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Cyclooxygenase-2 inhibitor celecoxib suppresses invasion and migration of nasopharyngeal carcinoma cell lines through a decrease in matrix metalloproteinase-2 and -9 activity

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Celecoxib is a selective inhibitor of COX-2, whose connection with the development and progression of human tumors has been extensively studied. So far, however, its anti-metastatic effect is poorly understood in nasopharyngeal carcinoma. The current study aimed to observe the effect of celecoxib on invasion and migration of nasopharyngeal carcinoma cell lines and investigate the potential mechanism *in vitro*. Human nasopharyngeal carcinoma cell lines HNE1, HONE1, SUNE1-5-8F were exposed to different concentrations of celecoxib. MTT assay was used to study its anti-proliferation effect, transwell assay wound healing repair assay were performed to investigate the invasiveness and migration capability after treatment with celecoxib. The activity of MMP-2 and MMP-9 was measured by gelatin zymography. MTT assay showed that celecoxib inhibited HNE1, HONE1, and SUNE1-5-8F cells growth. Wound healing repair assay and transwell assay showed that cell metastatic ability was suppressed after treatment with celecoxib. Celecoxib had a significant inhibitory effect on the activity of MMP-2/9 in a dose-dependent manner in HNE1, HONE1 and SUNE1-5-8F cell lines. These data demonstrated that celecoxib-induced suppression of MMP-2 and MMP-9 activity might be involved in the inhibition of nasopharyngeal carcinoma cell lines invasion and migration.

1. Introduction

Nasopharyngeal carcinoma (NPC) is a disease with striking geographic and ethnic distribution. The incidence reaches 25 to 50 per 100.000 persons in southern area of China, parts of Southeast Asia and the Mediterranean basin, while the disease is generally rare in Europe and the United States (Yu et al. 2002). In recent years, radiotherapy has been considered as the main treatment for patients with NPC in early stage, and adjuvant chemotherapy could significantly raise survival rate, progression-free survival rate, and prolong survival time (Mei et al. 2011). Molecular targeted therapy also has presented definite clinical effects *in vitro* and *in vivo* (Lee et al. 2012; Zhou et al. 2011). Unfortunately, 30–40% of NPC patients develop distant metastases and die of neoplasms at distant sites. The most common regions for distant metastases include bone, lung, and liver. The majority of cases present with stage III or IV disease at the initial diagnosis (Lee et al. 1992). As distant metastases are regarded as the leading causes of poor prognosis and mortality in patients with NPC (Wang et al. 2013), investigations into new effective medicines and their role in tumor invasion and metastases development may help to improve the prognosis of patients.

Numerous study results have indicated that cyclooxygenase-2 (COX-2) is overexpressed in several human cancers and contribute to tumorigenesis (Eberhart et al. 1994; Tucker et al. 1999; Zimmermann et al. 1999; Goulet et al. 2003). Celecoxib, a specific COX-2 inhibitor, is currently being evaluated for its mechanism of inhibition of cell proliferation, induction of apoptosis, inhibition of angiogenesis and metastasis (Amir et al. 2005; Grosch et al. 2006). Likewise, nearly 43%∼76.6% of the NPC patients have high expression of COX-2, and COX-2 overexpression is correlated with aggressive tumor behavior and worse prognosis (Chen et al. 2005; Pan et al. 2013). Research showed that celecoxib could inhibit tumor growth, induce apoptosis and cell-cycle arrest in NPC cells (Liu et al. 2012). Several reports recently suggested that COX-2 expression played a key role in lymphatic metastasis in nasopharyngeal (Fendri et al. 2008), but the mechanism underlying the role of COX-2 in this process has remained unknown.

Matrix metalloproteinases (MMPs) are zinc-dependent proteolytic enzymes, which play a crucial role in promoting angiogenesis, degrading and invading the extracellular matrix (ECM) or basement membrane, and increasing tumor cell motility (Coussens et al. 2002). Overexpression of MMPs is positively correlated with malignant potential in lung, gastric and colorectal cancer (Leinonen et al. 2008; Yan et al. 2011; Langers et al. 2012; Zhang et al. 2012). Therefore they were considered to be promising therapeutic targets for drug development; especially MMP-2 and MMP-9 are capable of degrading basement membrane collagen (Overall et al. 2006). Studies have indicated that

COX-2 inhibitors could inhibit invasion and migration by regulating the expression and activity of MMPs in most cancers (Ishizaki et al. 2006; Park et al. 2012), but little information is available as to the relationships between COX-2 inhibitors and MMPs in nasopharyngeal cancer.

