Introduction

Tumor metastasis is the primary cause of mortality in patients with hypopharyngeal squamous cell carcinoma (HSCC) (1,2). However, despite advances in diagnostic methods and treatments, the five-year survival rate remains only at 25–40% (3), and once distant metastasis occurs, the prognoses and the survival rates of HSCC patients remain unsatisfactory (4). Therefore, a better understanding of the molecular mechanisms driving HSCC metastasis is urgently needed.

CDH1 is the prototypical member of the classical...
cadherin family that mediates cell–cell adhesion. Loss of CDH1 indirectly regulates gene transcription by causing the translocation of β-catenin to the nucleus (5). CDH1 loss, the hallmark of epithelial to mesenchymal transition, is a prerequisite for subsequent tumor invasion and metastasis (6,7), but the specific mechanism of CDH1 in HSCC metastasis has not been fully elucidated.

Matrix metalloproteinase (MMP)-9 is a member of the MMP family and functions as a Zn2+-dependent endopeptidase, which plays a pivotal role in tumor dissemination and invasiveness (8,9). The over-expression of MMP-9 facilitates metastasis and tumor progression by degrading the extracellular matrix (ECM), which allows tumor cells to migrate and colonize host tissues (10). MMP-9 is associated with cell growth and metastasis in several neoplasms (11-13). Importantly, the enhanced expression of MMP-9 acts the progression of head and neck squamous cell carcinoma (14). In addition, increased expression of MMP-9 is tightly linked to lymph node metastasis of laryngeal cancer (15).

Loss of CDH1 leads to the upregulation of epidermal growth factor receptor (EGFR) in head and neck cancer and in non-small cell lung cancer cells (16,17). In addition, EGFR, which is one of the receptor tyrosine kinases, can phosphorylate signal transducer and activate transcription 3 (STAT3). Once activated, STAT3 modulates the transcription of a range of targeted genes such as MMP-9, thereby influencing the proliferation, migration, and invasion of tumor cells (18,19). MMP-9 cleaves CDH1 at the cell surface, and the resulting soluble form of CDH1 promotes tumor invasion (20). However, the precise mechanism by which the downregulation of CDH1 contributes to MMP-9 upregulation remains largely unknown. In this study, we demonstrate that loss of CDH1 is closely associated with postoperative lung metastasis and is accompanied by MMP-9 upregulation. Furthermore, CDH1 enhances MMP-9 expression via STAT3 activation, which subsequently facilitates cell proliferation, invasion, and metastasis of HSCC. Therefore, our research highlights the important role of CDH1 in HSCC metastasis and might provide potential therapeutic targets for HSCC therapy.

Methods

Patients

Paired tumor and adjacent normal tissue samples were obtained from 109 patients who were undergoing radical surgery without pre-operative adjuvant treatment and who were pathologically and/or cytologically diagnosed with HSCC in Shandong Provincial Hospital affiliated to Shandong University between 2010 and 2014. The 109 enrolled patients consisted of 31 post-operative lung metastases cases, whereas the remaining 78 cases did not have lung metastases. After receiving approval from the Shandong Provincial Hospital affiliated to Shandong University Ethical Committee (No. 2017018) and signing of informed consent by patients, samples were collected and used in subsequent assays.

Immunohistochemistry (IHC)

Formalin-fixed and paraffin-embedded blocks with HSCC tissue and non-cancerous tissue were used for IHC staining as previously described (21). The rabbit monoclonal antibodies against CDH1 and MMP-9 were used as primary antibodies, and the histopathological images were obtained with an Olympus BX53 microscope. The IHC staining of CDH1 and MMP-9 expression was analyzed independently by two pathologists who were blinded to the clinical data. The sections were graded according to the amount and intensity of immunoreactivity as follows: 0 (0–5% immunopositive cells), 1 (5–25% immunopositive cells), 2 (26–50% immunopositive cells), 3 (51–80% immunopositive cells), or 4 (≥80% immunopositive cells). The intensity was scored as follows: 0 (no coloration), 1 (yellow), 2 (light brown), or 3 (dark brown). A final score was obtained by multiplying the two scores as follows: ≤1, no expression (–); 2–3, weak expression (+); 4–7, moderate expression (++) ≥8, and strong expression (+++).

