Zoledronic acid suppresses metastasis of esophageal squamous cell carcinoma cells through upregulating the tight junction protein occludin

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Abstract We have previously demonstrated the radio-sensitizing effect of zoledronic acid (ZOL), a third generation bisphosphonate, on human esophageal squamous cell carcinoma (ESCC) cells. Here we show that ZOL suppresses metastatic progression of ESCC cells mainly through up-regulating the tight junction protein occludin. Exposure to ZOL at lower concentrations dramatically reduced migration and invasion of ESCC cells. In addition, ZOL treatment

decreased the expression of mesenchymal markers, vimentin and N-cadherin, while increased the expression of the tight junction protein occludin. Moreover, ectopic expression of Slug, a well-known transcriptional repressor of occludin, partially but significantly abrogated the effect of ZOL on occludin expression and subsequently rescued the malignant metastatic phenotype, suggesting that Slug is one of the mediators underlying the anti-metastatic effect of ZOL. The present study is the first to report the significance of ZOL on ESCC metastasis. These data are promising for the future application of this drug regimen in patients with ESCC.

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Introduction

Esophageal squamous cell carcinoma (ESCC), the major histologic subtype of esophageal cancer, is one of the most common malignancies and ranked as the sixth leading cause of cancer death worldwide (Pisani et al. 1999; Parkin et al. 2001; Kamangar et al. 2006). For the majority of patients the diagnosis of ESCC occurs during the advanced stage of the refractory disease. Although surgical techniques and perioperative management have progressed, ESCC is associated with a poor

prognosis, with a 5-year overall survival (OS) rate of only 10–20 %, largely owing to its rapid growth and metastasis (Parkin et al. 2001). Thus, prevention and repression of cancer metastasis become urgent in clinical ESCC therapy and more effective agents are being explored.

Zoledronic acid (ZOL), the third generation of bisphosphonates, is the most potent member in the bisphosphonate family as a standard treatment for preventing skeletal complications associated with bone metastases (Jantunen 2002). Its core bisphosphonate moiety attaches to bone and its imidazole ring containing two nitrogen atoms confers its potency. Recent evidences also suggest that ZOL has direct or indirect anti-tumor effects (Zhao et al. 2012; Matsumoto et al. 2005; Ge et al. 2014). The mechanism consists of inducing cell apoptosis, impairing cell migration and invasion, modulating immune response mediated by $\gamma\delta$ T cells and anti-angiogenesis effect (Zhao et al. 2012; Matsumoto et al. 2005; Ge et al. 2014; Hiroshima et al. 2014; Yuasa et al. 2007). To date, the clinical benefits of ZOL have been extended to patients with bone metastases secondary to a broad range of solid tumors (Hiroshima et al. 2014; Yuasa et al. 2007).

These previous findings suggest that chemotherapy with ZOL might be effective for the treatment or prevention of ESCC. Our previous report revealed the cytotoxic effect of ZOL on ESCC cells, as well as its potential as a radio-sensitizing reagent (You et al. 2014). Of note, combined treatment with ZOL plus irradiation produced synergistic suppressive effects on VEGF expression. These observations encourage us to further explore the anti-metastatic function of ZOL on ESCC cells. Interestingly, ZOL has been shown to reverse the epithelial–mesenchymal transition (EMT), a key process essential to the metastatic cascade (Schech et al. 2013). Exposure of breast cancer cells to ZOL decreased the expression of mesenchymal markers, including N-cadherin, Twist, and Snail, and subsequently up-regulated the expression of adheren junction protein E-cadherin. Therefore, the aim of the present study was to investigate the anti-metastatic effect of ZOL on ESCC cancer cells and subsequently the underlying mechanism. Our results show a novel significance of ZOL for therapeutic interventions in ESCC metastasis.

Materials and methods

Reagents and antibodies

ZOL was kindly provided by Novartis Pharma AG (Basel, Switzerland). Stock solution of ZOL was prepared at concentration 4 mM in phosphate-buffered saline (PBS) and aliquots were stored at -20°C . All dose formulations were prepared immediately before use. The antibodies included the followings: rabbit anti-vimentin, rabbit anti-N-cadherin, rabbit anti-GAPDH (Abcam, Cambridge, MA, USA), rabbit anti-E-cadherin, rabbit anti-occludin, goat anti-Slug and goat anti-Snail (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell culture and transfection

The two ESCC cancer cell lines, denoted EC109 and EC9706, were used in this study and routinely cultured as described previously (You et al. 2014). Both cell lines were kindly provided by Dr. Yonggang Ran (Medical NCO Academy of PLA, Shijiazhuang, China). A Slug expression vector, pCMV-myc-Slug, was generated by subcloning the PCR-amplified human slug coding sequence into pCMVMyc and confirmed by sequencing.

