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Protocadherin-7 induces bone metastasis of breast cancer

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ABSTRACT

Breast cancer had a propensity to metastasize to bone, resulting in serious skeletal complications associated with poor outcome. Previous study showed that Protocadherin-7 (PCDH7) play an important role in brain metastatic breast cancer, however, the role of PCDH7 in bone metastatic breast cancer has never been explored. In the present study, we found that PCDH7 expression was up-regulation in bone metastatic breast cancer tissues by real-time PCR and immunohistochemistry assays. Furthermore, suppression of PCDH7 inhibits breast cancer cell proliferation, migration, and invasion *in vitro* by MTT, scratch, and transwell assays. Most importantly, overexpression of PCDH7 promotes breast cancer cell proliferation and invasion *in vitro*, and formation of bone metastasis *in vivo*. These data provide an important insight into the role of PCDH7 in bone metastasis of breast cancer.

1. Introduction

Bone is the most common site for a distant metastasis in women with breast cancer. At least 80% of patients with metastatic breast cancer will develop bone metastases during the course of their disease [1,2]. Therefore, early detection of bone metastases will improve the quality of life and decrease morbidity and mortality [3]. Primary breast tumors express osteolytic and osteoblastic factors, stimulating different types of bone metastases, with osteolytic lesions occurring more commonly [4,5]. The development of osteolytic bone metastases has been described as a vicious cycle, with increased osteoclast activity implicated as the predominant mechanism of bone destruction [6]. The pathologic manifestations of osteolytic lesions can have devastating effects, including pain, pathologic fractures, spinal compression, and hypercalcemia. An increased understanding of the cellular and molecular mechanisms that contribute to bone metastasis is necessary for improving clinical management.

PCDH7 encodes an integral membrane protein that is believed to function in cell–cell recognition and adhesion, and its localization, 4p15, is a region of loss of heterozygosity in some head and neck squamous cell carcinomas [7,8]. A newly identified breast cancer tumorigenesis and metastasis related gene, has recently been identified PCDH7 mediates brain metastasis of breast cancer through cytoskeleton-mechanic loop mechanism [9]. However, PCDH7 has not been reported in breast cancer bone metastasis. Existing data regarding the involvement of PCDH7 in tumor metastasis also suggest its particular therapeutic impact.

In this study, we determined the effects of PCDH7 on human breast carcinogenesis. We found that there is a significant association between PCDH7 expression and bone metastasis of breast cancer. Functional analyses showed that suppression of PCDH7 led to the inhibition of breast cancer cell growth, migration, and invasion *in vitro*, whereas overexpression of PCDH7 in breast cancer cells results in bone metastasis *in vivo*. Thus, these results indicate that PCDH7 is involved in bone metastasis in human breast cancer cells and may be a potential target for breast cancer therapy.

2. Materials and methods

2.1. Cell and tumor specimens

MDA-MB-231 human breast cancer cells were cultured in in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2 and at 37 °C. Breast cancer samples obtained from patients undergoing surgical resection at Tianjin Medical University Cancer Institute and Hospital. All study subjects were treated according to

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current guidelines, including the use of adjuvant chemotherapy, irradiation, and endocrine therapy for ER-positive and/or PR-positive patients. This study was approved by the Human Ethics Review Committee of Tianjin Medical University Cancer Institute and Hospital. Documented informed consent was obtained from all patients in accordance with the Declaration of Helsinki and its later revision. Samples were classified as "Bone" when patients developed a tumor relapsing to bone, which included those who had additional metastases in other parts of the body. The remaining samples were labeled "Non-bone".

2.2. Plasmids, small interfering RNAs, transfection, and generation of stable cell line

The full-length PCDH7 cDNA were reverse transcriptase-polymerase chain reaction using total RNA from MDA-MB-231 cell line. The primer sequences were as following: 5′-CGGATCCAGATGCTGAGGATGCGGAC-3′ (forward) and 5′-GGATATCTCAGCCAAACACAGTAATG-3′ (reverse); The PCR products were cloned into BamH I and EcoR V sites of the mammalian expression vector pcDNA3.1 (+) (Invitrogen) (pcDNA3.1-PCDH7). siRNA were purchased from (RiboBio, Shanghai, China). For transient transfection, 2×10^5 cells were plated into 6-well plates and kept in antibiotic-free medium for 24 h before transfection. The cells were then transfected with and then transfected with the siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For generation of stable cell lines expressing PCDH7, cells were transfected with pcDNA3.1-PCDH7. Two days after transfection, cells were trypsinized, transferred to 10 cm cell culture dishes and selected by complete medium plus 1 mg/ml of G418 (Sigma–Aldrich, St Louis, MO, USA) for about 2 weeks.

