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Metadherin contributes to epithelial-mesenchymal transition and paclitaxel resistance induced by acidic extracellular pH in nasopharyngeal carcinoma

CHANGYUN YU[1], YONG LIU[2] and ZHAOBING QIN[1]

[1]Department of Otolaryngology Head and Neck Surgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450000; [2]Department of Otolaryngology Head and Neck Surgery, Xiangya Hospital, Central South University, Changsha, Hunan 410008, P.R. China

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Abstract. Paclitaxel resistance is a challenge to the treatment of nasopharyngeal carcinoma (NPC). An acidic extracellular pH (pH_e), a hallmark of solid tumors, is demonstrated to decrease the efficacy of chemotherapy. However, the precise function of acidic pH_e in mediating chemotherapy in NPC remains unknown. In the present study, acidic pH_e significantly decreased the cytotoxicity of paclitaxel in NPC cells. In addition, epithelial-mesenchymal transition (EMT)-like changes were observed in NPC cells cultured at acidic pH_e. Metadherin (MTDH), a novel oncogene, is expressed in multiple types of solid tumor, and is associated with several malignant cell characteristics, including malignant cell transformation, proliferation, angiogenesis, chemoresistance, invasion and metastasis. In the present study, MTDH expression was increased in NPC cells that had been cultured at an acidic pH_e. Furthermore, the silencing of MTDH expression reversed EMT molecular marker expression and sensitized NPC cells to paclitaxel. Taken together, the results of the present study provide evidence to support an association between acidic pH_e-induced paclitaxel resistance and MTDH-mediated EMT in NPC cells. Thus, targeting MTDH may provide a novel strategy for overcoming chemoresistance in NPC therapy.

Introduction

Paclitaxel is a cytotoxic agent that remains a first-line chemotherapy in the management of advanced nasopharyngeal carcinoma (NPC) (1,2). However, the development of intrinsic or acquired resistance limits the clinical efficacy of paclitaxel, which ultimately leads to tumor recurrence and a poor prognosis. Therefore, elucidation of the underlying molecular mechanisms that lead to paclitaxel resistance is an essential requirement to identify novel therapeutic strategies to overcome chemoresistance.

Extracellular pH (pH_e) is considerably more acidic in solid tumors compared with in normal tissue. Previous studies have identified that acidic pH_e is an important characteristic of tumor tissues and is involved in cancer progression (3-7). Furthermore, acidic pH_e has been reported to facilitate the development of resistance to chemotherapeutic drugs, including paclitaxel (8-10). Previous studies have provided evidence indicating that the 'ion-trapping' phenomenon mainly contributes to acidic pH_e-mediated drug resistance (11-13). However, paclitaxel is not ionizable and therefore the drug distribution should be unaffected by pH_e. Thus, it is important to understand the underlying molecular mechanisms responsible for the development of paclitaxel resistance.

Epithelial-mesenchymal transition (EMT) is a complex series of events, which was initially characterized as being essential during normal embryonic development (14). Previous studies have demonstrated that EMT is involved in physiological and pathological processes (15). Critically, previous studies have demonstrated an intricate association between chemoresistance and the changes associated with EMT (16). A previous study in ovarian cancer discovered that paclitaxel-resistant cells, which develop following continuous exposure to paclitaxel, exhibited the cellular and molecular characteristics of EMT (17).

Metadherin (MTDH), also known as astrocyte elevated gene-1 and lysine-rich carcinoembryonic antigen-related cell adhesion molecule-1-associated protein is an oncogene that is expressed in multiple tumors (18). Aberrant expression and dysfunction of MTDH is involved in tumor cell proliferation, survival and metastasis activity (19). Previous studies have confirmed that MTDH expression is associated with the chemoresistance of tumor cells by the regulation of a series of downstream target genes (20-24). The association between MTDH expression and EMT has been established in cervical

Correspondence to: Dr Changyun Yu, Department of Otolaryngology Head and Neck Surgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450000, P.R. China
E-mail: yuchangyun2007@163.com

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cancer, breast cancer and hepatocarcinoma, which may contribute to chemoresistance (25-27).

