An Adenovirus Expressing Mutant p27 Showed More Potent Antitumor Effects Than Adenovirus-p27 Wild Type

Kyung-Ho Park, Ja Young Seol, Tae-You Kim, Chul-Gyu Yoo, Young Whan Kim, Sung Koo Han, Young-Soo Shim, and Choon-Taek Lee


ABSTRACT

The main inhibitory action of p27, a cyclin-dependent kinase inhibitor (CDKI), arises from its binding with the cyclin E/cyclin-dependent kinase 2 (Cdk2) complex that results in G1-S arrest. Degradation of p27 is mediated by phosphorylation of Thr-187 of p27, which follows ubiquitination. In this study, we generated two adenoviruses expressing wild-type p27 (ad-p27wt) and mutant p27 (ad-p27mt), with mutation of Thr-187/Pro-188 (ACGCCG) to Met-187/Ile-188 (ATGATC), which were produced with the belief that mutant p27 would bind cyclin E/Cdk2 more stably and show more potent antitumor effects. Ad-p27wt and ad-p27mt expressed p27 proteins that were indistinguishable by anti-p27 antibody. A pulse-chase experiment showed that p27mt was more resistant to degradation than p27wt. In human lung cancer cell lines, ad-p27mt showed stronger growth inhibition than ad-p27wt. Both types of ad-p27 induced G1-S arrest and apoptosis; however, ad-p27mt induced stronger G1-S arrest and apoptosis. Intratumoral injection of ad-p27mt induced partial regression of established tumors and inhibited the growth of human lung cancer xenografts more strongly than ad-p27wt. From these results, we conclude that ad-p27mt has the potential to become a novel and powerful gene therapy tool.

INTRODUCTION

Cellular dedifferentiation and uncontrolled proliferation are the main characteristics of cancer cells. Uncontrolled proliferation of cancer cells results from the disruption of the normal cell cycle control, which is controlled by interactions among cyclin, Cdk, and CDKI (1). Two families of CDKIs are involved in cell cycle arrest. Inhibitors of the INK4 family (p16, p15, p18, and p19) bind Cdk4 and Cdk6 specifically and inhibit cyclin D binding. In contrast, inhibitors of the Cip/Kip family (p21, p27, and p57) act as broad, specific inhibitors of the cyclin D, E, and A complexes. The deregulation of these CDKIs is a common feature in tumor cells and mainly contributes to the disruption of cell cycle control (2). Since the cloning of the p27 gene (3, 4), many other functions have been associated with this cell cycle protein. p27Kip1 (p27) is a CDKI that exerts its inhibitory activity on many steps of the cell cycle. The main inhibitory action of p27 arises from its binding with the cyclin E/Cdk2 complex and its induction of the dephosphorylation of pRb (5). p27 mRNA levels are constant throughout the cell cycle, and p27 protein levels are regulated both by translational control (6) and by ubiquitin-mediated proteolysis. p27 protein levels and stability are high in quiescent cells and in the majority of other cell types but fall during G1 and reach a minimum in the S-phase (7). This regulation of the p27 level is linked to the phosphorylation on Thr-187, followed by ubiquitination (8–10). In addition, the cyclin E/Cdk2 complex causes the phosphorylation of p27 on Thr-187, which results in the degradation of p27. Therefore, p27 is both an inhibitor and a substrate of cyclin E/Cdk2 (11). Interestingly, mutation on Thr-187 of p27 to alanine made p27 resistant to the cyclin E/Cdk2-induced phosphorylation and induced G1-S arrest resistant to cyclin E/Cdk2 (11).

In addition to its role as a CDKI, p27 has the role of a putative tumor suppressor gene (12). p27 knockout mice develop multiorgan hyperplasia and parathyroid tumors (13), and p27 haplo-insufficient mice are more sensitive to tumor development by radiation and chemical carcinogens (14). Adenoviral gene transfer of p27 also induced the cell cycle arrest and apoptosis of breast cancer cell lines (15, 16). We have shown previously that the transduction of ad-p27 via adenoviral vector to human lung cancer cell lines induced cell growth suppression via G1-S arrest. Furthermore, intratumoral injection of ad-p27 induced growth suppression of established lung cancer xenografts (17).