2.3. Quantitative real-time PCR

Total RNA was isolated using the TRIzol reagent (Invitrogen). Quantitative real-time PCR was performed using SYBR Green qPCR Master Mix Kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instruction. The PCDH7 expression quantification in samples was accomplished by measuring the fractional cycle number at which the amount of expression reached a fixed threshold (CT). Triplicate CT values were averaged, and GAPDH CT was subtracted from GSTP1 CT to obtain ΔCT. The relative amount of GSTP1 mRNA was calculated as 2-ΔCT.

2.4. Western blot

Proteins were resolved by SDS–PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). The membranes were blocked in 5% skimmed milk and incubated with primary antibodies overnight (PCDH7 1:2000, Origene, Rockville, MD, USA; β-actin 1:5000, Sigma–Aldrich) and then with horseradish peroxidase-conjugated anti-mouse antibodies (1:3000, Sigma–Aldrich). Specific proteins were visualized with the enhanced chemiluminescence detection reagent (Pierce Biotechnology, Rockford, IL, USA).

2.5. Immunohistochemistry

Paraffin-embedded tissue sections were cut, deparaffinized and rehydrated with xylene and graded alcohols. Antigen retrieval was performed in 5 mM citrate buffer (pH 6.0). After inactivation of endogenous peroxidase with 5% hydrogen peroxide, the sections were incubated with normal serum diluted in PBS (1.5%) to block non-specific binding. After that, the sections were incubated overnight at 4 °C with the mouse monoclonal anti-PCDH7 antibody (western used) at dilution 1:200. The slides were then incubated with biotinylated secondary antibody and streptavidin–biotin–peroxidase, and diaminobenzidine (DAB) (Sigma–Aldrich) was used as a chromogen substrate. The slides were counterstained with Mayer's haematoxylin, washed, dehydrated, cleared and mounted. In the negative controls, the primary antibody was replaced with PBS. The immune-reactivity for PCDH7 was analyzed in the cytoplasm tumor cells taking into account the number of positively stained cells and intensity of staining. The number of positively stained cells was semi-quantified as follows: 0–5% of cells stained = (0); 5–25% of cells stained = (1); 26–75% of cells stained = (2); 76–100% of cells stained = (3). The intensity of nuclear staining was evaluated on a four grade scale: 0, negative; 1, weak; 2, moderate; 3, intensive. Percentage (*P*) and intensity (*I*)

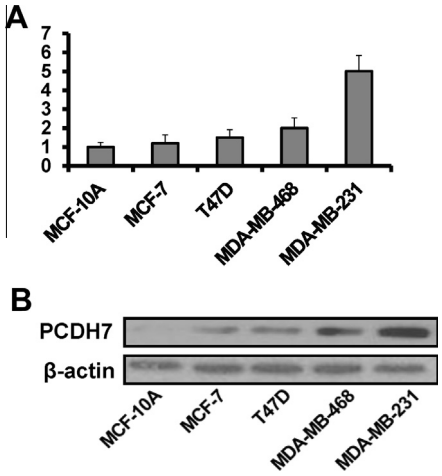

Fig. 1. PCDH7 expression in different breast cancer cell lines. (A) PCDH7 mRNA levels examined by Real-time PCR. (B) PCDH7 protein levels examined by western blot. Bone metastasis cell line: MDA-MB-231. Non-bone metastasis cell lines: MCF-7, T47D, MDA-MB-468. Normal breast cell line: MCF-10A.

