Carboxypeptidase E-ΔN promotes migration, invasion and epithelial–mesenchymal transition of human osteosarcoma cell lines through the Wnt/β-catenin pathway

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Title page

Carboxypeptidase E -ΔN promotes migration, invasion and epithelial-mesenchymal transition of human osteosarcoma cells via the Wnt/β-catenin pathway

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Abstract

Osteosarcoma (OS) is the most common malignant bone tumor in children and adolescents, and metastatic OS is the major cause of OS-related death. Carboxypeptidase E (CPE) is demonstrated to be highly expressed in some cancer types, and its N-terminal truncated form, CPE-ΔN, is implicated in tumor metastasis and poor prognosis. In this study, we investigated the effect of CPE-ΔN on cell migration, invasion and epithelial-mesenchymal transition (EMT) of OS cells, and illustrated the molecular mechanisms. We first constructed CPE-ΔN overexpressing human OS cell lines (143B and U2OS cells), and found that ectopic CPE-ΔN expression in OS cells enhanced cellular migratory and invasiveness abilities, and promoted EMT process. Further, overexpression of CPE-ΔN increased c-myc and nuclear β-catenin levels in OS cells, which suggested CPE-ΔN promoted the activation of Wnt/β-catenin pathway in OS cells. Treatment with β-catenin small interfering RNA (siRNA) inhibited the migratory and invasive abilities of CPE-ΔN-overexpressing cells, and reversed E-cadherin expression. Together, these results suggest that CPE-ΔN promotes migration, invasion and EMT of OS cells via the Wnt/β-catenin signaling.

Key words: carboxypeptidase E; osteosarcoma, β-catenin; metastasis; epithelial-mesenchymal transition
Introduction

Osteosarcoma (OS) is one of the most frequent malignant bone tumors among children and adolescents (Mirabello et al. 2009; Ritter and Bielack 2010). Currently, around 2/3 of OS patients could be cured with surgery and conventional chemotherapy strategies (Meyers et al. 2011). However, the prognosis of metastatic OS is still poor, with an overall 5-year survival rate of about 20% (Kansara et al. 2014). Therefore, identification of targeting genes and molecular signaling pathways that related to OS metastasis may provide novel insights and therapeutics.

Metastasis is a very important process in malignancies, accounting for high proportion of cancer deaths (Chaffer and Weinberg 2011; Mehlen and Puisieux 2006). Multiple sequential steps are involved in the metastatic process, among which epithelial-mesenchymal transition (EMT) is considered as a crucial initial step. During EMT, carcinoma cells lose epithelial characteristics and acquire increased migration and invasion abilities (Singh et al. 2018; Thiery et al. 2009). Therefore, the EMT process as a novel therapeutic avenue attracts increasing attentions in the treatment of malignancies.

Carboxypeptidase (CPE) is originally described as a neuropeptide-processing enzyme responsible for cleaving the C-terminally extended basic residues from peptide intermediates in endocrine cells or neuropeptides in peptidergic neurons (Cawley et al. 2012; Cool et al. 1997). Recently, CPE is demonstrated to have multifunctional properties, which may attribute to its different forms (Fricker and Snyder 1983; Horing et al. 2012; Lee et al. 2011). The full form of CPE is reported to exert anti-invasion and...
anti-migration abilities (Horing et al. 2012; Murthy et al. 2013). Whereas the newly identified N-terminally truncated splice variant of CPE (CPE-ΔN) is demonstrated highly expressed in metastatic cancers and it could be a biomarker for predicting future metastasis (Lee et al. 2011). For example, Sun et al have reported that lung adenocarcinoma patients with positive CPE-ΔN expression showing higher metastasis rates when compared with those with negative CPEΔN expression (Sun et al. 2016). Further, high CPE-ΔN mRNA expression is closely associated with tumor differentiation, recurrence, and lymph node metastasis in colorectal cancer patients (Zhou et al. 2013), and it may predict metastasis and recurrence in hepatic carcinoma (Lee et al. 2011). We previously showed that downregulation of CPE inhibited the migration, invasion and tumor growth of OS cells, and the splice variant form, CPE-ΔN could possibly be the inducer of migration and invasion (Fan et al. 2016). Here we further investigated the underlying mechanism. Our results showed that CPE-ΔN overexpression enhanced the migration and invasion, and facilitated EMT process of OS cells. Furthermore, the Wnt/β-catenin signaling pathway was related to CPE-ΔN-mediated metastasis. **Methods**