Cell viability assay

We assessed the growth rate of tumor cells under exposure of ZOL using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay, as described previously (You et al. 2014). Briefly, 1 day after plating in 96-well plates (Costar, Cambridge, MA, USA) at a density of 4×10^3 cells per well in triplicate, cells were exposed to ZOL at increasing concentrations (2, 4, 8, 16, 32 and 64 μM). MTT assays were performed after 48 h or 72 h incubation.

Cell motility assay

Cells were seeded at 1×10^5 cells per well in six-well tissue culture plates. After 12 h, wounds were incised by scratching the cell monolayers using 200 μl pipette tips, and then different concentrations of ZOL (2, 4 and 8 μM) were added to the DMEM medium

(Invitrogen). Cells were cultured routinely at 37 °C. Photographs were taken under phase-contrast microscopy (Carl Zeiss, Thornwood, NY, USA) immediately and 24 h after incision. The area of migrating cells was estimated by counting the number of pixels after the photographs had been converted to Photoshop data (Adobe, San Jose, CA, USA).

Cell invasion assay

Cell invasion assay using a Matrigel-coated chamber (Millipore, Billerica, MA, USA) has been previously described (You et al. 2013, 2014). Briefly, after 24 h treatment with ZOL at various concentrations (2, 4 and 8 μ M), 5×10^4 cells in 500 μ l of serum-free DMEM were seeded in the top chamber of the 24-well-size Matrigel-coated Transwell insert (Millipore) following the protocol indicated by the manufacturer. The bottom chamber was filled with 750 μ l DMEM medium containing 10 % fetal bovine serum (Hyclone, Logan UT, USA). Migrated cells were stained with crystal violet reagent (Sigma-Aldrich, St. Louis, MO, USA) and counted using an inverted microscope at 100 $\times$ magnification. Each experiment was performed in triplicate wells and repeated three times.

Western blot analysis

Western blot analysis was performed as described previously (You et al. 2013, 2014). In brief, approximately 50 μ g of total proteins from each sample was separated on 12 % polyacrylamide gels, transferred onto a PVDF membrane (Millipore, Billerica, MA, USA) and incubated with indicated antibodies. Immunoblots were then probed with the secondary antibodies conjugated to horseradish peroxidase (Santa Cruz) and detected using an enhanced ECL kit (Amersham, Arlington Heights, IL, USA). The blots were reprobed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (Abcam) to confirm equal loading of the different samples.

Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA (2 μ g) extracted from each sample was used for synthesis of the complementary DNAs with an M-MLV cDNA Synthesis Kit (Invitrogen) by using random hexamers. Semi-quantitative RT-PCR analysis

was performed as described previously (You et al. 2013, 2014). 2 μ l of prepared cDNA were used for PCR amplification in 50- μ l-reaction volume. The resulting products were separated on 2 % agarose gels and stained with ethidium bromide. GAPDH served as an internal control in all reactions. The gene-specific primers for different PCR products are listed in Table 1.

Fluorescence microscopy

Cells mounted on glass slides were permeabilized with PBS containing 0.1 % Triton X-100 and 0.1 M glycine for 15 min, blocked with 10 % BSA in PBS for 30 min at room temperature and then incubated with anti-occludin or anti-E-cadherin antibody for 1 h, followed by a FITC-labeled secondary antibody against rabbit IgG (Abcam) for 30 min at room temperature. The cells were then washed twice with PBS. Images were taken under a fluorescence microscope (Carl Zeiss).

Statistical analysis

Data were expressed as mean $\pm$ SD and analyzed using Student's *t* test, of at least three repeated experiments. Statistical analysis was carried out using the SPSS 16.0 software package and *P* < 0.05 was considered significant. All analyses were performed in triplicate.