Therefore, the main objective of this study was to investigate the invasive and metastatic capabilities of NPC cell lines after incubation with celecoxib *in vitro*, and explore the potential effect that celecoxib influences this process by regulating the activity of MMPs.

2. Investigations and results

2.1. Effect of celecoxib on proliferation of NPC cells

To study the anti-proliferation effect of celecoxib, MTT assay was performed as described below. A time and dose dependent inhibition of proliferation was observed in HNE1, HONE1, SUNE1-5-8F cell lines after incubation with celecoxib (Fig. 1).

2.2. Effect of celecoxib on migration of NPC cells

Migrating cells in the same area were photographed 12 and 24 h after scratching and the migrated distances were measured. Results were shown as wound healing rate which represents the difference between wound width at 24 h and original scratch width relative to the original width at 0 h. Data indicated that migration rate was decreased by 50.6%, 42.3% and 39.4% respectively after incubation with celecoxib in HNE1, HONE1 and SUNE1-5-8F cells, compared with control group (Fig. 2).

2.3. Effect of celecoxib on invasion of NPC cells

HNE1, HONE1, SUNE1-5-8F cell lines were then studied for their invasiveness of traversing an artificial basement membrane *in vitro* in response to celecoxib (30 μM). Compared with the control group, the study group displayed a substantially decreased ability to invade the filters. The invaded cell number was decreased by 57.8%, 42.1% and 49.0% respectively in HNE1, HONE1 and SUNE1-5-8F cell lines after treatment with celecoxib ($P < 0.01$, $P < 0.01$ and $P < 0.01$, respectively) (Fig. 3).

2.4. Effect of celecoxib on MMP-2 and MMP-9 activity of NPC cells

Matrix metalloproteinases (MMPs) are known to be crucial for degrading extracellular matrix components and promoting tumor cellular invasion *in vitro* and *in vivo*. So, we determined whether the decreased effects of celecoxib on the invasion of NPC cells were due to decreased activity of MMP-2 or MMP-9. NPC cells were treated with various concentrations of celecoxib (0, 25, 50 or 75 μM for 24 h, and then the supernatant of cells was collected to detect the activity of MMP-2 and MMP-9 using gelatin zymography. Fig. 4 shows that celecoxib had inhibitory effects on the activity of MMP-2/9 in a dose-dependent manner in HNE1, HONE1 and SUNE1-5-8F cell lines.

3. Disscussion

Prevention of cancer metastasis must be a major goal of cancer therapy, since metastasis is the most insidious and life-threatening aspect of cancer (Mehlen et al. 2006). It is a multi-step process, where local cancer cells infiltrate the adjacent tissue, invade the vasculature and are transported through the circulatory system. Finally disseminated cancer cells successfully develop distant metastatic colonization (van Zijl et al. 2011). Matrix metalloproteinases (MMPs) play a critical role in the processes involving degradation of extracellular matrix and basement membrane, so they are important in invasion and migration of malignancies. Particularly, MMP-2 and MMP-9 could degrade type IV collagen so that they are both prognostic factors in solid tumors (Piao et al. 2012). Most MMPs are synthesized and secreted from the cells as inactive proenzymes, and are then activated by extracellular serine proteinase. MMP-2 is also secreted as an inactive proenzyme (pro-MMP-2), and activated by type I membrane-type MMPs (MT1-MMP or MMP-14). Active-MMP-2 leads to the degradation of basement membrane. Thus, activation of these proenzymes is one of the critical steps that result in extracellular matrix breakdown (Nagase 1997). Previous research indicated that inhibition of MMP enzymatic activity resulted in suppression of cancer cell migration and invasion *in vitro* and *in vivo* (Fujisawa et al. 2012).