**Cell culture**

Human osteosarcoma cell lines 143B and U2OS cells were purchased from Zhong qiao Xin Zhou Biotechnology Co.,Ltd (Shanghai, China) and Procell Biotechnology Co.,Ltd (Wuhan, China). 143B cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO Life Technologies, Eggenstein, Germany) containing 10% fetal calf serum (FCS), and U2OS cells were maintained in RPMI-1640 medium (GIBCO) in a
humidified atmosphere of 5% CO\textsubscript{2} at 37°C.

**Stable clone selection and transfection of siRNA**

To generate CPE-ΔN-overexpressing vectors, the CPE-ΔN sequences were obtained by reverse transcription PCR then cloned into pcDNA3.1 vector. After sequencing confirmation, 143B and U2OS cells were transfected with CPE-ΔN-constructed vector-or empty vector using Lipofectamine 2000 Reagent (Invitrogen). After 24 h of transfection, the cells were selected with G418 (200 μg/mL, Invitrogen) for stable CPE-ΔN-overexpressing clones. For transient transfection, small interfering RNA (siRNA) against β-catenin was provided from GenePharma (Shanghai, China). Cells were transfected with β-catenin siRNA or a scrambled sequence using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. The efficiency of transfection was confirmed by western blot.

**Western blot**

The protocol of Western blot was performed following previous reports (Fan et al. 2016; Skalka et al. 2013). Total proteins from cells were extracted and protein concentrations were detected with BCA method. Equal amount of protein for each sample were separated by SDS-PAGE and then transferred to the polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) membranes. Thereafter, the membranes were blocked in 5% milk and incubated with relevant primary antibodies at 4°C overnight. The primary antibodies included anti-CPE(1:500, D124408, Sangon Biotech, Shanghai, China), anti-MMP2, and MMP9 (1:1000, Bioss, Beijing, China), anti-E-cadherin,
anti-β-catenin, anti-slug (1:1000, Cell Signaling Technology, Inc.), anti-vimentin, anti-snail, and anti-c-myc (1:2000, 1:1000, Proteintech, Wuhan, China). After that, membranes were incubated with HRP-conjugated secondary antibody and the blots were visualized with the ECL reagent (Biotime, Nanjing, China). Targeted blots were scanned and analyzed with ImageJ software. β-actin was served as a loading control.

**Wound healing assay**

The cells were cultured in 6-well plates in complete medium until they reached confluence. Each well was manually scratched with 200μL sterile pipette tips. The stripped cells were washed away, and then the cell plates were incubated at 37°C. Images were captured at 0 and 24h after incubation, and the distance of migration were calculated.

**Transwell assay**

Transwell chambers with 8.0 μm pore polycarbonate membrane (Corning, NY, USA) were used to analyze cell invasive ability. Cells (1×10⁴ cells/ml in 200μl serum-free medium) were seeded into the upper chamber, while the bottom chamber was filled with culture medium containing 20% FCS. After 24 h incubation, the cells that invaded to the bottom of the filter were fixed with 4 % formaldehyde solution for 20 min and stained with crystal violet for 5 min. The number of invaded cells was counted under a microscope. Statistical results of cell numbers per each image field were obtained from three independent experiments averaged from five image fields.

**Immunofluorescence assay**
143B and U2OS cells were grown on glass coverslips until 80% confluent and then fixed with 4% formaldehyde solution and permeabilized with Triton X-100 in PBS. After being blocked with goat serum, the cells were incubated with primary antibodies (anti-β-catenin and anti-E-cadherin, 1:200, cell signaling technology, Beverly, MA, USA) overnight at 4℃. Next, the cells were incubated with Cy3-labeled secondary antibody (1:200, Beyotime Institute of Biotechnology, Shanghai, China) at room temperature for 1h and then counterstained with DAPI. The slides were sealed and observed under a microscope (OLYMPUS BX53).