Results

ZOL inhibits the proliferation of ESCC cells

Using an MTT assay, we first investigated whether ZOL affected the proliferation of ESCC cells in vitro. Cells were treated with increasing concentrations of ZOL for either 48 or 72 h. As shown in Fig. 1, ZOL induces dose-dependent reduction of viability for both ESCC cell lines. Of note is that EC109 cells were more sensitive to a range of concentrations of ZOL, whereas EC9706 cells were relatively unaffected.

ZOL suppresses migration of ESCC cells

The wound-healing assay was performed on ESCC cells after exposure to ZOL. The concentration of 2, 4 and 8 μ M for drug treatment was chosen, because

Table 1 PCR primer sequences

Primers	Sequences	Product size (bp)
Vimentin-forward	5'-AATGGCTCGTCA CCTTCGTGAAT-3'	160
Vimentin-reverse	5'-CAGATTAGTTTCCCTCAGGTTTCAG-3'	
N-cadherin-forward	5'-CGCCAATCAACTTGCCAGAA-3'	110
N-cadherin-reverse	5'-TGGCCCAGTGACGCTGTATC-3'	
E-cadherin-forward	5'-TTAAGGGGTCTGTCATGGAAGGT-3'	106
E-cadherin-reverse	5'-GTGTAAGCGATGGCGGCATTGTA-3'	
Occludin-forward	5'-AAGGGAAGAGCAGGAAGGTC-3'	397
Occludin-reverse	5'-CTCCAACCATCTTCTTGATGTG-3'	
Snail-forward	5'-TTGGATACAGCTGCTTTGAG-3'	150
Snail-reverse	5'-ATTGCATAGTTAGTCACACCTC-3'	
Slug-forward	5'-GAGTCTGTAATAGGATTTCCCATAG-3'	122
Slug-reverse	5'-CTTTAGTTCAACAATGGCAAC-3'	
GAPDH-forward	5'-GACCCCTTCATTGACCTCAAC-3'	219
GAPDH-reverse	5'-CTTCT CCATGGTGGTGAAGAC-3'

Fig. 1 ZOL treatment inhibits cell proliferation of both EC109 (*left*) and EC9706 (*right*) cells in a dose-dependent manner. Cells were either untreated [vehicle control (PBS)] or treated with ZOL (dose range from 2 to 64 μM) for 48 and 72 h. Cell

viability was assessed by MTT assay. The results were representative of three independent experiments. * $P < 0.05$; ** $P < 0.01$, as compared to vehicle control group

these low doses of ZOL potentially allowed evidencing an anti-metastatic effect without exposing cells to excessive toxicity. The result showed that ZOL significantly decelerated the wound closure, as compared with the control (Fig. 2). Moreover, this inhibitory effect was presented in a dose-dependent manner. This finding indicates that treatment with ZOL would repress migratory behavior of ESCC cells.

ZOL suppresses invasiveness of ESCC cells

Next, an invasion assay was conducted to further confirm the inhibitory effect of ZOL on metastatic progression of ESCC cells. As expected, addition of

ZOL significantly diminished number of tumor cells that invaded crossing the Matrigel-coated membrane of chambers in a dose-dependent manner (Fig. 3).

Occludin, but not E-cadherin, is activated after ZOL treatment

Tumor metastasis is a complex process consisting of multiple individual steps. A key process in this metastatic cascade that converts an adherent epithelial cell into a migratory cell, which can invade through the extracellular matrix, is known as EMT (Thiery 2002). To better understand the molecular mechanism of ZOL on cancer metastasis, we examined the expression profiles of several EMT-related markers.

Fig. 2 ZOL decreases mobility of both EC109 and EC9706 cells. Cells were grown until 100 % confluent in a six-well plate. A straight line was scraped and different concentrations (2, 4 and 8 μM) of ZOL were added to the media. The percentage of wound healing after 24 h was calculated relative to the total

wound area at 0 h of the same wound spot. *Left* representative field ($\times 100$) of wound healing for EC109 cells treated with ZOL at 8 μM. *Right* quantitative analysis from three experiments. ** $P < 0.01$, as compared to control group