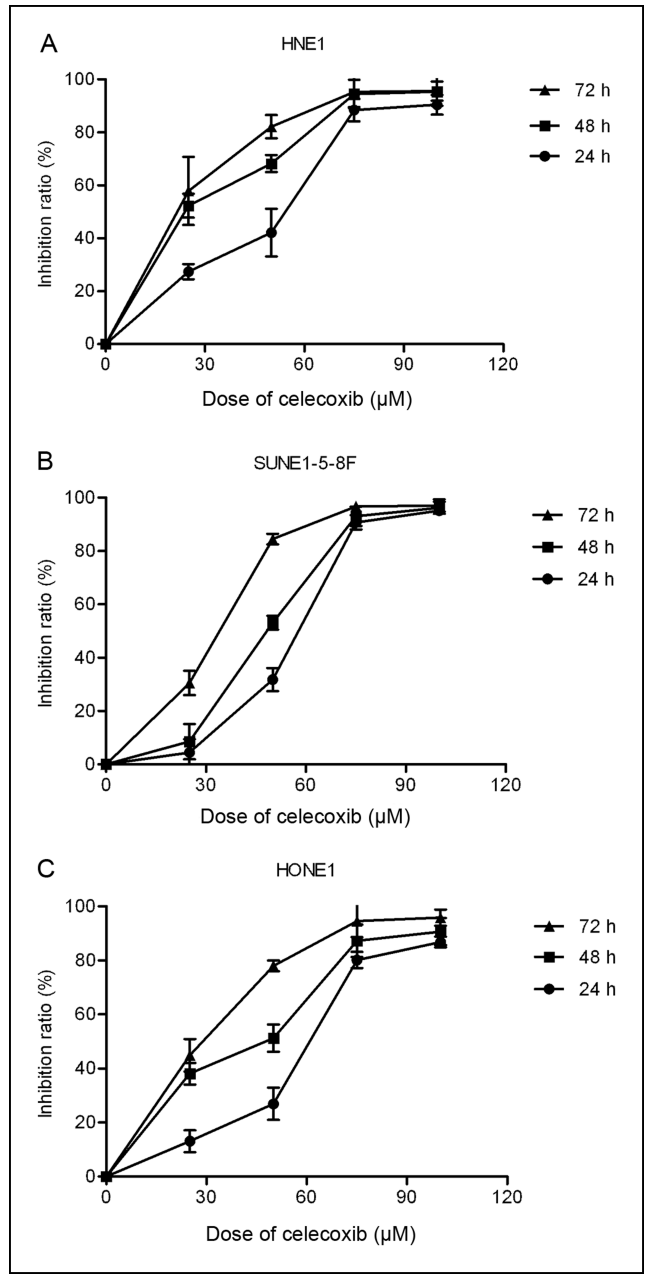

Fig. 1: Effect of celecoxib on proliferation of human nasopharyngeal carcinoma cells. After cultured with celecoxib at a concentration ranging from 25 to 100 μM for 24, 48 or 72 h, HNE1, HONE1 and SUNE1-5-8F cell lines were all inhibited growing in a time and dose dependent way. Values displayed are the mean ± SD (n = 3).