Statistics

All data were presented as the means ± standard deviation and analyzed using the GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). ANOVA with Bonferroni’s post-hoc test was used to compare the differences between multiple groups, and unpaired Student’s t test was used for the comparison between two groups. P < 0.05 was considered statistically significant.

Results

Construction of stable CPE-ΔN-overexpressing OS cells

To assess the role of CPE-ΔN in OS cells, we first constructed stable CPE-ΔN-overexpressing cells. Real-time PCR results showed that increased CPE-ΔN mRNA levels were found in both OS cells transfected with CPE-ΔN expressing construct (Fig. 1A, B). Western blot results also confirmed that the stable CPE-ΔN-expressing cells exhibited high levels of CPE-ΔN as compared to the vector control cells (Fig. 1C, D,
p<0.01). Of note, there were some endogenous CPE-ΔN and CPE in the parental cells and overexpression of CPE-ΔN did not affect CPE levels. Together, these results indicated that stable CPE-ΔN-overexpressing 143B and U2OS cells were constructed.

**Overexpression of CPE-ΔN increased the migratory and invasive abilities of OS cells**

Previously Lee TK et al (Lee et al. 2011) reported that CPE-ΔN expression was upregulated in several cancer cell lines, and CPE-ΔN expression was associated with hepatocellular carcinoma metastasis. To assess the effect of CPE-ΔN on OS cell motility, we further performed wound-healing and transwell invasion assays. Wound-healing results showed CPE-ΔN-overexpressing cells appeared increasing wound closure compared with control cells (Fig. 2A, B, E; p<0.01), which suggested CPE-ΔN could enhance migration of OS cells. The invasive capacity of parental or transfected OS cells were determined by transwell invasion assay (Fig. 2C, D). By quantification of the cells that passing through the matrigel, we found that CPE-ΔN significantly increased the invasive capability of OS cells (Fig. 2F, p<0.05 or p<0.01).

We further detected the levels of metastasis-related factors, including MMP2 and MMP9. Western blot results showed that CPE-ΔN overexpression led to significant increases in MMP2 and MMP9 expression compared to the control cells (Fig. 2G, H). Moreover, CPE-ΔN also increased the activities of MMP2 and MMP9 as determined by gelatin zymography (Fig. 2G, H; p<0.01 vs. control). Together, these results indicated that overexpression of CPE-ΔN promoted the migration and invasion of OS cells.
Overexpression of CPE-ΔN modulated the expression of EMT-related markers

Evidence is emerging that EMT is crucial for cell invasion and metastasis (Thiery et al. 2009). Therefore, we further investigated whether CPE-ΔN affects the EMT process in OS cells. Western blot results revealed that overexpression of CPE-ΔN led to significant reduction in E-cadherin while increased vimentin expression compared with the vector control cells (Fig. 3A-D; p<0.01). The transcription factors Snail and Slug were also upregulated in CPE-ΔN-transfected cells (p<0.01). Immunofluorescence results also revealed that CPE-ΔN-transfected cells exhibited lower intensity of E-cadherin compared with control cells (Fig. 3E, F). Taken together, these results suggested that overexpression of CPE-ΔN promoted the EMT process of OS cells.

CPE-ΔN overexpression promoted the activation of Wnt/β-catenin signaling

It has been reported that aberrant activation of Wnt/β-catenin signaling found in malignancies (Klaus and Birchmeier 2008), including OS. To investigate whether Wnt/β-catenin signaling is associated with CPE-ΔN-mediated invasion and migration in OS cells, we further detected key gene expression of Wnt/β-catenin signaling, including β-catenin and C-myc. Western blot results revealed that C-myc and nuclear β-catenin levels were dramatically increased in CPE-ΔN-overexpressing cells compared with control cells (Fig. 4A,B, p<0.01). Immunofluorescence staining further confirmed that CPE-ΔN overexpression increased the translocation of β-catenin from cytoplasm to nuclei (Fig. 4C, D). These results indicated that CPE-ΔN promoted the activation of Wnt/β-catenin signaling.
**Wnt/β-catenin signaling is responsible for CPE-ΔN-induced metastasis in OS cells**

