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Suppression of peripheral myelin protein 22 (PMP22) expression by miR29 inhibits the progression of lung cancer

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PMP22 is recently recognized as a key player in a variety of prevalent cancers. In this study, we sought to explore the correlation of peripheral myelin protein 22 (PMP22) expression with cell proliferation, invasion and apoptosis in lung cancer cell line. miR29 was transfected into Anip973 lung adenocarcinoma cell line to interfere the expression of PMP22 using Lipofectamine ® 2000 reagent. Real-time polymerase chain reaction (RT-PCR) and Western blot were performed to determine the expression level of PMP22 at mRNA and protein level. Then MTT, Matrigel transwell assay and flow cytometry were respectively used to explore the proliferation, invasion and apoptosis in Anip973 lung adenocarcinoma cell line *in vitro*. miR29 could significantly down-regulate the expression level of PMP22 in Anip973 cells not only at mRNA level but also at protein level. Moreover, the proliferation rate, invasive cell number and apoptosis rate of Anip973 cells in miR29 transfected group significantly decreased compared with blank group, while no significant difference existed between control group and blank group. Our study found that suppression of PMP22 expression could inhibit cell proliferation, invasion and apoptosis in lung cancer cells. All these findings suggest that PMP22 may be involved in progression of lung cancer and could be a new therapeutic target for this disease.

Key words: lung cancer, peripheral myelin protein 22, miR29, MTT, Matrigel transwell assay, flow cytometry

Lung cancer is the common leading cause of cancer-related death worldwide, in which non-small cell lung cancer (NSCLC) accounts for 80-85% of all [1, 2]. It is still increasing both in prevalence and mortality all over the world [2]. Radiation therapy or combined with chemotherapy is the common treatment for lung cancer, however, lung cancer has an extremely poor prognosis with a low overall 5-year survival rate (no more than 17%) [3, 4]. Although there has been considerable progress in therapies, the incidence and mortality in patients with lung cancer are still high over the years [5]. As a result, it is of great importance to elucidate the molecular mechanisms for promoting cell proliferation, invasion and apoptosis in lung cancer cells.

Peripheral myelin protein 22 (PMP22) is a disease-associated protein and contributes to peripheral myelination in the peripheral nervous system [6]. Recently, accumulated evidences suggest that PMP22 is emerging as a key player in a variety of prevalent cancers. PMP22 is found to play an important role in the neoplastic transformation process from the normal pancreas to pancreatic cancer [7]. Moreover, PMP22 is proved to be frequently amplified in osteosarcomas and may be involved in cell cycle to aberrant regulate cell growth in osteosarcoma tumorigenesisn [8, 9]. Besides, PMP22 is observed to be differentially expressed in breast cell lines and is defined as an independent prognostic factor for overall survival in patients with breast cancer [10]. Irshad *et al.* also have confirmed that PMP22 is a molecular signature in newly diagnosed prostate cancer with independent prognostic value [11]. The roles of PMP22 in various prevalent cancers have aroused extensive attentions, however, its molecular pathogenesis underlying lung cancer remains unclear.

In the present study, we sought to explore the expression level of PMP22 in Anip973 lung adenocarcinoma cell line with highly metastasis potential. Meanwhile, suppression of PMP22 expression was performed to further elucidate the role of PMP22 in Anip973 lung adenocarcinoma cell line by miRNA interference. Then MTT, Matrigel transwell assay and flow cytometry were respectively used to investigate the cell

proliferation, invasion and apoptosis *in vitro*. The objective of our study was to evaluate the correlation of PMP22 expression with cell proliferation, invasion and apoptosis in Anip973 lung cancer cell line, as well as its underlying mechanism. All of our efforts will provide theoretical basis and new insights into the treatment of lung cancer.