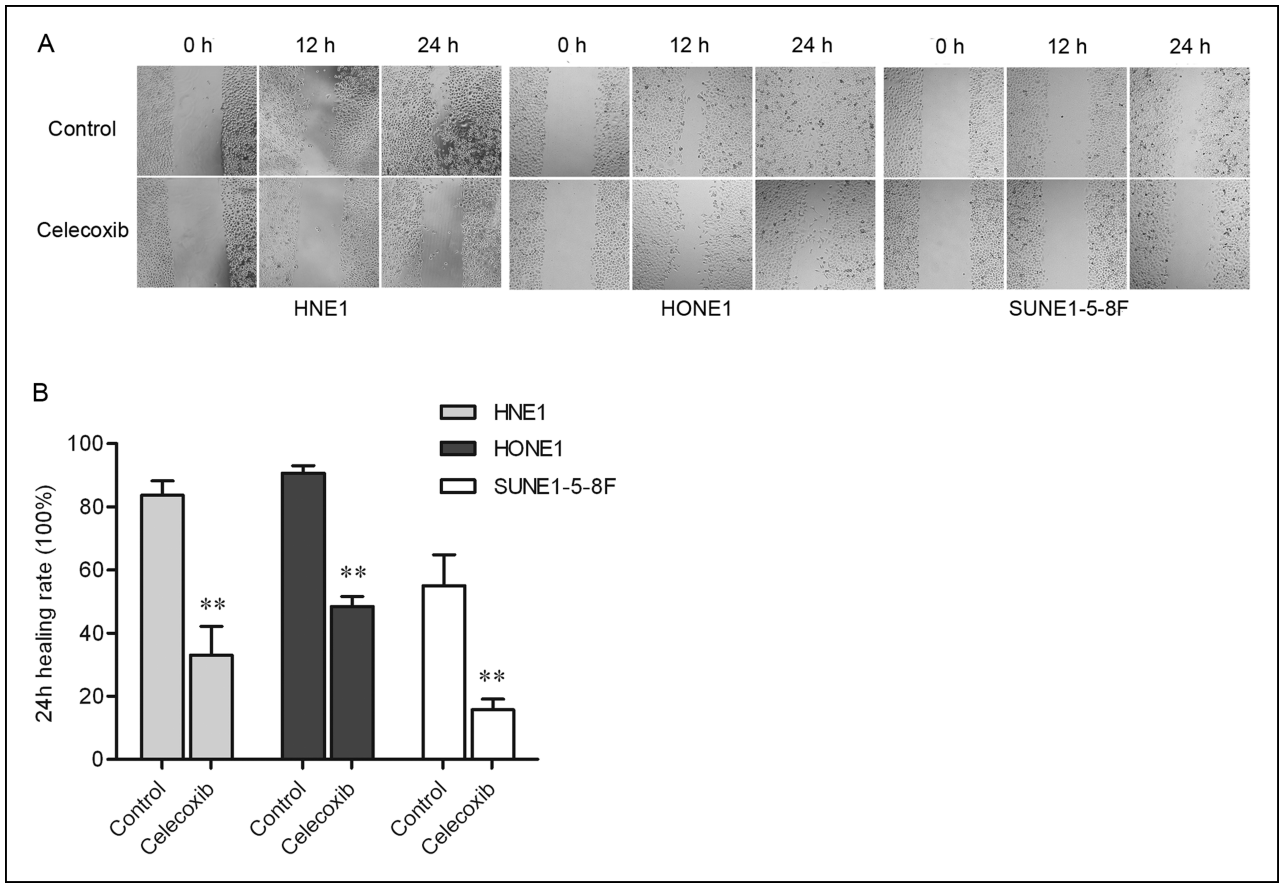

Fig. 2: Effect of celecoxib on migration of human nasopharyngeal carcinoma cells. Images of migrating cells were taken 12 and 24 h after scarification (A and B). Wound healing rate was detected. Data indicated that untreated cells migrated faster than cells incubated with celecoxib. Experiments were carried out for three times. ** $P < 0.01$ vs control (Student's t-tests).

There is a large number of studies analyzing the relationship between COX-2 expression and the outcome of tumor patients. In those studies, overexpression of COX-2 indicated stronger metastasis possibility and worse prognosis in various cancers (Khor et al. 2007; Zerkowski et al. 2007; Lambropoulou et al. 2010). Liver-transplanted tumor growth is inhibited by RNA interference-mediated COX-2 inhibition in nude mice (Liu et al. 2012). However the connection between COX-2 and nasopharyngeal carcinoma is still controversial. Pan et al. (2013) have demonstrated that a high level of COX-2 indicates worse long-term outcomes in non-metastatic NPC. Kim et al. (2004) also suggested that overexpression of COX-2 is associated with treatment failure.

Consequently, the selective COX-2 inhibitor celecoxib is thought be a potential chemoprevention agent for various kinds of cancer (Harris 2009). In spite of playing an important role in the treatment of pain, inflammation and fever, celecoxib could inhibit cancer cell growth, angiogenesis, apoptosis, and metastasis in a rectal xenograft model (Ninomiya et al. 2012). Celecoxib could inhibit the enzymatic activity of MMP-2 and MMP-9, as well as synthesis of the MMP-2 precursor protein. Sansone et al. (2009) found that, after knocking down the COX-2 gene by RNAi in colon cancer cells, the production of active MMP-2 was decreased obviously acccompanied by a decrease in cell invasion ability, which indicated that COX-2 gene expression was closely related to MMP-2 production and invasion abil-