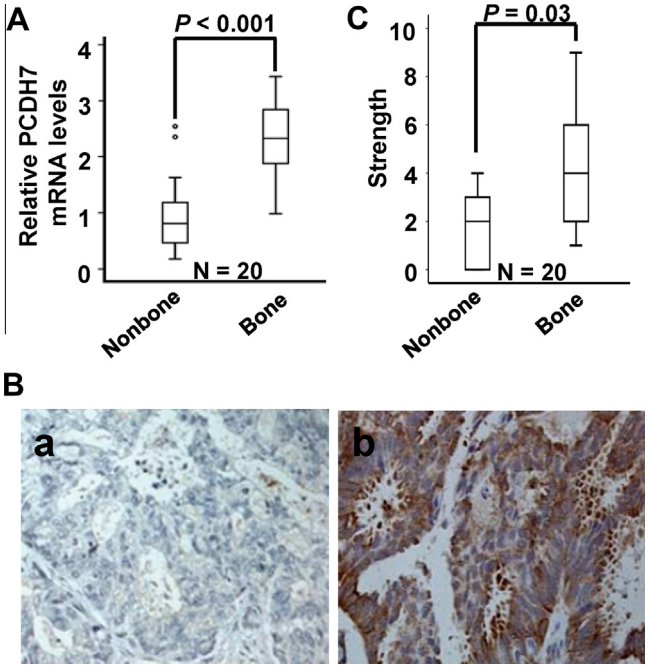

Fig. 2. PCDH7 expression in breast cancer tissues. (A) PCDH7 mRNA levels in non-bone metastasis and bone tissues by real-time PCR ($n = 20$). (B) PCDH7 protein levels in non-bone metastasis (a) and bone metastasis (b) tissues by immunohistochemistry. (C) PCDH7 protein expression as in (B) was calculated by Student *t* test ($n = 20$).

of cytoplasm expression were multiplied to generate numerical score (Strength = $P \times I$).

2.6. Migration and invasion assays

Cells were plated in 6-well plates and formed a fluent monolayer. Cell layer was scratched with a 200-μl pipette tip and detached cells were removed. For each sample, at least three scratched fields were photographed immediately and at the time points indicated after the scratch were made.

The invasion of cells *in vitro* was measured by the invasion of cells through Matrigel-coated transwell inserts (8 μm pore size, BD Biosciences, San Jose, CA, USA). 1×10^5 cells in 500 μl of serum-free medium were added to the upper chamber, Medium (DMEM/F12) containing 20% FBS were added into the lower chamber. After incubation for 24 h, cells that invaded the Matrigel were stained and counted under a microscope in six predetermined fields.

2.7. Proliferation assay

MTT assay were used to observe and compare cell proliferation ability. Briefly, 2×10^3 cells in 200 μl culture medium were plated into a well of 96-well plates. After culturing cell for an appropriate time, 10 μl of 5 mg/ml MTT was added into each well and continued to culture for 4 h. Then, the cell culture medium was replaced by 100 μl of dimethyl sulfoxide. Thirty minutes after dimethyl sulfoxide addition, the plates were placed on a micro-plate auto-reader (Bio-Rad, Richmond, CA, USA). Optical density was read at 570 nm wavelength and cell growth curves were determined according to the optical density value.

2.8. Bioluminescence imaging and analysis

1×10^6 MDA-MB-231-luc-PCDH7 and control cells were washed and harvested in 0.1 ml PBS and injected into the left cardiac ventricle of 4-week-old, female BALB/c-nu/nu nude mice. Mice were anesthetized with ketamine (100 mg/kg of body weight) and xylazine (10 mg/kg of body weight) before injection. Anesthetized mice were injected with 100 mg/kg D-Luciferin in PBS. Bioluminescence images were obtained by using the Xenogen IVIS system 5 min after injection. Analysis was performed with LIVINGMAGE software (Xenogen, Alameda, CA, USA). Survival time was defined as the time interval between cells injected and mice died. Survival plots were created using Kaplan–Meier analysis and Log-rank test were used to assess statistical significance.