In the present study, the influence of pH_e on the cytotoxicity of paclitaxel in NPC cells *in vitro* was determined, and the associated mechanisms involved were investigated further. The results indicated that the cytotoxic efficacy of paclitaxel was markedly decreased at an acidic pH_e. Furthermore, it was revealed that MTDH-mediated EMT may confer acidic pH_e-induced paclitaxel resistance in NPC.

Materials and methods

Cell culture. The human NPC 5-8F cells were provided by the Cell Center of Central South University (Changsha, China). 5-8F cells were maintained as monolayer cultures in RPMI-1640 medium (Hyclone; GE healthcare, Chicago, IL, USA) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 IU/ml streptomycin (all purchased from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in a humidified atmosphere with 5% CO_2. Culture media with different pH values were prepared by the addition of 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid or 20 mM 2-(N-morpholino)ethanesulfonic acid (both purchased from Thermo Fisher Scientific, Inc.) to RPMI-1640 medium and pH values were adjusted accordingly, using NaOH or HCl to pH 7.4 or 6.8, respectively. The actual pH in the media was determined prior to and following each experiment. Cells in the exponential growth phase were used for all subsequent experiments.

Paclitaxel cytotoxicity assays. The viability of NPC 5-8F cells was analyzed using a Cell Counting Kit-8 (CCK8; Beyotime Institute of Biotechnology, Shanghai, China) to produce cell viability curves according to the manufacturer's protocol. The IC_{30} value of paclitaxel in NPC 5-8F cells was confirmed as 2.947. IC_{30} is defined as the concentration of paclitaxel required to produce 30% inhibition of 5-8F cells. Subsequently, NPC 5-8F cells were seeded at a density of $3x10^3$ cells/well into 96-well plates in triplicate. Following 24 h of culture, the culture medium was aspirated and replaced with 100 μl medium, buffered to pH 7.4 or 6.8, and supplemented with paclitaxel at its IC_{30} value. After 48 h of culture at 37˚C, the optical density values of each group were determined at a wavelength of 490 nm. Each experiment was representative of three independent repeats.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. In total, 1 μg RNA was used for reverse transcription to synthesize cDNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems; Thermo Fisher Scientific Inc.). In total, 20 μl of reverse-transcription reaction components [10 μl 2X RT Buffer, 1 μl 20X RT Enzyme mix, 7 μl Nuclease-free H_2O (all from Applied Biosystems; Thermo Fisher Scientific, Inc.) and 2 μl RNA] were established and incubated for 60 min at 37˚C, 5 min at 95˚C and then incubated at 4˚C for further investigation. iQ™SYBR® Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to PCR amplification in a 20 μl reaction system. The reaction system was prepared according to the following system: 10 μl iQ™SYBR® Green Supermix, 0.5 μl PCR forward primer, 0. 5 μl PCR reverse primer, 8 μl Nuclease-free H_2O (Bio-Rad Laboratories, Inc.) and 1 μl cDNA template. Primer sequences used in the present study were as follows: E-cadherin forward, 5'-GCTGGACCGAGAGAGTTTCC-3' and reverse, 5'-CAAAATCCAAGCCCGTGGTG-3'; vimentin forward, 5'-TGTCCAAATCGATGTGGATGTTTC-3' and reverse, 5'-TTGTACCATTCTTCTGCCTCCTG-3'; N-cadherin forward, 5'-TGGGAAATGGAAACTTGATGGC-3' and reverse, 5'-AGTTGCTAAACTTCACTGAAAGGA-3'; MTDH forward, 5'-GATGATGAATGGTCTGGGTTAAA-3' and reverse, 5'-GACCTTTTGATCATCAGGAATTG-3'; GAPDH forward, 5'-GAGTCAACGGATTTGGTCGT-3' and reverse, 5'-TTGATTTTGGAGGGATCTCG-3'. PCR thermocycling conditions were as follows: 3 min at 95˚C followed by 40 cycles of 15 sec at 95˚C and 30 sec at 60˚C. The PCRs for each gene were performed in triplicate, and the mean values of fold changes were used to calculate mRNA expression. The fold change in expression of each gene was calculated using the $2^{-\Delta\Delta Cq}$ method (28).