Reagents

Mouse monoclonal antibodies against CDH1 (#610181) and STAT3 (#610189) were obtained from BD Transduction Laboratories. Rabbit monoclonal anti-MMP-9 (#13667) and rabbit polyclonal anti-phosphor-STAT3 (#9131) antibodies were obtained from Cell Signaling Technology. Mouse anti-β-actin monoclonal antibody (#TA-09) and the rabbit monoclonal anti-CDH1 (#ZA-0565) and anti-MMP-9 (#ZA-0562) antibodies used in the IHC assay were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. Purified AG490 powder (#T3434) was purchased from Sigma Aldrich.
Cell lines and cell culture

Human FaDu cell line was originally obtained from the American Type Culture Collection and was routinely tested for mycoplasma infection prior to the initiation of the experiments. The cell line was cultured in DMEM/F12 (Gibco, C11330500BT) supplemented with 10% fetal bovine serum (Ausbian, VS500T) and was maintained in a monolayer culture at 37 °C in a humidified atmosphere composed of 5% CO₂ and 95% air.

Gene silencing by siRNA

All small interfering RNAs (siRNAs) were synthesized by GenePharma (Shanghai, China). Control siRNA duplexes targeted the sequence 5’-UUC GAA CGU GUC ACG UTT-3’. STAT3 siRNA duplexes targeted the sequence 5’-GCA AGA UUC AGA CCC UCA ATT-3’. CDH1 siRNA #1 and #2 duplexes targeted the sequences 5’-CAG ACA AAG ACC AGG ACT A-3’ and 5’-GCA CGU ACA CAG CCC UAA U-3’, respectively. MMP-9 siRNA duplexes silenced the sequence 5’-CAU CAC CUA UUG GAU CCA A-3’. The FaDu cell line was transfected with the siRNAs by using the Lipofectamine® RNAiMAX Reagent according to the manufacturer’s instructions.

Western blot analysis

Whole-cell protein lysates and Western blot assays were prepared described previously (22).

Sulforhodamine B (SRB) assay

FaDu cells were transfected with siRNA in six-well plates for 24 h and further reseeded in 96-well plates with 5,000 cells/well for 48 h. Cell viability was assessed using the SRB assay as described previously (23).

CCK-8 assay

After treatment with siRNA for 24 h, the cells were reseeded in 96-well culture plates at a density of 5,000 cells/well and incubated for another 48 h. Then, 10 μL CCK-8 solution was added to each well and incubated for another 2 h at 37 °C. The absorbance was analyzed at 450 nm by utilizing a microplate reader (BioTek).

Cell migration assay

FaDu cells were transfected with siRNA for 48 h and reseeded in trans-well chambers consisting of inserts with 8 μm-pores. After further incubation for 24 h, the cell migration assay was performed as described previously (24).

Cell invasion assay

The metastatic ability of the cells was evaluated by performing invasion assay in the trans-well chambers mentioned above coated with Matrigel following the manufacturer’s protocol (Corning Incorporated).

Statistical analysis

Statistical analyses were performed using SPSS 17.0. The Mann-Whitney U-test was used to compare CDH1 and MMP-9 expression in the tumor tissues to expression in the paired adjacent normal tissues. The association between CDH1 expression and lung metastases was analyzed using Spearman’s rank correlation coefficients. Western blot, cell proliferation, migration, and invasion assays were analyzed by Student’s t-test. Statistical significance was deemed at P<0.05.