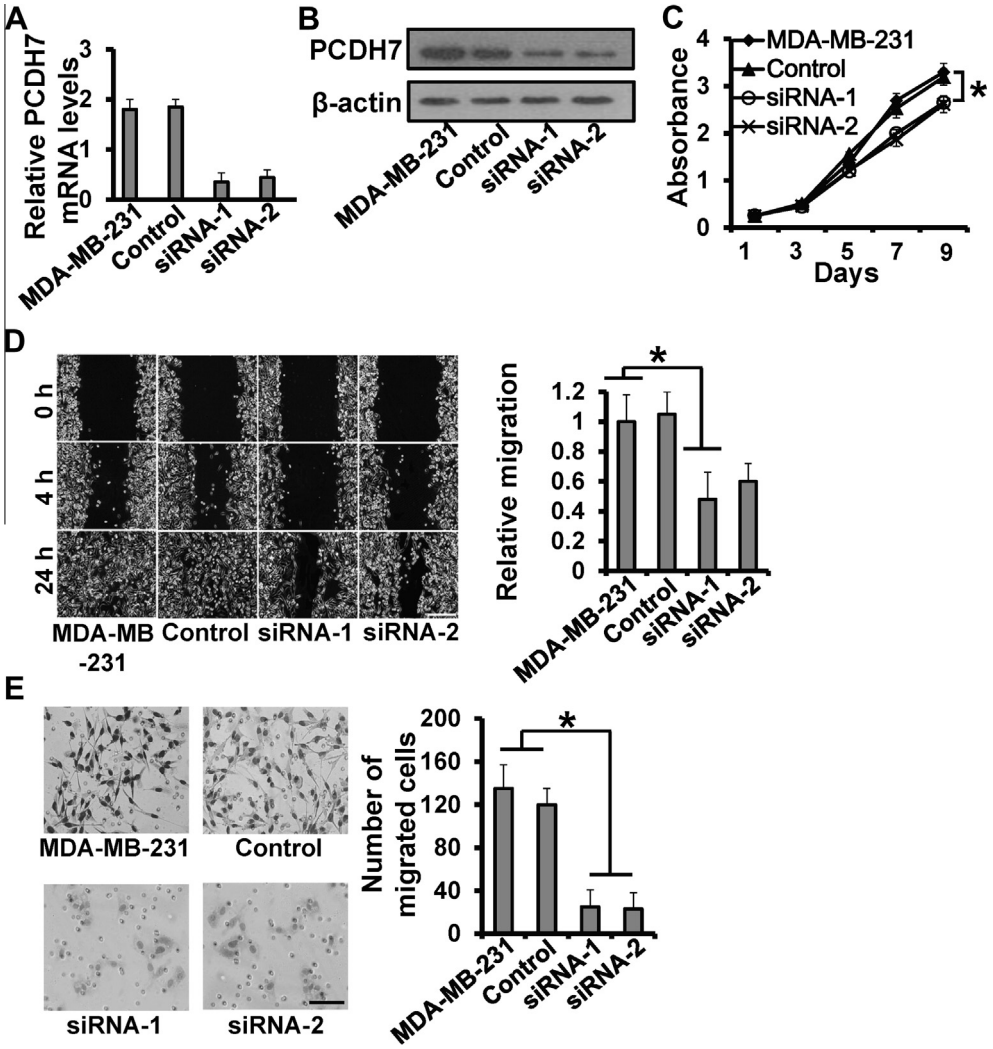

Fig. 3. Suppression of PCDH7 inhibits breast cancer cell proliferation, migration, and invasion. (A) Real-time PCR analysis of PCDH7 expression in cells transfected with control and PCDH7 siRNAs. (B) Western blot analysis of PCDH7 and β-actin expression in cells transfected with control and PCDH7 siRNAs. (C) MTT analysis of PCDH7 siRNAs-transfected and control cells. (D) Scratch analysis of PCDH7 siRNAs-transfected and control cells. (E) Transwell analysis of PCDH7 siRNAs-transfected and control cells. Scale bar = 200 μm. $^*P < 0.01$.

2.9. Statistics

Data are present as the mean ± s.d. Statistical analyses were conducted by Student *t* test. $P < 0.01$ was considered statistically significant. All data quantification and statistical analysis were performed using SPSS 18.0 software (Chicago, IL, USA).

