer. Five wells were assayed for each condition.

Western blotting. According to the manufacturer’s protocol, total protein samples for immunoblots were extracted from cells using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). Following quantification of the protein extracts using a BCA protein assay, equivalent amounts of protein (40 µg/lane) were resolved using 10% SDS-PAGE and then transferred onto a polyvinylidene fluoride membrane (both Beyotime Institute of Biotechnology) for 1 h at 4˚C. Next, the blots were incubated with the appropriate primary antibody for 12 h at 4˚C and then with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h at 37˚C. Signals were detected using an enhanced chemiluminescence reagent (EMD Millipore, Billerica, MA, USA). The results were analyzed by Quantity One software (version 4.6.2, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Antibodies directed against GAPDH (cat. no. 560005) and zinc finger protein SNAI1 (Snail, cat. no. 550589) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Antibodies directed against epithelial cell adhesion molecule (EpCAM, cat. no. ab71916), the homeobox protein Nanog (Nanog, cat. no. ab102950), the transcription factor sex determining region Y-box 2 (Sox2, cat. no. ab92494), multidrug resistance protein 1 (MDR1, catalog no. ab170904), ATP-binding cassette sub-family G member ABCG1...
Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted from the cells using RNAiso (Takara Bio, Inc., Otsu, Japan) and complementary DNA (cDNA) was then synthesized using the PrimeScript II First Strand cDNA Synthesis kit (Takara Bio, Inc.) according to the manufacturers’ protocols. PCR (100 ng cDNA used per qPCR) was performed using a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) with SYBR® Premix Ex Taq™ II (Takara Bio, Inc.). The PCR conditions were as follows: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. Data were normalized against β-actin messenger RNA (mRNA). The sequences of the PCR primers were as follows: Notch1 forward, 5’-TGC CGA ACCA ATAC AACC CTC-3’ and reverse, 5’-TGTG TTAG CTATC ATCTCGGACA-3’; Hes1 forward, 5’-GTTGCAT GAAGG AGGTGACC-3’ and reverse, 5’-GTATTA ACG CCCTG CGACGT-3’; and β-actin forward, 5’-CCACG AAC TACCTT CAACTCC-3’ and reverse, 5’-GTATG CTCCCTTC TGCA TCGCTG-3’. qPCR was performed according to the 2-ΔΔCq method (23).

Histological examination. Tumor tissues were fixed in 10% neutral-buffered formalin for 24 h at room temperature, embedded in paraffin, and then the sections (5 um) were stained with hematoxylin for 10 min and eosin (both Beyotime Institute of Biotechnology) for 5 sec at room temperature. Histological differences were examined using an inverted microscope.

Statistical analysis. All experiments were repeated three times and the results were analyzed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation. The statistical significance of the differences among the groups was evaluated using the Student’s t-test (two-tailed). P<0.05 was considered to indicate a statistically significant difference.

Results

Sphere-forming cells isolated from gastric cancer MKN45 cells exhibit the characteristics of CSCs. Tumors contain a small number of CSCs that possess self-renewal and tumor-initiating abilities (24). To isolate sphere-forming cells, MKN45 cells were grown in ultralow attachment plates with sphere culture medium for 7 days. Subsequently, the expression levels of CSC markers, including EpCAM, Nanog and Sox2, were determined using western blotting. Concordant with previous studies the protein expression levels of these CSC markers were significantly higher in sphere-forming cells than in parental cells (Fig. 1A). Furthermore, in the spheroid colony formation assay, sphere-forming cells formed significantly more spheroids compared with those in parental cells (Fig. 1B).

In the tumorigenicity assay, nude mice were injected with 5x10^2-5x10^3 sphere-forming or parental cells. Transplantation of 5x10^3, 5x10^4 or 5x10^5 parental cells consistently failed to form tumors in all mice, while 5x10^5 parental cells led to tumor formation in 1/5 mice (Table I). By contrast, transplantation of 5x10^3 sphere-forming cells resulted in tumor formation in 2/5 mice, while transplantation of 5x10^5, 5x10^4 or 5x10^3 sphere-forming cells resulted in tumor formation in all mice (Table I). Subsequently, the chemotherapy susceptibility of sphere-forming and parental cells to 5-Fu, which is generally used for the treatment of gastric cancer, was examined. Sphere-forming cells were significantly more chemoresistant to 5-Fu than parental cells, as determined using an MTT cell viability assay (Fig. 1C; P<0.05). These data suggest that sphere-forming cells are tumorigenic and possess CSC characteristics.

Notch1 signaling pathway is activated in sphere-forming MKN45 cells. To investigate the role of the Notch1 signaling pathway in CSCs, the expression of Notch1 and Hes1 in sphere-forming and parental cells was analyzed. It was revealed that Notch1 and Hes1 mRNA and protein expression levels were significantly higher in sphere-forming cells compared with those in parental cells (Fig. 2). These data suggest that the Notch1 signaling pathway is activated in sphere-forming cells.