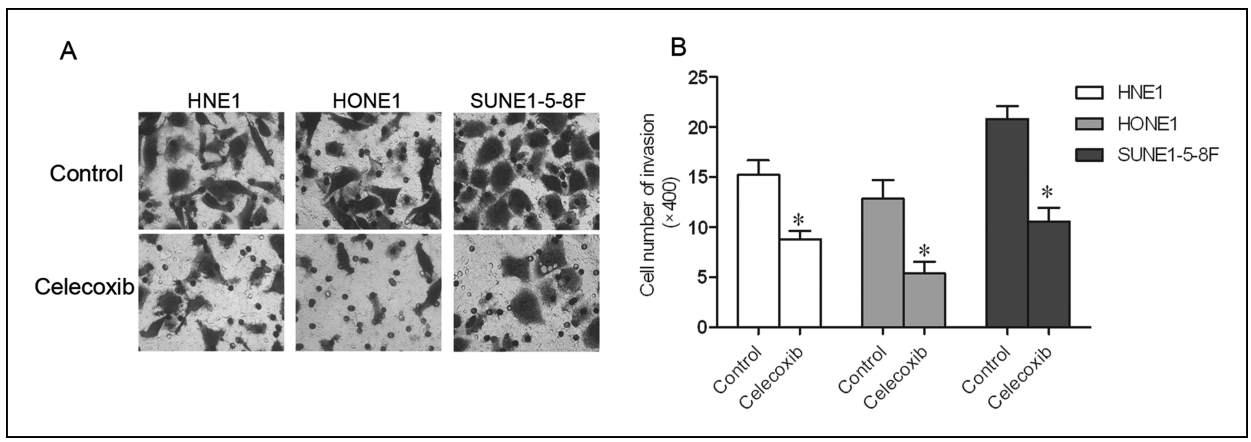

Fig. 3: Effect of celecoxib on invasion of human nasopharyngeal carcinoma cells. HNE1, HONE1, SUNE1-5-8F cell lines were treated with celecoxib (30 μM) for 24 h, transwell assay was carried out to measure their ability to traverse an artificial basement membrane in *vitro*. Finally, the number of cells was counted from 5 random fields (magnification, 400 ×). Compared with control group, research group displayed markedly decreased ability to invade the filters in HNE1, HONE1 and SUNE1-5-8F cells. Data was expressed as cell number of the mean ± SD. * $P < 0.01$ vs control (Student's t-tests).