3. Results

3.1. The expression of PCDH7 in breast cancer cell lines

To examine the expression of PCDH7 in breast cancer, we firstly detected the PCDH7 mRNA and protein expression in normal breast cell line and breast cancer cell lines. According to the results of qRT-PCR, the PCDH7 mRNA was significantly increased in the bone metastatic breast cancer cell line, MDA-MB-231 compared to the normal breast cancer cell line, MCF-10A, and the non-bone metastatic breast cancer cell lines, MCF-7, T47D, and MDA-MB-468 (Fig. 1A). The expression levels of PCDH7 protein was also increased in bone metastatic breast cancer cell line, MDA-MB-231 by western blot (Fig. 1B).

3.2. Up-regulation of PCDH7 expression levels in bone metastatic breast cancer tissues

We detected the PCDH7 mRNA expression in bone-metastatic primary breast cancer tissues of patients (Bone) and non-bone metastatic primary breast cancer tissues (Non-bone). Real-time PCR analysis indicated that PCDH7 mRNA was significantly increased in the bone metastatic tissues (Fig. 2A). We next determined the PCDH7 protein expression by immunohistochemistry. Consistently, the protein levels of PCDH7 in bone metastatic tissues were dramatically higher than those in the non-bone metastatic tissues (Fig. 2B). The altered expression of PCDH7 between bone and non-bone metastatic tissues appeared statistically significant (Fig. 2C).

3.3. Suppression of PCDH7 inhibits breast cancer cell proliferation, migration, and invasion in vitro

We then examined the effect of PCDH7 deficiency on the proliferation of breast cancer cells. We used two specific siRNAs targeting PCDH7, and both of them could efficiently reduce PCDH7 expression in MDA-MB-231 cells (Fig. 3A and B). By MTT assay, we found that transfection of PCDH7 siRNAs inhibited cell growth in MDA-MB-231 cells (Fig. 3C). Next, we performed scratch assay and transwell assay to examine the role of PCDH7 in the migration and invasion of breast cancer cells. A scratch assay indicated that siPCDH7 cells were defective in migration (Fig. 3D). Furthermore, MDA-MB-231 cells displayed a clear invasion; whereas siPCDH7 cells were defective in invasion (Fig. 3E). Taken together, these results indicated that PCDH7 induces breast cancer cell proliferation, migration, and invasion.

3.4. PCDH7 contribution to breast cancer bone metastasis formation

To better understand the role of PCDH7 in breast cancer cells, we stably overexpressed PCDH7 in MDA-MB-231-luc (231-PCDH7), using mammalian vector. After G418 selection, stable transfectants were obtained and examined by real-time PCR (Fig. 4A) and western blot (Fig. 4B). The MTT and transwell assay showed that overexpression of PCDH7 induced cell proliferation and invasion in MDA-MB-231-luc (Fig. 4C and D). The 231-PCDH7 and control cells were inoculated into the left cardiac ventricle of immune-deficient mice to allow the formation of bone metastasis. As determined by bioluminescence imaging of luciferase activity, 231-PCDH7 cells caused a significant bone and brain metastasis than control cells (Fig. 4E). These results showed that PCDH7 contributed to breast cancer bone metastasis.

4. Discussion

The protocadherins comprise the largest subfamily of the cadherin superfamily and are predominantly expressed in the nervous system [10]. Protocadherins are predominantly expressed in the