Sphere-forming MKN45 cells are resistant to the EGFR-TKI lapatinib. The sensitivities of sphere-forming and parental cells to lapatinib were next assessed. The results of the MTT assays demonstrated that the viability of sphere-forming cells was significantly increased compared with that of parental cells (P<0.05; Fig. 3). Therefore, this suggests that...
sphere-forming cells are more resistant to lapatinib than parental cells.

Inhibition of Hes1 expression attenuates the characteristics of CSCs and resistance to lapatinib in sphere-forming cells.

To examine the effects of the Notch1 signaling pathway in GCSCs, sphere-forming cells were infected with lentivirus containing Hes1-siRNA or scrambled control-siRNA. The protein expression levels of Hes1 were determined using western blot analysis. Infection of sphere-forming cells with
lentivirus containing Hes1-siRNA significantly reduced Hes1 protein expression levels compared with those in control-siRNA-treated and untreated cells, while the control siRNA group exhibited no significant effect compared with the untreated control group (Fig. 4A). Simultaneously, the protein expression levels of the CSC markers EpCAM, Nanog and Sox2 were significantly downregulated in Hes1-siRNA lentivirus-infected sphere-forming cells compared with those in the control-siRNA group (Fig. 4B).

In the spheroid colony formation assay, sphere-forming cells infected with Hes1-siRNA lentivirus formed significantly less spheroids compared with those in the control-siRNA lentivirus-infected cells (Fig. 4C). In addition, the results of the MTT assay revealed that the susceptibility of Hes1-siRNA lentivirus-infected cells to the chemotherapeutic agent 5-Fu was significantly increased compared with that of the control-siRNA group (Fig. 4D).

To investigate the influence of the Notch1 signaling pathway on the susceptibility of sphere-forming cells to EGFR-TKIs, the susceptibility of Hes1-siRNA lentivirus-infected sphere-forming cells to lapatinib was assessed. Data from the MTT assays demonstrated that the survival rate of Hes1-siRNA lentivirus-infected sphere-forming cells was significantly decreased compared with that of control-siRNA lentivirus-infected cells (P<0.05; Fig. 4E). These data indicate that inhibition of Hes1 expression attenuates the characteristics of CSCs and increases susceptibility to lapatinib in sphere-forming cells.

Inhibition of Hes1 prevents EMT and decreases the expression of chemoresistance-associated proteins in sphere-forming MKN45 cells. To further investigate the molecular mechanisms of the Notch1 signaling pathway on the susceptibility of sphere-forming cells, the expression of EMT markers and chemoresistance-associated proteins was examined. Western blot analysis demonstrated that the expression levels of the epithelial markers E-cadherin and ZO1 were significantly upregulated, while the expression levels of the mesenchymal markers N-cadherin, vimentin and Snail were significantly downregulated in Hes1-siRNA lentivirus-infected sphere-forming cells compared with those in the control-siRNA group (Fig. 5A). Furthermore, the expression levels of the chemoresistance-associated proteins MDR1, ABCG1, ABCG2 and RAD51 were significantly decreased in Hes1-siRNA lentivirus-infected sphere-forming cells (Fig. 5B). Together, these results indicate that inhibition of Hes1 can impair EMT and decrease the expression of chemoresistance-associated proteins in sphere-forming MKN45 cells.

Discussion

The CSC hypothesis suggests that cancer is maintained by a subpopulation of stem cells with an indefinite life span, which raises the possibility that targeting CSCs could provide an approach for cancer treatment (25). The sphere-forming assay in which cells are cultured in non-adherent conditions in a serum-free medium supplemented with bFGF and EGF is a practical approach for identifying stem cells in individual solid tumor tissue samples or cancer cell cultures (26,27). In the present study, sphere-forming cells were developed by cultivating the human gastric cancer cell line MKN45 in defined serum-free medium. The sphere-forming cells were able to generate significantly more spheroid bodies than their parental cells. This phenomenon indicates that sphere-forming cells are capable of self-renewal and proliferation, which are important characteristics of CSCs (25). Chemoresistance is another important characteristic of CSCs (18). To assess whether the self-renewing sphere-forming cells possessed a hypothetical CSC chemoresistant property, the sensitivity of sphere-forming cells to chemotherapeutic agents was examined. Sphere-forming cells exhibited significantly greater resistance to 5-Fu compared with that of their parental cells, as determined using cell viability assays. Xenotransplantation is generally regarded as the gold standard for evaluating the tumorigenicity of tumor cells. In the tumorigenicity assay, nude mice were injected with sphere-forming or parental cells. As few as 500 sphere-forming cells were able to generate tumors in mice. Additionally, sphere-forming cells generated subcutaneous tumors with larger volumes in shorter time periods compared with those generated from parental cells. To further investigate the CSC properties of sphere-forming cells, the expression of the CSC markers Sox2, Nanog and EpCAM was investigated. Western blot analysis demonstrated that the expression levels of Sox2, Nanog and EpCAM were significantly higher in sphere-forming cells than in parental cells. In summary, sphere-forming cells from the human gastric cancer cell line MKN45 possess GCSC properties, which is in agreement with previous studies (28).