Western blot analysis. The western blot assay was performed as described previously (29,30). In brief, the total protein (50 μg) was separated by 10% SDS-PAGE and the separated proteins were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% skimmed milk at room temperature for 1 h, then incubated with the following primary antibodies at 4˚C overnight: Primary antibodies used in the present study were: Mouse monoclonal antibody against E-cadherin (1:400; cat. no. sc-8426; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), mouse monoclonal antibody against vimentin (1:200; cat. no. sc-32322; Santa Cruz Biotechnology, Inc.) and rabbit polyclonal antibody against MTDH (1:800; cat. no. 13860-1-AP, Proteintech Group, Inc., Chicago, USA). Subsequent to washing three times in TBST, membranes were incubated with horseradish peroxidase (HRP)-labeled goat Anti-mouse IgG (H+L; 1:1,000; cat. no. A0216) or HRP-labeled Goat Anti-rabbit IgG (H+L; 1:4,000; cat. no. A0208) (both purchased from Beyotime Institute of Biotechnology, Shanghai, China) for 1 h at room temperature. Bands were visualized using the BeyoECL Plus Detection System (Beyotime Institute of Biotechnology) and the images were obtained by X-ray film exposure. For normalization of protein loading, mouse monoclonal antibody against β-actin (1:1,000; cat. no. AF0003; Beyotime Institute of Biotechnology) was used. All antibodies were diluted using primary antibody dilution buffer or secondary antibody dilution buffer (both purchased from Beyotime Institute of Biotechnology). Each experiment was performed in triplicate.

Transient transfection. NPC 5-8F cells were transiently transfected with MTDH small interfering (si)RNA (cat. no. sc-77797) or control siRNA (cat. no. sc-37007; Santa Cruz Biotechnology, Inc.), according to the manufacturer's protocol. MTDH siRNA is a pool of three target-specific 19-25 nt siRNAs designed to knock down MTDH expression. Control siRNA is a non-targeting 20-25 nt siRNA designed as a negative control. 5-8F cells ($2x10^5$ cells) were seeded on a six well plate 24 h prior to transfection. 8 μl of siRNA duplex (80 pM

siRNA) or control siRNA was diluted into 100 μl siRNA transfection medium (cat. no. sc-36868; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) as solution A. Furthermore, 6 μl of siRNA transfection reagent (cat. no. sc-29528; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was diluted into 100 μl siRNA transfection medium as solution B. Solution A and B were mixed and incubated for 45 min at room temperature. In total, 0. 8 ml siRNA transfection medium was added to each tube containing the siRNA transfection reagent mixture (solution A and B), and overlayed onto the washed cells. Cells were incubated for 5-7 h at 37˚C in a 5% CO_2 incubator. To determine the efficiency of the siRNA knockdown, the transfected cells were collected 3 days after transfection and the protein levels of MTDH were assessed using western blotting, as aforementioned.

Statistical analysis. All statistical analyses were conducted using SPSS 17. 0 software (SPSS, Inc., Chicago, IL, USA). Quantitative data are expressed as the mean ± standard deviation. Statistical differences between groups were compared using two-tailed unpaired Student's t-tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Acidic pH_e decreases the cytotoxicity of paclitaxel in NPC cells. To confirm the association between acidic pH_e and the sensitivity of NPC to paclitaxel, the IC_{30} value for paclitaxel in NPC 5-8F cells was confirmed as 2.947 (Fig. 1A). The viability of NPC 5-8F cells, incubated in normal (pH 7.4) or acidic (pH 6.8) medium, following paclitaxel stimulation at its IC_{30} for 48 h, was evaluated using CCK-8 assays. As presented in Fig. 1B, the survival rate of NPC 5-8F cells was 30.32±3.34 compared with 45.58±6.34 (P<0.05), incubated in normal (pH 7.4) or acidic (pH 6.8) medium, respectively. These results indicated that acidic medium enhanced the survival rate of NPC 5-8F cells and that acidic pH_e significantly decreased the cytotoxicity of paclitaxel in NPC cells.