Results

Depletion of CDH1 is associated with HSCC lung metastasis and increased MMP-9 expression

We used IHC staining to measure the expression of CDH1 and MMP-9 in 109 paired cancer and adjacent non-cancer tissues, of which 31 cases exhibited postoperative lung metastases. IHC staining showed that besides prominent localization to the membrane of tumor cells (Figure S1A), CDH1 also showed cytoplasmic expression (Figure S1B,C). However, only membrane expression was mostly found in the adjacent non-tumor cells (Figure 1A). Positive staining for CDH1 was found in 104 of 109 normal tissues, while 93 of 109 tumor tissues presented positive staining. CDH1 positive staining was significantly different between the normal (Figure 1A) and tumor tissues (Figure 1B) (P=0.012, Mann-Whitney U-test). MMP-9 expression was observed in both the adjacent non-tumor and tumor tissues, which was mainly seen as diffuse staining in the cytoplasm with occasional localization in the membrane of tumor cells (Figure S1D,E,F). The overexpression of MMP-9 protein
in IHC staining was detected in 61 of 109 tumor tissues, whereas only 14 of 109 non-tumor tissues showed positive staining for MMP-9. The positive staining of MMP-9 immunoreactive protein was significantly decreased in non-tumor tissues (Figure 1C) compared with tumor tissues (Figure 1D) (P<0.001, Mann-Whitney U-test). Intriguingly, the expression of CDH1 was significantly decreased in HSCC lung metastases tissues (Figure 2A) compared with non-metastatic samples (Figure 2B). Eleven of 16 HSCC patients with no expression of CDH1 had lung metastases. The lung metastasis rate was 68.75%. Six of the 12 HSCC patients with weak (+) expression of CDH1 had lung metastases. The lung metastasis rate was 50%. For patients with moderate (++) and strong (+++) expression of CDH1, the lung metastasis rates were 24.3% (9/37) and 11.4% (5/44), respectively, which significantly differed (P=0.006, χ² test; Table 1). The differences between the negative expression and moderate or strong expression of CDH1 were significant (P=0.000, χ² test, α=0.008; Table 1). However, no difference was found from the comparisons between other groups. Spearman’s rank correlation coefficients showed that the expression of CDH1 was negatively correlated with HSCC lung metastases (Spearman rho =−0.431, P<0.01). Moreover, when CDH1 was downregulated, the expression of MMP-9 was correspondingly increased in the same HSCC sample (Figure 1B,D), which indicates that the expression of CDH1 might be directly associated with MMP-9 expression in HSCC.

**Loss of CDH1 induces MMP-9 upregulation in FaDu cells**

To test whether MMP-9 expression was enhanced when
CDH1 was depleted in FaDu cells, we suppressed CDH1 expression with siRNA treatment in FaDu cells and measured the level of MMP-9 by Western blot assay. FaDu cells with decreased CDH1 expression showed elevated expression of MMP-9 compared with the controls (Figure 3).

Table 1 HSCC lung metastases and occurrence of expression of CDH1 immunoreactive protein

| CDH1 expression | Non-lung metastases (%) | Lung metastases (%) | n   | P     |
|-----------------|-------------------------|---------------------|-----|-------|
| −               | 5 (31.3)                | 11 (68.7)           | 16  | 0.000 |
| +               | 6 (50.0)                | 6 (50.0)            | 12  | 0.539 |
| ++              | 28 (75.7)               | 9 (24.3)            | 37  | 0.006 |
| +++             | 39 (88.6)               | 5 (11.4)            | 44  | 0.000 |

Note: *P=0.000 (− vs. + vs. ++ vs. +++), *P=0.539 (− vs. +), *P=0.006 (− vs. ++), *P=0.000 (− vs. +++), α=0.008. HSCC, hypopharyngeal squamous cell carcinoma.

CDH1 was depleted in FaDu cells, we suppressed CDH1 expression with siRNA treatment in FaDu cells and measured the level of MMP-9 by Western blot assay. FaDu cells with decreased CDH1 expression showed elevated expression of MMP-9 compared with the controls (Figure 3).

**Loss of CDH1 upregulated MMP-9 expression via STAT3 phosphorylation**

Considering that p-STAT3 contributes to MMP-9 expression (18), we further explored the effect of CDH1 depletion on the activation of STAT3. Depletion of CDH1 elicited a significant increase in p-STAT3 (Figure 4A,B). To determine whether p-STAT3 acts in MMP-9 up-regulation when CDH1 is depleted in FaDu cells, we inhibited STAT3 and CDH1 expression by siRNA transfection and found that blocking STAT3 attenuated the induction of MMP-9 caused by CDH1 depletion (Figure 4C,D). In parallel, we treated CDH1-siRNA-transfected FaDu cells with AG490 to suppress STAT3 activation and found that the pharmacological inhibition of STAT3 also prevented the induction of MMP-9 triggered by CDH1 loss (Figure 4E), which was consistent with the results of the co-transfection assay. Hence, the suppression of CDH1 leads to MMP-9 upregulation via increased phosphorylation of STAT3.