Materials and methods

Cell culture and plasmid transfection. Anip973 lung adenocarcinoma cell line with highly metastasis potential is a subline of maternal cell line AGZY83-a after consecutive batches in abdominal cavity of nude mice, which was cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Welgene Ltd.) in a 5 % CO2 incubator at 37°C. In our study, miR-29 was purchased from Ambion (Austin, TX) and transfected into Anip973 cells for interfering the expression of PMP22, which had previously confirmed to post-transcriptionally target PMP22 [12]. The miR-29a sequences are: sense, 5'-UAGCACCAUCUGAAAUCGGUUA-3'; antisense, 5'-ACCGAUUUCAGAUGGUGCUAUU-3'. Control miRNA sequences are: sense, 5'-UUCUCCGAACGUGUCACGUTT-3', antisense: 5'-ACGUGACACGUUCGGAGAATT-3'. Twenty-four hours before transfection, an appropriate concentration of resuspended Anip973 cells were seeded on 6-well plates. Then miR-29 was transfected into Anip973 cells with Lipofectamine® 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Meanwhile, no plasmid transfection and Anip973 cells transfected with out-of-order miRNA were respectively set as blank and control groups. All the treated cells were continued to incubate for 4 h, and normal media was added. After 48 h, the cells of each group were harvested for further detection.

Real-time polymerase chain reaction (RT-PCR) analysis. Following the manufacturer's instructions, total RNA of Anip973 cells of each group was firstly extracted by TRIzol® reagent (Invitrogen, Burlington, ON, Canada). The quality of RNA (1.8 < A260/A280 < 2.0) and RNA concentrations were determined by a spectrophotometer (NanoDrop 2000, Thermo Scientific, USA). Reverse transcription was carried out using the PrimeScriptTM RT Master Mix Kit (Takara, RR036A). The total reaction volume was 20 μl including 10 ng RNA. Finally, the expression level of PMP22 was determined using fluorescent quantitative RT-PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), using the $2^{-\Delta\Delta CT}$ method [13]. Detailed speaking, the primers specific for PMP22 were as follows: forward primer: 5'-GCCACCATGATCCTGTCGAT-3'; reverse primer: 5'-CCCTTGGTGAGGGTGAAGAGT-3'. Each sample was analyzed in triplicate using the Applied Biosystems 7500 RT-PCR system (Applied Biosystems, CA, USA). The PCR reaction was pre-incubated at 95°C for 10 min, incubated at 95°C for 30 s followed by 40 cycles of 95°C for 15 s, 60°C for 1 min and a final dissociation stage.

Western blot analysis. The expression of PMP22 and caspase-3 was detected by western blot at protein level. For western blot analysis, protein samples were obtained by harvesting cells in 4–20% Tris-glycine polyacrylamide gel buffer and protein concentrations were examined by bicinchorinic acid (BCA) assay kit (Pierce, Rockford, IL). Then 25μg protein was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose membranes. The membranes were blocked using 5% nonfat dry milk for 1 h. The membranes were firstly probed using appropriate primary antibodies overnight at 4°C and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotech) at room temperature for 1 h. The bands in membrane were finally detected using chemiluminescence ECL kit (GE Healthcare Biosciences). Meanwhile, the membrane was probed with appropriate loading controls for equal loading of proteins. Densitometric measurements of the bands were performed by digitalized scientific software program in Kodak 2000R imaging system.

Measurements of cell proliferation rate. Total $5 \times l0^3$ cells at logarithmic phase of each group were respectively seeded on 96-well plates and grown in DMEM supplemented with 10% FBS overnight in a 5 % CO2 atmosphere at 37°C. Supernatant was removed after 24 h of cell culture and 20ul of MTT solution (Roche) was added to each well at intervals of 24 h. All determinations were performed in triplicate. Then the plates continued to incubate for 4 h. After terminating the reaction, the supernatant was removed by centrifugation and 150 μL of dimethyl sulfoxide (DMSO) was added into each well to sufficiently solubilize the formazan crystals for 10 min. Finally, the absorbance at 570 nm wavelength (A570) was measured with a microplate reader (BioTek, USA). The inhibtory rate Y (%) was calculated as follows: $Y = (1\text{-}A_{570T}/A_{570C}) \times 100\%$, where A_{570T} refers to absorbance of treatment groups and A_{570C} refers to the absorbance of control groups.