EMT-like features of NPC cells grown in acidified medium. In order to investigate whether EMT is involved in acidic pH_e-induced paclitaxel resistance, the key markers of EMT were measured in NPC 5-8F cells grown in acidified medium for 48 h. Phase-contrast microscopy revealed that NPC 5-8F cells cultured in acidic medium underwent several morphological changes, with some loss of adherence and cell-to-cell contact and the induction of a spindle-like form (Fig. 2A). The results of qPCR and western blot analysis demonstrated that expression of the characteristic mesenchymal markers vimentin and N-cadherin were upregulated in NPC 5-8F cells cultured at pH_e 6.8, compared with that of pH_e 7.4 (Fig. 2B and C). In addition, a significant decrease in the expression of the epithelial maker E-cadherin was observed when NPC 5-8F cells were cultured in acidic medium (Fig. 2B and C). These results indicated that the acidic pH_e-induced paclitaxel-resistant NPC cells acquired the EMT phenotype.

Acidic pH_e enhances the expression of MTDH in NPC cells. In the present study, the potential molecular mechanism responsible for the EMT-like phenotypic changes in acidic

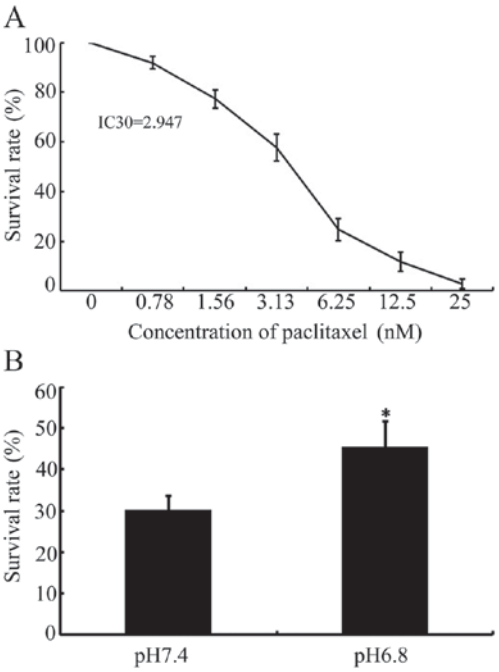

Figure 1. Influence of acidic pH_e on the cytotoxicity of paclitaxel in NPC cells. (A) Determination of the IC_{30} value for paclitaxel in NPC 5-8F cells. (B) Cell viability was evaluated in NPC 5-8F cells, incubated in normal (pH 7.4) or acidic (pH 6.8) medium, following paclitaxel stimulation at its IC_{30} value for 48 h. *P<0. 05 vs. pH 6.8. pH_e, extracellular pH; NPC, nasopharyngeal carcinoma; IC_{30}, concentration of paclitaxel required to produce 30% inhibition in NPC 5-8F cells.

pH_e-induced paclitaxel resistance in NPC cells was explored. In a previous study, it was demonstrated that MTDH was increased and promoted EMT in squamous cell carcinoma of the head and neck (SCCHN) (30). Another previous report revealed that increased MTDH expression is also associated with drug resistance, including that of paclitaxel, in breast cancer, hepatocellular carcinoma and prostate cancer (23,31). Therefore, the differences in MTDH expression in NPC cells cultured at a pH_e of 6.8 compared with that at a pH_e of 7.4 were investigated further. As presented in Fig. 3A, MTDH mRNA levels were increased when cultured in acidic medium. In addition, western blot analysis demonstrated that MTDH protein expression was significantly increased in response to an acidic culture environment (Fig. 3B). The results from the present study indicated that MTDH may be associated with an acidic pH_e-mediated EMT and paclitaxel resistance in NPC cells.