The Notch signaling pathway serves an important role in cellular processes during embryonic and postnatal development, including stem cell renewal, cell fate determination and apoptosis (15). However, dysregulation of the Notch signaling pathway also contributes to tumorigenesis (29). The interaction of Notch ligands with their receptors promotes γ-secretase-dependent cleavage of the Notch receptor and releases the Notch intracellular domain, which results in the activation of the signaling pathway and induces target genes, including Hes1 (30). The role of the Notch1 signaling pathway in the maintenance of CSCs has been described in preclinical models and in clinical studies (31,32). Using gain-and
loss-of-function approaches, the maintenance of CSCs has been attributed to Notch signaling regulation through Hes1, which dictates cell fate decisions (33). In the present study, the expression of Notch1 and its downstream target Hes1 were significantly higher in sphere-forming cells compared with those in parental cells.

To the best of our knowledge, the role of the Notch1 signaling pathway in lapatinib resistance has not been investigated previously. Therefore, in the present study, the sensitivities of sphere-forming and parental cells to lapatinib were assessed, and it was demonstrated that sphere-forming cells were significantly more resistant to lapatinib than parental cells. To investigate the potential molecular mechanisms that influence the Notch1 signaling pathway in lapatinib resistance, the expression of Hes1 was inhibited via transfection with Hes1-siRNA lentivirus. Following the downregulation of Hes1 expression, the CSC properties of sphere-forming cells were significantly impaired and the expression levels of CSC markers were significantly downregulated compared with those of the corresponding control siRNA groups.

The activation of EGFR family proteins is regulated by ligand binding, with the exception of HER2, which dimerizes independently of ligand binding (34,35). Once dimerization occurs, intracellular tyrosine kinases are fully activated and induce autophosphorylation of their tyrosine residues. These phosphorylated tyrosines function as docking sites for several adapter proteins, including growth factor receptor bound
protein 2 and SHC adaptor protein, which further transduce the signaling pathways through protein-protein interactions and post-translational modifications (36). Cross-regulation between EGFR/HER2 and Notch signaling pathways has long been observed in genetic studies (37). For instance, activation of the Notch1 signaling pathway is associated with EGFR-TKI resistance in PC9 cells expressing mutated EGFR (38). In the present study, MKN45 cells were selected, as HER2 is highly activated in these cells (39). It was revealed that the inhibition of Hes1 significantly increased the sensitivity of sphere-forming cells to lapatinib.

To further investigate the role of the Notch1 signaling pathway in lapatinib resistance, EMT and resistance-associated protein expression levels were examined. During EMT, epithelial cells lose several of their epithelial characteristics, including E-cadherin and ZO1 expression, and acquire properties that are typical of mesenchymal cells, including the expression of vimentin (40). In the present study, the inhibition of Hes1 decreased Snail expression and impaired EMT in sphere-forming cells. Additionally, it is known that CSCs can effectively increase drug resistance through upregulating the expression of drug efflux transporter genes and various multidrug resistance genes (41). CSCs isolated from the sphere-forming culture of cancer cells were previously demonstrated to possess high expression of the ABCB1 gene and were identified to be significantly resistant to various chemotherapeutic agents (42). In the present study, the expression levels of the resistance-associated proteins MDR1, ABCG1, ABCG2 and RAD51 were significantly downregulated in sphere-forming cells infected with Hes1-siRNA lentivirus compared with those in the control-siRNA group. Protein expression levels were normalized to GAPDH. Data are presented as the mean ± standard deviation (n=5). P<0.05. Notch1, neurogenic locus notch homolog protein 1; Hes1, transcription factor Hes1; siRNA, small interfering RNA; Snail, zinc finger protein SNAI1; MDR1, multidrug resistance protein 1; ABCG, ATP-binding cassette sub-family G member; RAD51, DNA repair protein RAD51 homolog 1; ZO1, tight junction protein ZO-1; EMT, epithelial-mesenchymal transition.

Figure 5. Infection with Hes1-siRNA lentivirus results in impaired EMT and decreased expression of chemoresistance-associated proteins in sphere-forming MKN45 cells. (A) EMT was impaired in sphere-forming cells infected with Hes1-siRNA lentivirus, as demonstrated by a significant decrease in the expression levels of the EMT-associated proteins Snail, N-cadherin and vimentin, and an increase in expression level of E-cadherin and ZO1 compared with those in the control-siRNA group. (B) The expression levels of the chemoresistance-associated proteins MDR1, ABCG1, ABCG2 and RAD51 were significantly downregulated in sphere-forming cells infected with Hes1-siRNA lentivirus compared with those in the control-siRNA group. Protein expression levels were normalized to GAPDH. Data are presented as the mean ± standard deviation (n=5). *P<0.05. Notch1, neurogenic locus notch homolog protein 1; Hes1, transcription factor Hes1; siRNA, small interfering RNA; Snail, zinc finger protein SNAI1; MDR1, multidrug resistance protein 1; ABCG, ATP-binding cassette sub-family G member; RAD51, DNA repair protein RAD51 homolog 1; ZO1, tight junction protein ZO-1; EMT, epithelial-mesenchymal transition.

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