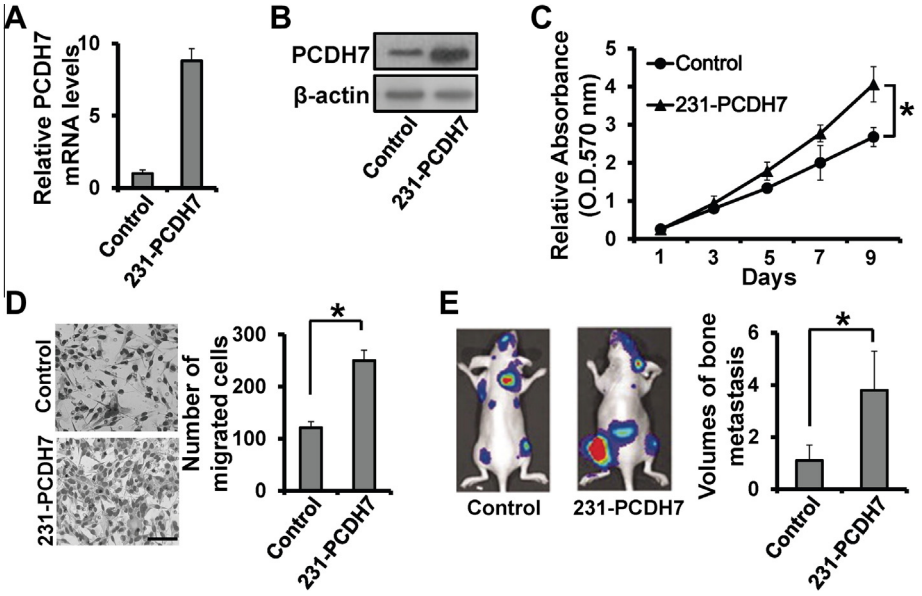

Fig. 4. PCDH7 contribution to breast cancer bone metastasis formation. (A) Real-time PCR analysis of PCDH7 expression in MDA-MB-231-luc transfected with control and pcDA3.1-PCDH7. (B) Western blot analysis of PCDH7 and β-actin expression in MDA-MB-231-luc transfected with control and pcDA3.1-PCDH7. (C) MTT analysis of 231-PCDH7 and control cells. (D) Transwell analysis of 231-PCDH7 and control cells. (E) Representative photos of general physical condition of xenograft mice carrying MDA-MB-231-luc cells expressing either control or PCDH7 on 32 days after injection are shown ($n = 6$). Scale bar = 200 μm.*$P < 0.01$.

brain and they function as cell–cell recognition molecules [8,11]. They are divided into two groups (the clustered and non-clustered PCDH families) based on their genomic structure. *PCDH7*, also known as BH-PCDH, is located on the p-arm of chromosome 4. It is belonged to the non-clustered families [12]. PCDH7 is predominantly expressed in the somatosensory and visual cortices, and external granule cell layer of the cerebellar cortex, and the brainstem starting from embryonic day 17, and PCDH7 exhibits a critical period for the establishment of specific synaptic connections [8,13]. In addition, PCDH7 is expressed in the ganglion cell layer of the retina [14], and its overexpression leads to a morphological change and Ca^{2+}-dependent cell adhesion in mouse fibroblast L cells [7]. However, there is less known about the function of the PCDH7 in breast cancer except Ren et al., which reported that PCDH7 mediates brain metastasis of breast cancer [9].

In the present study, we investigated the involvement of PCDH7 in the human breast cancer bone metastasis. We have identified PCDH7 significantly up-regulated in bone metastatic breast cancer cell line (MDA-MB-231), compared to non-bone metastatic breast cancer cell lines by real-time PCR and western blot. In a subsequent study, we expanded our investigation of PCDH7 expression in human bone metastatic breast cancer and non-bone metastatic breast cancer by real-time PCR and immunohistochemistry. We demonstrated that the PCDH7 expression was significantly increased in human bone metastatic breast cancer tissues compared to human non-bone metastatic breast cancer. This important finding supports our initial cell-line-based results. These results indicated that a novel biomarker, PCDH7, is up-regulation in breast cancer cells with bone metastasis compared to in-breast cancer cells without bone metastasis.

Cell proliferation, migration and invasion of cancer cells at a primary site are important steps in the process of bone metastasis [15]. In this study, we determined the proliferation, migration and invasion activities of breast cancer cells by *in vitro* assays. We knock down PCDH7 expressions in MDA-MB-231. Our results showed that the migration and invasion activities were significantly decreased in the PCDH7-depleted MDA-MB-231 cells compared to the control cells. Furthermore, the migration and invasion activities were significantly increased in the PCDH7-overexpressed MDA-MB-231 cells both *in vitro* and *in vivo*.

In conclusion, we have demonstrated that PCDH7 expression is significantly increased in bone metastatic breast cancer. Our results suggest that PCDH7 may be a potential biomarker in bone metastatic breast cancer. Whether the PCDH7 is dependent on the presence of other proteins is an issue that will be investigated in future studies.

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