Fig. 4 showed that CPE-ΔN overexpression increased nuclear translocation of β-catenin, which implied the activation of Wnt/β-catenin pathway. Considering that Wnt/β-catenin signaling is an important regulator for EMT process, we further investigated whether Wnt/β-catenin signaling participated in CPE-ΔN-induced EMT and motility. Control and CPE-ΔN-overexpressing OS cells were transfected with control siRNA or β-catenin siRNA. Western blot showed that CPE-ΔN overexpression significantly increased nuclear β-catenin expression and decreased E-cadherin levels in both OS cells (Fig. 5A-D, p<0.01, vs. vector control cells). The changes were reversed by β-catenin silencing (Fig. 5A-D, p<0.01 vs. the CPE-ΔN+control siRNA cells). These results suggested that inhibition of β-catenin was able to reverse the promoting effect of CPE-ΔN on EMT. We further examined the effect of β-catenin inhibition on CPE-ΔN-induced migration and invasion. As expected, silencing of β-catenin abolished the increased migratory and invasive abilities of OS cells induced by CPE-ΔN (Fig. 5E-F, p<0.01 vs. CPE-ΔN+control siRNA control cells). Taken together, these results suggested that Wnt/β-catenin signaling played an important role in CPE-ΔN-induced EMT and motility of OS cells.

**Discussion**

Osteosarcoma is the most prevalent primary tumor of bone among children and adolescents. With multimodal therapies like complete surgery and chemotherapy, around 60%-70% of OS patients with localized osteosarcoma can be cured. However, the
survival rate of patients with metastatic OS remains very low (Kager et al. 2010), and metastasis is the main cause of mortality of the OS patients (Kansara et al. 2014; Messerschmitt et al. 2009). Therefore, understanding the mechanisms involved in OS metastasis is of great importance and may provide promising therapeutic targets for OS treatment.

Previous studies have demonstrated that the splice variant of CPE, CPE-ΔN is highly expressed in metastatic cancers and it is a promising biomarker for predicting future metastasis (Zhou et al. 2013). We previously also showed that CPE downregulation had an inhibitory effect on migration and invasion and tumor growth of OS cells, and CPE-ΔN could possibly be the inducer of migration and invasion (Fan et al. 2016). To further confirm the role of CPE-ΔN in OS metastasis, we first detected the effect of CPE-ΔN overexpression on OS cell motility. Wound-healing and transwell results indicated that CPE-ΔN overexpression enhanced the migratory and invasive abilities of OS cells. Furthermore, MMP2 and MMP9 expression and activities were also upregulated in CPE-ΔN overexpressing cells. MMP2 and MMP9 belong to matrix metalloproteinase family, they play important roles in tumor cell invasion and migration (Velinov et al. 2010). Over all, these results suggested that CPE-ΔN promoted the migration and invasion in OS cells.

The metastatic process contains several sequential and discernable cellular events, including loss of cellular adhesion, cell migration and invasion into surrounding tissue, cell survival in the circulation system, and finally colonization in distant sites (Gupta and
Massague 2006). EMT is a crucial initial step in the metastatic process. During EMT, epithelial cells lose cell-cell junctions, the epithelial marker like E-cadherin is downregulated by several contributors, while the mesenchymal markers like N-cadherin and vimentin are upregulated, which renders cancer cells with increased motility and invasion (Singh et al. 2018). Additionally, some transcription factors like Slug and Snail can also result in repression of E-cadherin and induce loss of cell-cell junctions (Lamouille et al. 2014). To clarify whether CPE-ΔN contributes to the process of EMT, we further detected EMT-related genes in CPE-ΔN-overexpressing OS cells. Our results showed that enforced CPE-ΔN expression significantly downregulated E-cadherin expression and upregulated the vimentin, Slug and Snail levels in OS cells, which confirmed the involvement of CPE-ΔN in EMT regulation.