Matrigel transwell assay. The invasive ability of lung cells was determined using transwell chamber with Matrigel-coated membrane (24-well insert; pore size, 8mm; BD Biosciences) *in vitro*. Forty-eight hours after transfection, 500 μL of cells (1 × 105 cells/mL) of each group were added to each insert of the upper well of the chamber containing serum free media. DMEM supplemented with 10% FBS as a chemoattractant was added into the lower well of the chamber. After 12 hours of incubation, cells that did not invade through the pores were removed with a cotton swab and cells that had invaded the membrane were fixed with 70% ice ethanol, stained with 0.1% crystal violet and sealed on slides. The migrated cells were counted in 8 random high-power fields per chamber under a light microscope and the average of invasive cell number per field was calculated to measure the invasion rate. All determinations were carried out in triplicate.

Detection of cell apoptosis by flow cytometry. Cells (5×10^6) were incubated for 48 h and harvested for flow cytometric analysis which was described by Shang *et al*. [14]. The Annexin

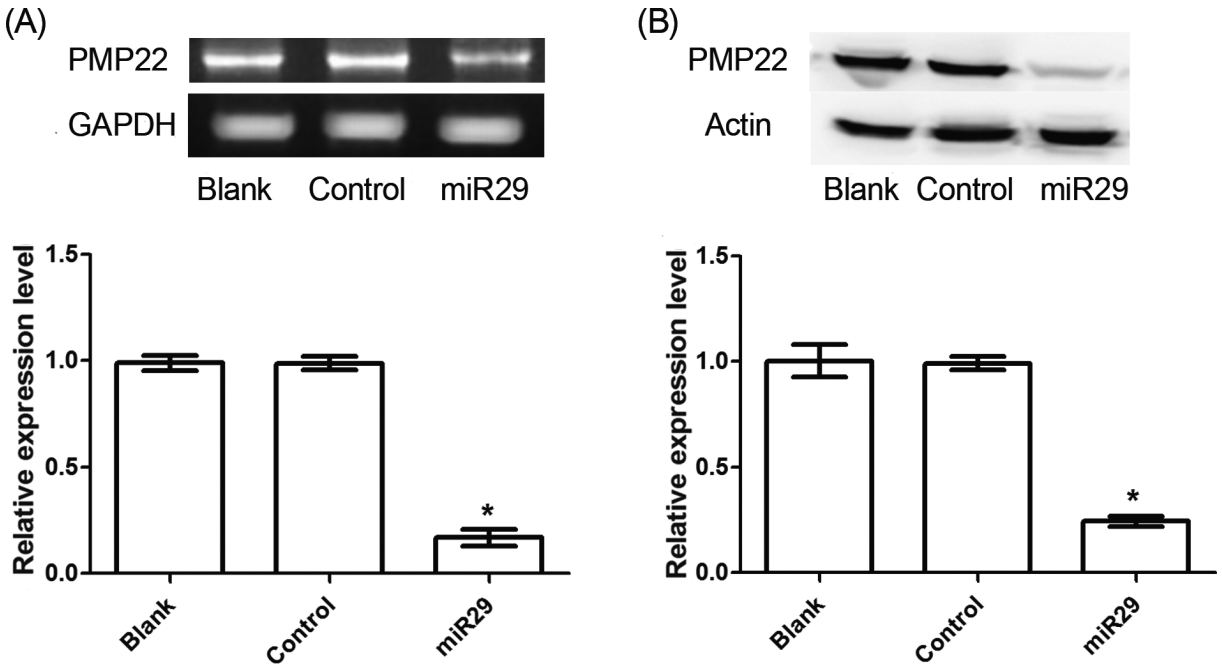

Figure 1. The expression level of PMP22. (A) The expression level of PMP22 analyzed by RT-PCR. (B) The expression level of PMP22 analyzed by western blot. Error bars indicate means ± SD and * indicates significant difference compared with blank group ($p < 0.05$).

V-FITC apoptosis detection kit (Biosea, China) was used to examine the apoptosis rate in accordance with the manufacturer's instruction. Briefly, cells were firstly resuspended in 96 μL of ice-cold mixed with Annexin V binding buffer. Afterwards, 10 μL Annexin V-FITC and 5 μL propidium iodide (PI, 10 mg/L) was added to each. The cells were then incubated away from light for 15 min on ice. Then the cells were read by flow cytometry (FACS Aria, Becton Dickinson, San Jose, CA, USA) and scatter plots were analyzed with CellQuest 3.0 software (BD Biosciences, San Jose, CA). Annexin V-positive cells were regarded as apoptosis cells and were counted by a dual-color flow cytometric method.