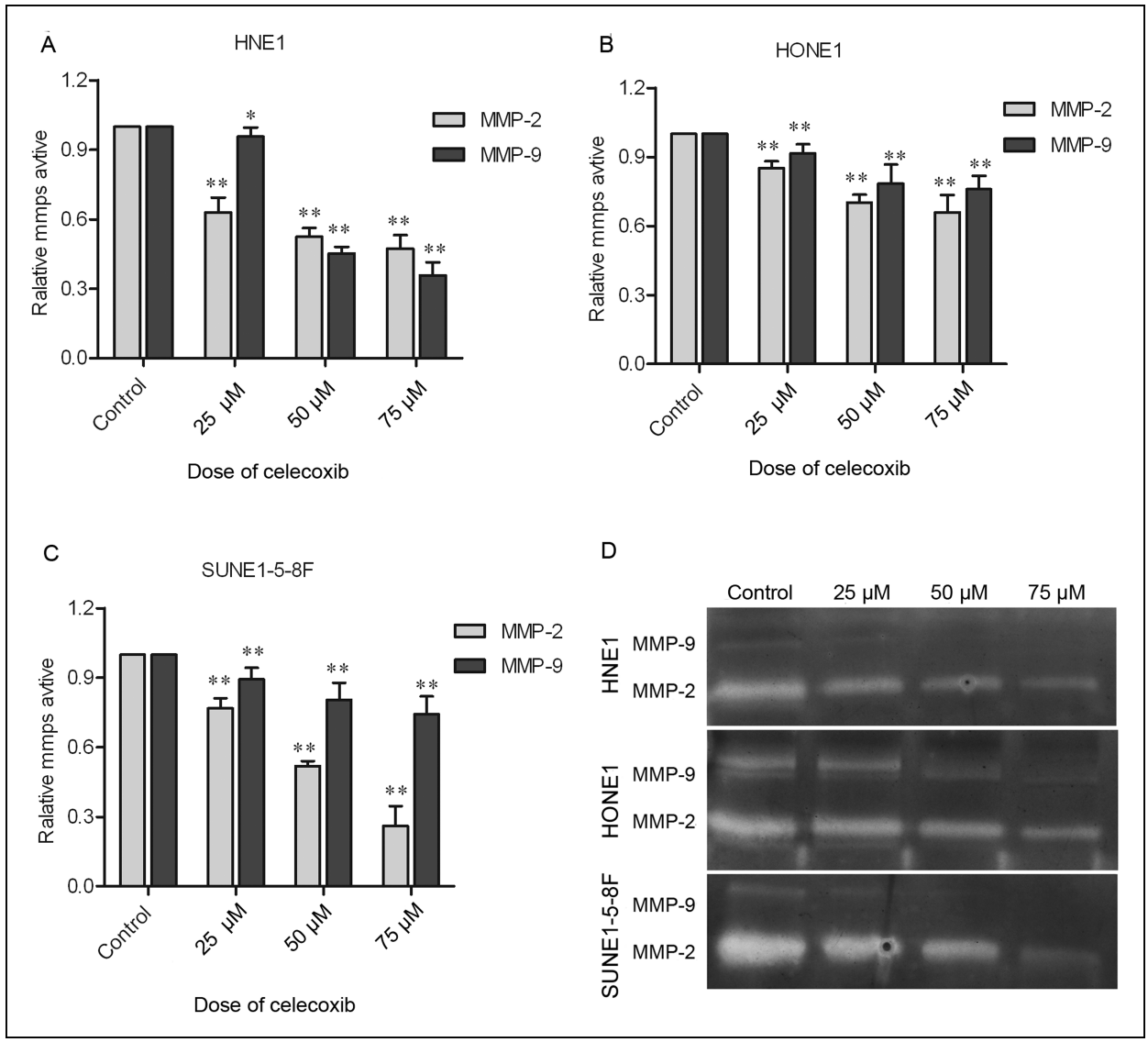

Fig. 4: Effect of celecoxib on MMP-2 and MMP-9 activity of human nasopharyngeal carcinoma cells. Gelatin zymography assays were used to determine the activities of MMP-2 or MMP-9 secreted by NPC cell lines after cultured with celecoxib (0, 25, 50, 75 μM) for 24 h. Datas showed celecoxib had significantly inhibitory effect on the activities of MMP-2/9 in a dose-dependent manner in HNE1, HONE1 and SUNE1-5-8F cell lines. $*P < 0.05$, $**P < 0.01$ vs control (Student's t-tests).

ity of cancer cells. In our research, celecoxib inhibited both migration and invasion in HNE1, HONE1 and SUNE1-5-8F cell lines. Through gelatin zymography assay, we observed that the activities of MMP-2 and MMP-9 were both suppressed in a dose-dependent manner after HNE1, HONE1 and SUNE1-5-8F cell lines were cultured with celecoxib for 48 h, which could partly explain the inhibition of tumor cell migration and invasion by inhibiting the activity of MMP-2/9. Further experiments are needed to explore the potential mechanism behind this process. Previous studies also suggested that COX-2 inhibitor was able to suppress cancer invasion and metastasis by inhibiting MMP-2/9 expression. An optical molecular imaging approach was used to determine the effect of celecoxib on MMP activity. The results indicated that control groups had bigger xenograft tumor volumes and higher MMP activity than treated groups (Sheth et al. 2012). Kurihara et al. (2009) found that selective COX-2 inhibitors act against invasion of oral squamous cell carcinoma cell lines. Such an effect may result from inhibiting MMP-2 protein expression and down-regulating MMP-2 activity through suppression of the expression of tissue inhibitors of metalloproteinases (TIMP-2), which plays an essential role in regulating pro-MMP-2 activation (Jiang et al. 2002). Clinical research has suggested that the positive rates of TIMP-2 are significantly higher in nasopharyngeal carcinoma than in inflammation tissues or normal controls and is closely related to lymph node metastasis (El Badry et al. 2007; Yao et al. 2010). It is therefore inferred that the inhibition of invasion and metastasis of NPC cell lines may be due to the down-regulation of TIMP-2 so that activation of pro-MMP-2 is suppressed. In order to elucidate the underlying molecular mechanisms of this process, further research is needed to investigate whether TIMP-2 is down-regulated after treatment with celecoxib in NPC cell lines.

From the above discussion, can be concluded that celecoxib inhibited invasion and migration in NPC cells *in vitro*, and this is partially associated with the suppression of MMP-2 and MMP-9 acitcity. Thus, celecoxib seems to be a promising anti-metastatic agent in the treatment of nasopharyngeal carcinoma and further studies on the expression of metastasis-related proteins are needed to fully elucidate its mechanisms.

4. Experimental

4.1. Reagents

Lyophilized celecoxib (International Laboratory, South San Francisco, CA, USA) was dissolved in DMSO (Sigma–Aldrich, St. Louis, MO, USA) and

stored at -20 °C. Drug was diluted into culture medium to the appropriate concentrations before each experiment and the final concentrations of DMSO were kept below 0.1%. ECM gel (E1270) was bought from Sigma Chemical Co. (St. Louis, MO, USA).