Fig. 3 Effect of ZOL on invasiveness of EC109 and EC9706 cells. Cells were pretreated with or without ZOL (2, 4 and 8 μM) for 24 h and then subjected to cell invasion assay. *Left* representative field ($\times 100$) of invaded cells for EC109 cells

treated with ZOL at 8 μM. *Right* quantitative analysis from three experiments. Data are expressed as mean $\pm$ SD. * $P < 0.05$; ** $P < 0.01$, as compared to control group

Figure 4a illustrates representative western blot and semi-quantitative RT-PCR results. Treatment with ZOL for 24 h resulted in significant down-regulation of representative mesenchymal markers, such as vimentin and N-cadherin, with concomitant up-regulation of tight junction protein occludin, at protein and mRNA levels. However, the expression level of E-cadherin appeared to be little affected, which is contradictory with published data in breast cancer (Schech et al. 2013). Immunofluorescence analysis further confirmed that occludin protein levels, but not that of E-cadherin, was dramatically increased at cell–cell junctions concurrently with ZOL treatment (Fig. 4b).

Since occludin mRNA levels as measured by semi-quantitative RT-PCR was also elevated, we hypothesized that ZOL induces transcriptional up-regulation of occludin. Sequence analysis of occludin promoter fragment, using MatInspector (Gnomatix), revealed putative-binding E-BOX sites for the zinc-finger transcription factors Snail and Slug. Therefore, we investigated the expression levels of Snail and Slug to identify whether Snail or Slug expression was affected by ZOL. In contrast to the untreated control, ZOL significantly down-regulated the expression of Slug, but not Snail, at both the protein and mRNA levels (Fig. 4a). Thus, these data suggest that ZOL may repress transcription factor Slug to restore occludin expression.

Fig. 4 ZOL treatment induces up-regulated expression of occludin, but not E-cadherin. **a** EC109 cells were incubated with ZOL (8 μ M) for 24 h. Total cell lysates and RNA were isolated and subjected to western blot and semi-quantitative RT-

PCR analyses, respectively, with the indicated antibodies or gene-specific primers. GAPDH was used as a loading control. **b** Immunofluorescence staining of occludin and E-cadherin in EC109 cells after exposure to ZOL (8 μ M) for 24 h

Twist overexpression rescues metastatic phenotype inhibited by ZOL

Previous report has shown that Slug represses the transcription of occludin through direct binding to its promoter (Wang et al. 2007). Having observed that treatment with ZOL stimulated expression of occludin with a concomitant decrease of Slug, we sought to address whether ectopically expressed Slug could abolish the effects of ZOL on occludin expression and cancer metastatic phenotype. EC109 cells were transfected with pCMV vector or pCMV-myc-Slug and treated with ZOL, and then examined for migration and invasion. Overexpression of Slug significantly attenuated the induction of occludin expression by ZOL, at protein and mRNA levels (Fig. 5a). Exposure to ZOL dramatically suppressed both migration and invasion abilities of EC109 cells, which were partially but significantly rescued by ectopic expression of Slug (Fig. 5b, c). Together these results suggest that Slug is required for ZOL-induced occludin expression and ZOL exerts its anti-metastatic function, at least in part, through Slug.

Discussion

Although a number of studies have shown that ZOL reduces migration and invasion of multiple types of cancer cells, this effect of ZOL on ESCC remains to be elucidated. In the present study, we discovered, for the

first time, that ZOL suppressed migration and invasion of ESCC cells and this anti-metastasis effect of ZOL strongly correlated with upregulation of the tight junction protein occludin. Our results suggest the clinical potential of ZOL as an anti-metastasis agent for ESCC therapy.

Uncontrolled cell proliferation and angiogenesis are key characteristics of initiation and early growth of epithelial origin cancers. However, it is the subsequent acquisition of motility and invasiveness that leads to the metastatic growth (Thiery 2002). Tumor metastasis is a complex process consisting of multiple individual steps. Among them, a key process in this metastatic cascade that converts an adherent epithelial cell into a migratory cell, which can invade through the extracellular matrix, is known as EMT (Thiery 2002). Multiple studies have shown that tumor cells of epithelial origin can acquire mesenchymal phenotype and that these cells are typically seen at the invasive front of primary tumors. The role of EMT in tumor metastasis is further highlighted by acquisition of mesenchymal markers, such as vimentin or N-cadherin, concomitantly with loss of cell–cell interaction (Thiery 2002; Uchikado et al. 2005; Natsugoe et al. 2007; Hajra et al. 2002).