Statistics analysis. All collected data were shown as mean ± SD and tested for the normal distribution using one-sample K-S test. Measurement data was tested by student t-test (only for two groups) or one-way ANOVA. Further comparison between groups was performed by post-hoc Tukey test. A value of $P < 0.05$ was defined to have statistical significance.

Results

Detection of the expression level of PMP22. RT-PCR analysis and western blot analysis displayed the expression of PMP22 at mRNA level and protein level, respectively. As shown in Figure 1, miR29 could significantly down-regulate the expression level of PMP22 in Anip973 cells not only at mRNA level but also at protein level ($P < 0.05$). Moreover, there was no significant difference between control group and blank group ($P > 0.05$).

Detection of cell proliferation/viability. MTT assay was used to determine the proliferation/viability of Anip973 cells after 24 h of transfection. The results showed that the cell proliferation/viability of Anip973 cells transfected with miR29 significantly decreased ($P > 0.05$) compared with blank group, while no significant difference existed between control group and blank group ($P > 0.05$) (Figure 2).

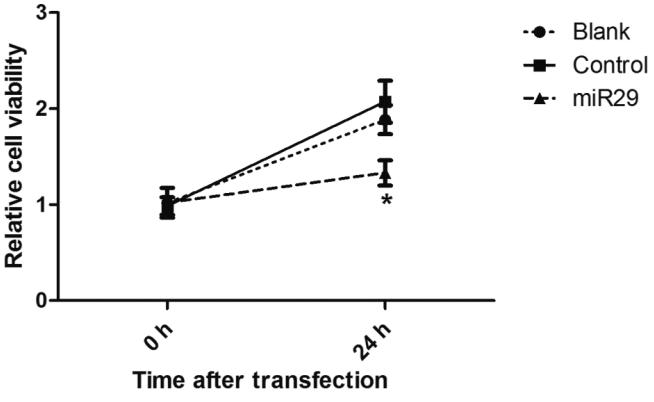

Figure 2. Correlation of PMP22 expression with cell proliferation/viability. Error bars indicate means ± SD and * indicates significant difference compared with blank group ($p < 0.05$).

Correlation of PMP22 expression with cell invasion. Matrigel transwell assay was used to determine the invasive ability of Anip973 cells in each group (Figure 3). The results showed that the average of invasive cell number per field in blank group and control group respectively were 14.3 and 14.5 while the average of invasive cell number in miR29 treated group only was 5.1. There was significant difference between miR29 treated group and blank group ($P > 0.05$), suggesting that the suppression of PMP22 expression could significantly decrease the invasive ability of Anip973 cells.

Correlation of PMP22 expression with cell apoptosis. The results of flow cytometry displayed that the apoptosis rates of Anip973 cells in blank group, control group and miR29 transfected group were 11.8%, 9.9% and 3.4% (Figure 4A). Moreover, the apoptosis rate of Anip973 cells in miR29 treated group significantly decreased compared with that of blank group ($P < 0.05$). Besides, Figure 4B illustrated that the expression level of caspase-3 in Anip973 cells of miR29 transfected group obviously decreased by western blot analysis.

Discussion

Lung cancer is one of the leading causes of death without effective treatment strategy. PMP22 is recently recognized as a key player in a variety of prevalent cancers. However, limited data are available on the role of PMP22 in the molecular pathogenesis of lung cancer. In the present study, miR29 was successfully transfected into Anip973 lung adenocarcinoma cell line to interfere the expression of PMP22. The results showed that suppression of PMP22 expression significantly decreased the proliferation, invasion and apoptosis in Anip973 lung adenocarcinoma cells, indicating that PMP22 may be a key player involved in the progression of lung cancer.