Silencing of MTDH reversed the EMT phenotype and sensitized NPC cells to paclitaxel in an acidic pH_e environment. To determine whether increased MTDH expression was associated with EMT in NPC cells, siRNA was used to downregulate MTDH expression in NPC cells cultured at a pH_e of 6.8. Cells transfected with MTDH siRNA demonstrated decreased MTDH expression, accompanied by decreased vimentin expression and increased E-cadherin expression, compared with NPC 5-8F cells transfected with the control vector (Fig. 4A). The results demonstrated that E-cadherin downregulation and vimentin upregulation, induced by acidic pH_e were attenuated by MTDH RNA interference in NPC 5-8F

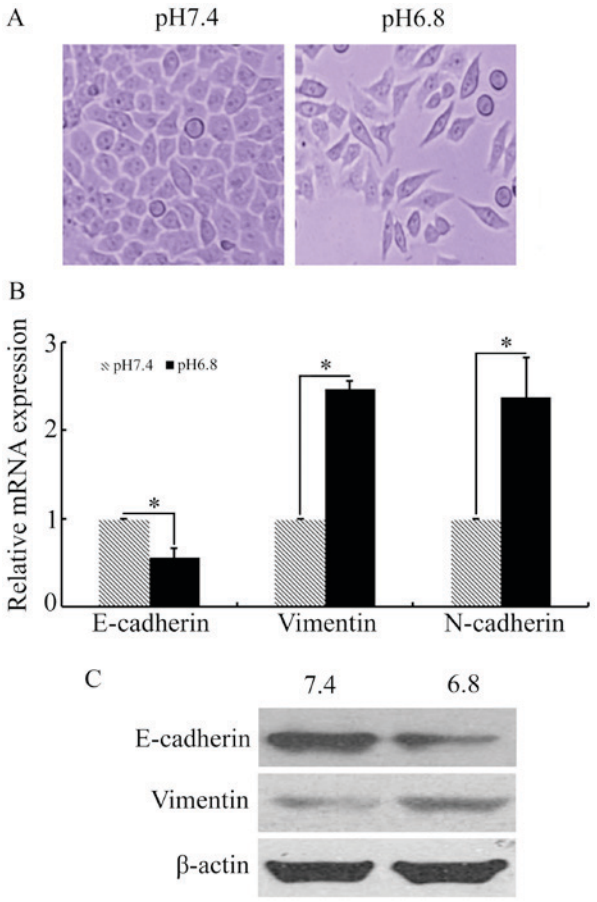

Figure 2. EMT-like changes are induced by acidity in NPC 5-8F cells. (A) Morphological changes induced by acidity in NPC 5-8F cells. Images captured at x100 magnification. (B) Reverse transcription-quantitative polymerase chain reaction and (C) western blot analysis assessing the mRNA and protein levels of EMT makers. *P<0. 05 vs. pH 6.8. EMT, epithelial-mesenchymal transition; NPC, nasopharyngeal carcinoma.

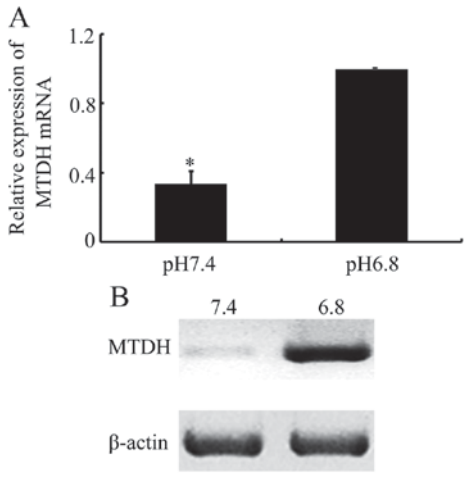

Figure 3. Acidic pH_e enhances MTDH expression in NPC cells. (A) Reverse transcription-quantitative polymerase chain reaction and (B) western blot analysis of the mRNA and protein levels of EMT in NPC 5-8F cells cultured at a pH_e of 6.8 or 7.4, respectively. *P<0. 05 vs. pH 6.8. pH_e, extracellular pH; MTDH, metadherin; EMT, epithelial-mesenchymal transition; NPC, nasopharyngeal carcinoma.