4.2. Cell lines culture

Because of the different characteristics of invasiveness and metastasis, three human nasopharyngeal cancer cell lines (HNE1, HONE1 and SUNE1-5-8F) which were purchased from Xiang Ya Central Experiment Laboratory, Central South University (Changsha, China) were chosen. HNE1 and HONE1 were both derived from a poorly differentiated NPC and Epstein-Barr virus (EBV) negative. SUNE1-5-8F keeps high tumorigenic and high metastasis characterization, carry EBV continuously and express latent membrane protein (LMP1) (Song et al. 2002). These cell lines were all cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Grand Island, USA) supplemented with 10% newborn calf serum (Gibco, Grand Island, USA). Cells were grown at 37 °C in 5% CO_2 atmosphere. Control groups were cultured with DMSO at the same concentration with research groups.

4.3. Cell growth assay (MTT)

Cells were seeded into 96-well plates at 5×10^3, 4×10^3, 3×10^3 cells/well and incubated overnight to allow attachment. Various concentrations of celecoxib were subsequently added. After 24 h, 48 h or 72 h, 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added into each well. Supernatant was then removed after 4 h incubation at 37 °C. Then, the crystal products were solubilized in 100 µL DMSO, and colorimetric analysis was performed (wavelength 570 nm). Each experiment was performed three times.

4.4. In vitro wound healing repair assay

For cell migration assay, the cells were seeded in 24-well plates and grew to confluence overnight. The monolayers were straight scratched using the fine end of a 10 µL pipette tip (time 0). Cellular debris was removed by washing with PBS, and then the cells were incubated in serum-free medium in the absence and presence of celecoxib (30 µM). The wound regions were photographed at 0, 12 and 24 h to measure the migrated distances by a phase contrast microscope (TE2000, NIKON). Migration distances were measured by ImageJ analysis software (National Institutes of Health, USA). The experiments were repeated three times.

4.5. Transwell migration assays

Transwell chambers (Corning, NY, USA) with 8-µm diameter filters were used in this study. In brief, the ECM gel (E1270, Sigma Chemical Co., St. Louis, MO, USA) was diluted to the desired final concentration (10 mg/mL) with cold distilled water in order to form a matrix barrier. Then 25 µg ECM gel was applied to each polyvinylpyrrolidone-free polycarbonate filter and dried under a hood as basement membrane. After that each filter with basement membrane was reconstituted with serum-free medium. Thereafter $2x10^5$ cells were placed in the upper compartment of the transwell chamber, suspended in 0.1% serum medium with celecoxib (30 µM). Serum medium (30%) was put into the lower compartment of the transwell chamber as a chemoattractant. Assays were carried out in 5% CO_2 at 37 °C. After 24 h of incubation, the cells on the upper surface of the filter were removed by wiping with a cotton swab. The filters were fixed with cold methanol and then stained with 2% ethanol containing 0.2% crystal violet powder. Twenty four hours later, cells from five random views of the lower surface were counted under a microscope (TE2000, NIKON) at x400 magnification. Each assay was performed in triplicate and repeated 3 times.

4.6. Gelatin zymography assay

Cells were seeded in culture flasks and allowed to grow to 80% confluency. The cells were then cultured in serum-free medium in the absence or presence of varying concentrations of celecoxib (25, 50, or 75 µM) for 24 h. Afterwards, the supernatants were collected, cleared by centrifugation, and mixed with 4 × SDS sample buffer, followed by electrophoresis in polyacrylamide gel containing 0.1% (w/v) gelatin (Sigma). Following electrophoresis, the gels were incubated in renaturing buffer (2.5% Triton X-100) with gentle agitation to remove SDS, followed by incubation in developing buffer (50 mM Tris-HCl buffer, pH 7.4, and 10 mM $CaCl_2$) overnight at 37 °C to allow digestion of the gelatin. Gels were then stained with 0.1% Coomassie Brilliant Blue R-250 (Invitrogen Corporation, Carlsbad, CA, USA) until clear bands suggestive of gelatin digestion appeared.

4.7. Statistical analyses

The data were presented as mean ± standard deviations (SD). Statistical comparison was performed using Student's t test. $P < 0.05$ was considered statistically significant.

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