PMP22 has been shown to be implicated in a number of cellular roles including the regulation of proliferation [15]. Winslow *et al.* demonstrated that PMP22 protein could regulate cell spreading and affect cell proliferation in breast cancer cells [16]. Furthermore, it has been demonstrated previously that PMP22 can interact with members of the

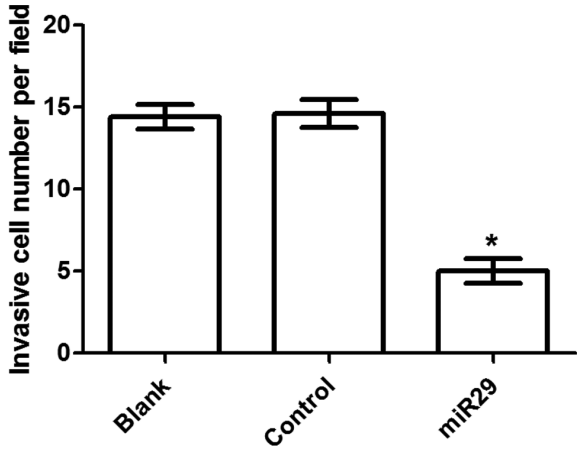

Figure 3. Correlation of PMP22 expression with cell invasion. Error bars indicate means ± SD and * indicates significant difference compared with blank group ($p < 0.05$).

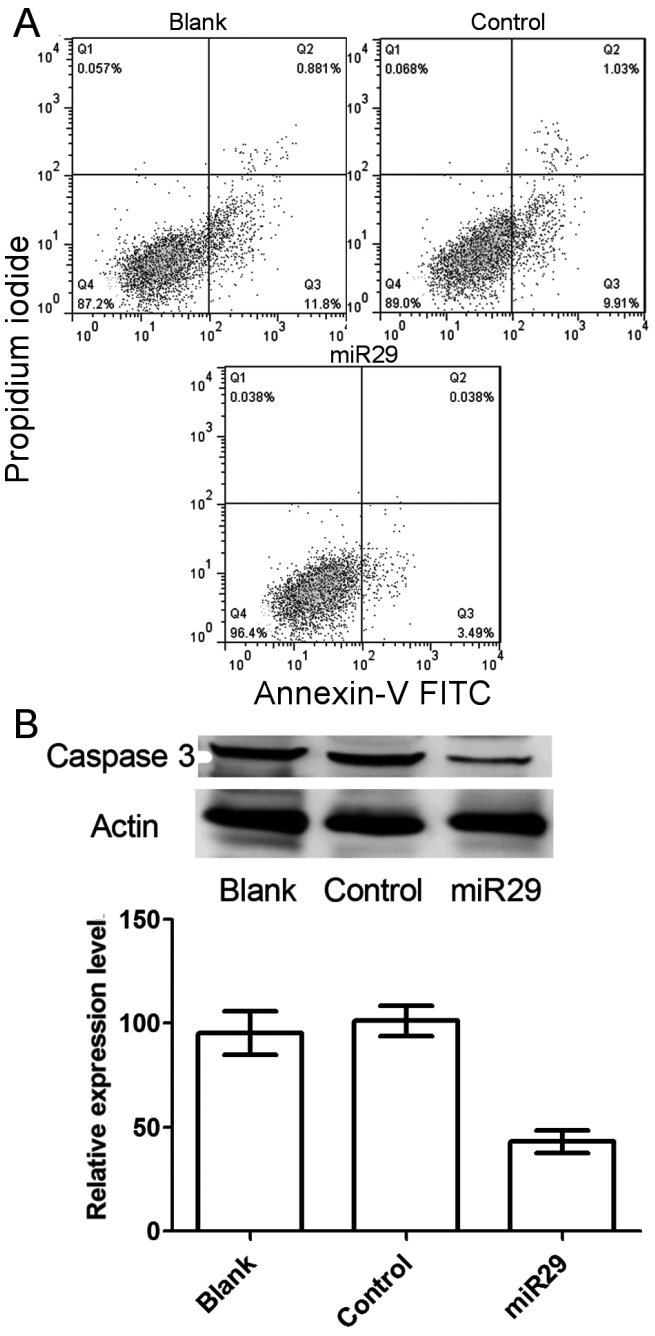

Figure 4. Correlation of PMP22 expression with cell apoptosis. A: The apoptosis rates of Anip973 cells analyzed by flow cytometry. X-axis represents the intensity of Annexin-PE and Y-axis represents the intensity of Annexin-FITC. B: The expression level of caspase-3 in Anip973 cells analyzed by western blot. Error bars indicate means ± SD and * indicates significant difference compared with blank group ($p < 0.05$).