**STAT3 and MMP-9 enhanced the proliferation and metastasis induced by CDH1 depletion in HSCC cells**

Our data showed that loss of CDH1 might contribute to tumor progression and metastasis by altering STAT3 and MMP-9 levels. To determine the roles of CDH1, STAT3, and MMP-9 in HSCC cells, a cell proliferation assay was conducted in FaDu cells. As shown in Figure 5A,B, the growth and proliferation of FaDu cells treated with CDH1 siRNA were enhanced, whereas an obvious decline was observed in the cells transfected with STAT3 or MMP-9 siRNAs. Moreover, additional knockdown of STAT3
Figure 3 Loss of CDH1 promotes MMP-9 expression in FaDu cells. Treating FaDu cells with CDH1 siRNA 1# and 2# for 72 h increased the levels of MMP-9 as measured by Western blot and quantified using the Image J software (**p<0.01, ***p<0.001 versus control).

Figure 4 Loss of CDH1 upregulates MMP-9 by activating STAT3 in FaDu cells. (A and B) Treating FaDu cells with CDH1 siRNA 1# and 2# for 72 h increased the phosphorylation of STAT3 compared with control siRNA. (C and D) FaDu cells were treated with CDH1 siRNA 1# and 2# and STAT3 siRNA for 72 h, and the levels of p-STAT3 and MMP-9 were measured by Western blot analysis. (E) FaDu cells were transfected with CDH1 siRNA 2# for 48 h and then treated with 20 μM AG490 for 24 h. The levels of p-STAT3 and MMP-9 were measured by Western blot. Each experiment was carried out at least thrice, and the representative Western blots are shown.

or MMP-9 weakened the enhanced cell growth and proliferation induced by CDH1 depletion (Figure 5A,B). In addition, the metastatic potential was determined using a cell migration and invasion assay. In comparison with the control cells, knockdown of CDH1 significantly enhanced the motility of FaDu cells, whereas the migration of FaDu
Figure 5 STAT3 and MMP-9 are responsible for the enhanced proliferation promoted by CDH1 suppression. (A) In addition to CDH1 siRNA 1# treatment, FaDu cells were transfected with STAT3 or MMP-9 siRNA. The SRB assay was then performed when the cells were re-seeded and incubated in 96-well plates for 48 h (mean ± SEM, n=5). (B) FaDu cells were used with the identical transfection as in (A), and the proliferation of FaDu cells was measured with a CCK-8 assay when the cells were re-seeded and incubated in 96-well plates for 48 h. Each experiment was performed at least thrice (mean ± SEM, n=4). ***, P<0.001; **, P<0.01; *, P<0.05.

cells was greatly decreased when treated with STAT3 or MMP-9 siRNA. Furthermore, increased migration was observed in the CDH1-depleted cells compared with the cells co-transfected with CDH1 siRNA and either STAT3 or MMP-9 siRNA (Figure 6A,B). Finally, cell invasiveness was measured using Matrigel-coated chambers, and these results were consistent with the cell migration assay (Figure 6C,D). Hence, CDH1 depletion facilitates the proliferation, migration, and invasiveness of FaDu cells, which is accompanied by the phosphorylation of STAT3 and upregulation of MMP-9.

Discussion

HSCC has the poorest outcome among the malignant head and neck tumors, and once patients present lung metastasis, the average time from diagnosis to death is only approximately 5 months (3,25). Thus, understanding the molecular mechanisms involved in HSCC metastasis is important. Loss of CDH1 triggers active signals that support invasion and metastasis in various epithelial tumors (6), implying that it might also play an important role in HSCC lung metastases; Berx and Bircheimer et al. reported that loss of CDH1 is functionally correlated with increased incidence of metastasis (6,26). Here, we show that CDH1 downregulation is associated with HSCC lung metastases. Moreover, loss of CDH1 upregulates MMP-9 by phosphorylating STAT3 and thus contributes to the proliferation, migration, and invasiveness of FaDu cells, which might be responsible for lung metastasis and serve as an indicator for HSCC lung metastasis in clinical treatment.