Epithelial cell–cell interaction is mediated through a series of intercellular junctions referred to as adherens junctions, tight junctions and desmosomes. Formation of cell–cell adhesions is mainly dependent on the E-cadherin system and functional loss of E-cadherin in epithelial cell has been considered a

Fig. 5 Slug mediates the inhibitory effects of ZOL on cell migration and invasion. EC109 cells were transfected with pCMV vector or pCMV-myc-Slug and treated with 8 μ M of ZOL. Subsequent analyses were performed 24 h post-treatment. **a** Protein and total RNA were isolated from the cells. Equal amounts of protein lysates were separated on SDS-PAGE and blotted with the indicated antibodies (*top three panels*). RNA

was subjected to semi-quantitative RT-PCR analysis for the expression of occludin. **b**, **c** Cells were subjected to wound healing assay (**b**) or invasion assay (**c**) as described under “Materials and methods”. The experiments were repeated three times in triplicate. Data are expressed as mean $\pm$ SD. * $P < 0.05$; ** $P < 0.01$, as compared to control group

hallmark of EMT (Uchikado et al. 2005; Natsugoe et al. 2007; Hajra et al. 2002). During tumor progression, E-cadherin can be functionally inactivated or silenced by a number of different mechanisms including somatic mutations (Berx et al. 1995), downregulation of gene expression through promoter hypermethylation (Ran et al. 2011), histone deacetylation (Peinado et al. 2004), or transcriptional repression by several transcription factors including the zinc-finger factors Snail and Slug, basic helix–loop–helix (bHLH) factors Twist and the two-handed zinc-finger factors of δ EF1 family proteins (δ EF1/ZEB1 and SIP1) (Uchikado et al. 2005; Natsugoe et al. 2007; Hajra et al. 2002; Shirakihara et al. 2007).

Tight junctions are the most apical cell–cell contacts, forming the primary barrier to solute diffusion through the paracellular pathway and providing the boundary between the apical and basolateral plasma membrane domains characteristic of polarized epithelia (Furuse et al. 1994). The transmembrane tight junction proteins are involved in a number of cellular functions, including paracellular barrier formation, cell polarization, differentiation and proliferation (González-Mariscal et al. 2003). Major families of tight junction proteins include occludin and claudins (Tobioka et al. 2004). The protein occludin was first identified and characterized as an integral membrane protein localized at tight junction strands (Tobioka et al. 2004). Altered expression of tight junction proteins was reported in various epithelial tumors. For example, down-regulation of

occludin expression has been shown to correlate with invasiveness and metastatic potential of human endometrial carcinoma and pancreatic cancer (Tobioka et al. 2004; Tan et al. 2004). A recent study reported that occludin is also involved in adhesion, apoptosis, differentiation and Ca^{2+} -homeostasis of human keratinocytes (Rachow et al. 2013).

To our knowledge, the role of ZOL on EMT has so far only reported in breast cancer (Schech et al. 2013). Exposure of triple-negative breast cancer cells of ZOL decreased the expression of N-cadherin, Twist, and Snail, and subsequently upregulated expression of E-cadherin, which was further linked to inhibition of self-renewal phenotype. In our study, we found that ZOL treatment, even at relative lower concentrations, could significantly attenuated migration and invasion of ESCC cells. This alteration was characterized by decreased expression of vimentin and N-cadherin with a concomitant increase of occludin, but not E-cadherin. We also detected a decreased level of Slug and demonstrated its role in regulation of occludin. Overexpression of Slug abolished the induced occludin expression by ZOL and significantly rescued malignant metastatic phenotypes, suggesting that Slug is one of regulatory targets in ESCC cells responding to ZOL treatment. The mechanism underlying may depend on the function of ZOL to suppress the established EMT regulator NF- κ B activation, which results in directly down-regulated levels of Slug (Schech et al. 2013; Zhang et al. 2006).

Altogether, our present study elucidates a mechanism by which ZOL increased expression of occludin and thereby inhibits migratory and invasive behaviors of ESCC cells. These findings might be relevant for expanding application of ZOL in cancer treatment for patients with metastatic ESCC, although further in vivo and clinical studies are necessary to confirm these results in the future.

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Conflict of interest The authors declare no conflicts of interest.

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