The mechanisms underlying CPE-ΔN regulating cell motility and EMT were further investigated. Researchers have shown that multiple signals are involved in the metastatic process, such as the transforming growth factor-β (Zhang et al. 2016), the nuclear factor-kappa B (Chu et al. 2014), the Notch (Chang et al. 2014), and the Wnt (DiMeo et al. 2009) signaling. Here we focused on whether Wnt signaling contributes to CPE-ΔN-mediated migration, invasion and EMT in OS cells. The canonical Wnt/β-catenin signaling pathway is most extensively studied (Cai et al. 2014). In this signaling pathway, β-catenin functions as a pivotal signaling mediator, nuclear accumulation of β-catenin leads to activation of specific target genes and regulates a diverse array of biological process. Here we found that overexpression of CPE-ΔN
resulted in significant increase in nuclear β-catenin and c-myc expression, which implied an activation of Wnt/β-catenin signal in OS cells. Moreover, silencing of β-catenin by siRNA was able to reverse the motility and EMT induced by CPE-ΔN. Together, these results indicated that CPE-ΔN promoted invasion and EMT partially through the Wnt/β-catenin pathway. Our results were in line with an earlier research showing that CPE-ΔN as a positive regulator of the oncogenic Wnt pathway by colocalizing in the nucleus with β-catenin (Skalka et al. 2013).

In summary, our study demonstrated that overexpression of CPE-ΔN promoted the migration and invasion and facilitated EMT process in OS cells. Furthermore, CPE-ΔN-induced invasion and EMT were partially mediated by the Wnt/β-catenin signaling. The results provide new insights showing CPE-ΔN as a potential target for OS therapy.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

References


Figure legends

Figure 1 Construction of stable CPE-ΔN-overexpressing OS cells. (A, B) Real-time PCR analysis was performed to detect CPE-ΔN mRNA expression in parental, vector-transfected, and CPE-ΔN -transfected cells. (C, D) Western blot analysis was performed to detect CPE and CPE-ΔN protein expression in parental, vector-transfected, and CPE-ΔN -transfected cells. Relative expression of CPE-ΔN was quantified by a gray analysis with normalization to β-actin. Data were presented as the mean ± standard deviation (n=3 experiments). Compared to the vector control cells, **P <0.01.
Figure 2 Overexpression of CPE-ΔN promotes cell migration and invasion in OS cells. (A,B,E) Cell migration was determined by the wound-healing assay. The percent of wound closure was calculated as the cell migration distance to the initial wound distance and the migration rate was determined. (C, D, F) The invasive capacity of 143B and U2OS cells with/without transfection was evaluated by transwell assay, the invaded cells were counted under an inverted light microscope. Data were presented as mean ± standard deviation of triplicate experiments; compared with the vector control cells, *P< 0.01. (G, H) The protein levels of MMP2 and MMP9 in OS cells were determined by Western blot, relative protein level was quantified by a gray analysis with normalization to β-actin. (G,H) The activities of MMP2 and MMP9 in OS cells were assessed by gelatin zymography. Data were presented as the mean ± standard deviation (n=3 experiments). Compared to the vector control cells, **P <0.01.

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Figure 4 Overexpression of CPE-ΔN promotes the activation of Wnt/β-catenin signaling.
Expression levels of nuclear β-catenin and c-myc in nontransfected or CPE-ΔN-transfected 143B and U2OS cells were detected by Western blot. Quantitative analysis was performed by densitometric scanning, and relative β-catenin protein level was normalized to Histone H3 and relative c-myc protein level was normalized to β-actin. Data were presented as the mean ± standard deviation (n=3 experiments). Compared to the vector control cells, **P <0.01. (C, D) Immunofluorescence staining was performed to detect the expression of β-catenin in 143B and U2OS cells with/without transfection. Magnification 400×, scale bar = 50 μm.

**Figure 5** Inhibition of Wnt/β-catenin signaling represses CPE-ΔN-induced EMT, invasion. Control and CPE-ΔN-overexpressing cells were transfected with control or β-catenin siRNA for 48h. (A, B) The expression of nuclear β-catenin, and E-cadherin were detected by Western blot. (C, D, G) Cell migration was determined by the wound-healing assay. The percent of wound closure was calculated as the cell migration distance to the initial wound distance and the migration rate was determined. (E, F, H) The invasive capacity of 143B and U2OS cells with/without transfection was evaluated by transwell assay, the invaded cells were counted under an inverted light microscope. Data were presented as the mean ± standard deviation (n=3 experiments). Compared to vector+control siRNA cells, #P<0.05 or ##P<0.01; compared to the CPE-ΔN +control siRNA cells, *P<0.05 or**P<0.01.
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