Proteolytic enzymes, such as MMP-9, degrade various structural components of the ECM, thus facilitating tumor invasion into surrounding connective tissues (27). The over-expression of MMP-9 indicates metastatic potential in head and neck squamous carcinomas (28). In the present investigation, we found that reduced expression of CDH1 was concomitant with increased expression of MMP-9 in the same HSCC tissue sample. In addition, loss of CDH1 activates EGFR, which in turn phosphorylates STAT3 (16,29). Activated STAT3 upregulates the transcription of MMP-9 (18). Hence, the reduction of CDH1 affects the expression of MMP-9 through p-STAT3 in HSCC. In this study, we used two CDH1 siRNAs to transfect the FaDu cells and then measured the level of MMP-9 in FaDu cells. Our data showed that CDH1 inhibition upregulated the level of p-STAT3 and MMP-9 in FaDu cells, and silencing STAT3 attenuated the
**Figure 6** STAT3 and MMP-9 contribute to the increased migration and invasion induced by CDH1 suppression. (A) After transfecting with different combinations of CDH1, STAT3, and MMP-9 siRNAs for 48 h, FaDu cells were cultured in transwell chambers for another 24 h, migrated cells were stained with crystal violet for 15 minutes in migration assay. (B) Quantitative analysis of migrated FaDu cells. (C) FaDu cells were transfected with the same siRNAs as in (A) and re-cultured in transwell chambers coated with a 1:3 dilution of Matrigel for another 36 h, invaded cells were stained with crystal violet for 15 minutes in invasion assay. (D) Quantitative analysis of invaded FaDu cells. Images of the migrated (A) and invaded (C) cells were obtained under a light microscope. Magnification, 20×. Each assay was performed thrice in independent experiments (mean ± SEM, n=5. ***, P<0.001; **, P<0.01; *, P<0.05).

Induction of MMP-9 caused by the suppression of CDH1, which indicates that CDH1 downregulation promotes MMP-9 expression in a p-STAT3-dependent manner.

Excessive cell proliferation, migration, and invasion are positively correlated with tumorigenesis and progression. Our work shows that depleting CDH1 significantly enhanced cell growth, proliferation, and invasiveness compared with the control, and silencing STAT3 and MMP-9 prevented the increased proliferation in CDH1-silenced FaDu cells. In addition, suppression of STAT3 and MMP-9 impaired the cell migration and invasion capacity caused by the loss of CDH1 in HSCC cells. Thus, the reduction of CDH1 plays a vital functional role in HSCC progression by upregulating MMP-9 via p-STAT3, hinting that p-STAT3 and MMP-9 might serve as crucial targets for the clinical treatment of HSCC.

**Conclusions**

In summary, we showed that CDH1 is a powerful indicator for HSCC lung metastasis, loss of CDH1 elevates MMP-9 expression by activating STAT3. Subsequently, the proliferative, invasive, and metastatic capacity of FaDu cells are promoted, which might contribute to the malignant...
development and lung metastases of HSCC. Taken together, our study might provide novel therapeutic targets for metastatic HSCC and introduce new avenues for the further development of clinical treatments.

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Footnote
Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.07.51). The authors have no conflicts of interest to declare.

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). The study was approved by the Shandong Provincial Hospital Affiliated to Shandong University Ethical Committee (No. 2017018) and all patients signed informed consent.

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Figure S1 Representative IHC staining of CDH1 and MMP-9 in HSCC. (A) Membrane expression of CDH1 (indicated by arrows); (B) cytoplasmic expression of CDH1 (indicated by arrows); (C) arrows indicating the membrane and cytoplasmic expression of CDH1; (D) membrane immunostaining of MMP-9 (indicated by arrows); (E) diffuse cytoplasmic expression of MMP-9 (indicated by arrows); (F) membrane and cytoplasmic staining of MMP-9 (indicated by arrows). Magnification, 200x. Scale bar =200 μm.