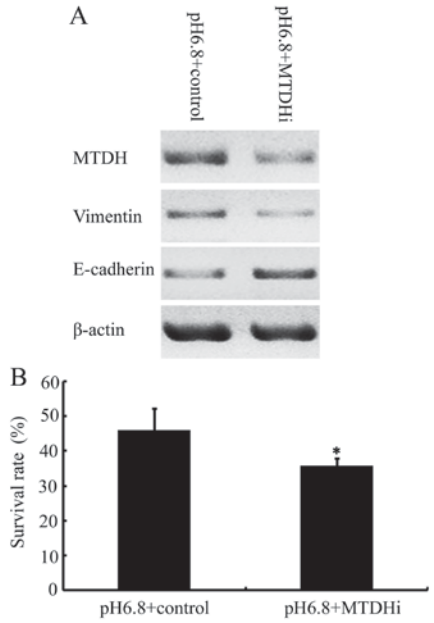

Figure 4. Silencing of MTDH reverses the EMT phenotype and sensitized NPC cells to paclitaxel in an acidic pH_e environment. (A) Downregulation of E-cadherin and upregulation of vimentin induced by acidic pH_e were attenuated by RNA interference with MTDH in NPC 5-8F cells. (B) MTDH knockdown significantly restored sensitivity of NPC 5-8F cells to paclitaxel. *P<0.05 vs. pH 6.8 control. MTDH, metadherin; EMT, epithelial-mesenchymal transition; NPC, nasopharyngeal carcinoma; pH_e, extracellular pH; MTDHi, metadherin interference; E-cadherin, epithelial cadherin.

cells. This is indicative of a crucial function for MTDH in the induction of the EMT phenotype in NPC cells, stimulated by an acidic pH_e environment. Finally, the effects of inhibiting MTDH on the cytotoxicity of paclitaxel in NPC cells in acidic pH_e environment were investigated. As presented in Fig. 4B, the cell survival rate of NPC 5-8F cells at an acidic pH_e in the presence of paclitaxel at its IC_{30} was 45.92±6.3, compared with 35.72±2.27 in the MTDH-downregulated 5-8F cells (P<0.05). These results revealed that MTDH knockdown significantly restored the sensitivity of NPC 5-8F cells to paclitaxel, previously decreased in response to an acidic pH_e environment.

Discussion

NPC is one of the most common malignant tumors in southern China and Southeast Asia, with ~70% of newly diagnosed NPC cases, and thus is classified as locoregionally advanced disease (32). Paclitaxel is now routinely used to treat advanced NPC. However, the development of paclitaxel resistance is a significant barrier to the treatment of NPC. Therefore, elucidation of the molecular mechanisms underlying paclitaxel resistance is crucial to enhance the efficacy of treatment and to improve the survival rates of patients with NPC.

Acidic pH_e, a hallmark of solid tumors, is thought to decrease the efficacy of chemotherapeutic regimens (33-35). However, the exact function of acidic pH_e in mediating chemotherapeutic resistance in NPC remains unclear. In the present study, it was demonstrated that acidic pH_e decreased the cytotoxicity of paclitaxel in NPC 5-8F cells *in vitro*. The results from the present study are consistent with those of a previous study that identified that acidic pH_e leads to decreased paclitaxel sensitivity in murine EMT6 cells and the human bladder

carcinoma cell line MGH-Ul (36). However, other previous *in vitro* studies have revealed no significant differences in the paclitaxel toxicity of MCF-7 cells cultured at a pH_e of 6.8 compared with 7.4 (12,13). The discrepancies in the results between distinct cancer cell lines indicated that the effects of acidic pH_e on paclitaxel resistance may be cell line-specific.