transforming growth factor-beta (TGF-β) family [17]. TGF-β has been proved to control cell proliferation [18]. Gallelli *et al.* also verified that TGF-β was involved in fibrotic events and induced cell proliferation in human lung fibroblasts [19]. Besides, decreased expression of PMP22 is associated with the pathogenesis of urethan-induced lung tumors in mice [20]. In our study, MTT assay showed suppression of PMP22 expression decreased the cell proliferation of Anip973 cells obviously. It is thus intriguing to speculate that PMP22 expression may have a role in promoting cell proliferation in lung cancer.

As another aspect of the present analysis, our results verified that suppression of PMP22 decreased the ability of cell invasion significantly by Matrigel transwell assay. PMP22 is previously confirmed to interact with members of TGF-β family [17]. A work of Willis and Borok indicated that TGF-β could induce EMT, which had implications for fibrotic lung disease [21]. Moreover, EMT has been shown to have a role in promoting carcinoma invasion in a variety of cancers [22, 23]. Besides, PMP22 can modulate alpha 6 integrin expression in the human endometrium [15]. The expression of alpha 6 integrin has an important function in the induction of cell migration and invasion [24]. A peptide selected by biopanning also demonstrates that integrin αvβ6 is identified as a prognostic biomarker for non-small cell lung cancer [25]. In view of the correlation of PMP22 with cell invasion reported previously, we speculate that PMP22 may play a crucial role in the progression of lung cancer via enhancing the ability of invasion.

Besides, cell apoptosis is also an important mechanism involved in the progression of cancers. In our study, flow cytometric analysis showed that the apoptosis rate of miR29-treated cells in which PMP22 expression was suppressed significantly decreased compared with other groups, suggesting PMP22 may induce cell apoptosis. Nestler *et al.* previously confirmed that PMP22 could interact with the zinc containing pro-apoptotic protein siva [26]. High expression of PMP22 is also proposed to lead to growth arrest, cell cycle stop and consequently apoptosis in fibroblasts [7]. Moreover, PERP (p53 apoptosis effector related to PMP22) is a novel member of the PMP-22/gas3 family and shown to be a apotosis-associated target of p53 [27]. Bax, a Bcl-2 family member, is proved to have p53 binding sites in its promoter and consequently leads to apoptosis via activation by p53 [27, 28]. Thus, our results are in accordance with previous findings and imply that PMP22 may induce apoptosis in lung cancer cells via Bax activation by p53. Besides, accumulated studies show that Bcl-2 family proteins could contribute to lung tumor formation and associated with radio- or chemoresistance of lung cancers [29, 30]. Bcl-2 inhibitor and Bax activator are thought to be a promising approaches for cancer therapy [31, 32]. It can therefore be speculate that Bcl-2 inhibitor/or Bax activator could synergize with the function of PMP22, and co-targeting PMP22 with Bcl2 and/or Bax may have feasibility and application prospects in cancer therapy. On the other hand, previous studies have suggested that caspases-3 is a key mediator of mitochondrial events of apoptosis [33]. Activation of caspases-3 is an important mechanism for the induction of apoptosis in human lung cancer A549 cells [34]. In our study, western blot analysis indicated that the expression of caspases-3 was inhibited followed by the decrease of PMP22 expression levels due to miR29 interference. It can therefore be hypothesized that PMP22 may induce cell apoptosis in lung cancer cells via activation of caspases-3.

Taken together, our study found that suppression of PMP22 expression could inhibit cell proliferation, invasion and apoptosis in lung cancer cells. All these findings suggest that PMP22 may be involved in progression of lung cancer and could be a new therapeutic target for this disease. However, only one cell line (Anip973 lung adenocarcinoma cell line) was used to verify the role of PMP22 in cell viability, invasion and apoptosis *in vitro*. Moreover, there was no experimental validation *in vivo* in our study. Further studies are still needed to verify our observation.

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