Low pH_e forms a physiological drug barrier, namely ion trapping, which has a negative effect on the efficacy of weakly basic chemotherapies, yet is more suited to weaker acidic therapeutics (9,12). Previous studies have demonstrated that ion trapping mainly contributes to acidic pH_e-mediated chemoresistance. Owing to the complex structure of paclitaxel, which is composed of acidic and basic domains, the drug is not ionizable under physiological conditions, and thus its efficacy should be unaffected by ion trapping (12,37). EMT is considered to be an essential feature of epithelial malignant tumor cells and is accompanied by increased vimentin and N-cadherin expression, and decreased E-cadherin expression (38-40). Previous studies have revealed that acidic pH_e induces EMT-like changes in human melanoma cells and Lewis lung carcinoma, which further promotes tumor progression (41,42). In the present study, the transformation of fibroblast-like morphology, epithelial marker E-cadherin downregulation, and upregulation of mesenchymal markers vimentin and N-cadherin, were observed in NPC 5-8F cells cultured at a pH_e of 6.8, suggesting that acidic pH_e may induce EMT-like changes in NPC cells.

EMT is thought to be involved in wound healing, stem cell behavior, development and the progression of cancer. Emerging evidence has revealed a strong link between resistance to chemotherapy and the induction of EMT in cancer (43). A number of studies have indicated that chemoresistant cancer cells undergo morphological and molecular changes similar to EMT. In colorectal cancer, oxaliplatin-resistant cells acquired the ability to migrate and invade with phenotypic changes resembling those of EMT (44). In pancreatic and ovarian cancer, stable cell lines resistant to gemcitabine and paclitaxel established by continuous exposure were able to undergo EMT with increased Snail and Twist expression (17,45). A previous study also revealed that EMT is necessary for acquired resistance to cisplatin and increases the metastatic potential of NPC cells (46). In addition, another *in vitro* and *in vivo* study demonstrated that paclitaxel-resistant NPC cells exhibited characteristic EMT phenotypes, and forkhead box C2 promoted chemoresistance in NPC via the induction of EMT (47). Taken together, this evidence, together with the results of the present study supports the conclusion that EMT is responsible for acidic pH_e-induced paclitaxel resistance in NPC cells.

Previous results indicated that acidic pH_e was able to promote tumor progression through the increase in the expression of specific genes, including those encoding vascular endothelial growth factor, interleukin-8 and matrix metalloproteinase-9 (48-51). MTDH, a novel oncogene, is prevalently expressed in numerous solid tumors, including SCCHN, and is involved in multiple cellular behaviors associated with malignant cells, including malignant cell transformation, proliferation, angiogenesis, invasion and metastasis (30,52-55). There is further evidence to indicate that MTDH modulates the sensitivity of cancer cells to chemotherapeutic agents (20,56). In the present study, the results demonstrated that MTDH expression was significantly enhanced in NPC cells cultured in an acidic medium. In a previous study, our group identified that MTDH promoted EMT in SCCHN (30). In the present study, the potential function of MTDH in acidic pH_e-mediated EMT was investigated further. The data demonstrated that knockdown of MTDH abrogated the acidic pH_e-induced suppression of E-cadherin and increased vimentin expression in NPC 5-8F cells; which indicated that MTDH is involved in acidic pH_e-mediated EMT-like changes in NPC cells. Furthermore, the cytotoxicity of paclitaxel in NPC cells recovered, whereas MTDH was knocked down, under acidic conditions. Taken together, these results supported the existence of an association between acidic pH_e-induced paclitaxel resistance and MTDH upregulation-mediated EMT in NPC cells.

In conclusion, the results of the present study indicated that MTDH-mediated EMT may be an alternative mechanism through which acidic pH_e promotes paclitaxel resistance in NPC. Thus, normalization of pH_e may be a reasonable strategy for tumor therapy. Furthermore, targeting MTDH may provide a novel strategy for overcoming chemoresistance in NPC therapy. However, further *in vivo* experiments are required to confirm whether MTDH is a viable target for therapy.

Acknowledgements

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