On the basis of these two findings of the role of p27 as a tumor suppressor gene and the process of p27 degradation by phosphorylation on Thr-187, we hypothesized that p27mt at Thr-187 would be resistant to degradation and bind to the cyclin E/Cdk2 complex more stably and, consequently, demonstrate a more potent antitumor effect.

To confirm this hypothesis, we constructed two adenoviruses expressing wild p27 (ad-p27wt) and mutant p27 at the Thr-187/Pro phosphorylation site (ad-p27mt) and compared their antitumor effects.

MATERIALS AND METHODS

Cell Lines and Animals. Five human lung cancer cell lines (NCI H157, NCI H358, NCI H460, NCI H126, and A549) were purchased from American Type Culture Collection (Manassas, VA) and the Korean Cell Line Bank (Seoul, Korea). Cells were maintained in RPMI 1640 with 8% fetal bovine serum and penicillin/streptomycin. Six-week-old female nude mice (BALB/c) were purchased from SLC, Inc. (Hamamatsu, Japan).

Construction of ad-p27wt and ad-p27mt. We have described the construction of ad-p27wt (17) and ad-p16 (18) previously. These viruses are replication-defective, E1-deleted adenoviruses with CMVie promoter. Briefly, the cDNA of human p27 (kindly provided by J. Massagué, Memorial Sloan Kettering Cancer Center, New York, NY) was subcloned into the KpnI sites of the polylinker of the adenoviral shuttle vector, pAC CMVplp (kindly provided by Robert Gerard, University of Texas Southwestern Medical Center, Dallas, TX). The resulting pAC CMV-p27wt and pCMV17 (also kindly provided by R. Gerard) were cotransfected into 293 cells using the standard calcium phosphate precipitation method. The 293 cells were maintained in RPMI 1640 with 2% fetal bovine serum until the onset of the cytopathic effect. Generating adenovirus was purified three times by plaque method, and the titer was determined by plaque assay (19). The titer was determined by plaque assay (19).

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4 The abbreviations used are: Cdk, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; CMV, cytomegalovirus; MOI, multiplicity of infection; NSCLC, non-small cell lung cancer; PI, propidium iodide; PARP, poly(ADP-ribose) polymerase; ad, adenovirus; wt, wild type; mt, mutant; Rb, retinoblastoma; pRb, Rb protein.

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ADENOVIRUS-p27 MUTANT SHOWS POTENT ANTITUMOR EFFECTS

Transduction of Lung Cancer Cells with ad-p27(wt/mt). Exponentially growing lung cancer cells were transduced with ad-p27wt or mt at 20 MOI for 1 h with frequent gentle shaking and then incubated with complete medium for the experiment.

Western Blot for p27 and pRb. Lung cancer cells (NCI H358) were transduced with 20 MOI of ad-p27wt and incubated for 48 h. Western blots of p27 and pRb were performed using the ECL Western blotting system according to its protocol (Amersham). Monoclonal mouse antihuman p27 and pRb antibodies (PharMingen, San Diego, CA) were used as the primary antibodies for p27 and pRb.

Pulse Chase Experiment. To demonstrate the stability of p27mt produced in ad-p27mt transduced cells, a pulse chase experiment for p27 was performed. Lung cancer cells (NCI H460) were transduced with 20 MOI of ad-p27wt and ad-p27mt. After 24 h, the medium was replaced with serum-free medium without methionine/cysteine for 16 h. Cells were then incubated with complete medium containing 1 μCi of [35S] methionine/cysteine for 4 h (pulse). Proteins were extracted and immunoprecipitated for p27 from cells at 0, 15, 60, and 240 min and 24 h. The stabilities of p27wt and p27mt were compared by 10% SDS-PAGE electrophoresis.

Analysis of the Growth Inhibition on Lung Cancer Cell Lines. To compare the growth-suppressing effect of ad-p27wt and ad-p27mt, four NSCLC cell lines (NCI H460, large cell carcinoma of the lung; NCI H157, squamous cell carcinoma of the lung; NCI H1264, adenocarcinoma of the lung; and NCI H358, bronchioloalveolar cell carcinoma) were plated in six-well plates (5 × 104/well). After 24 h of incubation, transductions were performed with ad-p27wt/mt; 20 MOI; ad-lacZ, or PBS for 1 h, and the cells were maintained in complete medium. Cell numbers were counted in triplicate on a daily basis using a hemocytometer.

Cell Cycle Analysis after Transduction with ad-p27wt and ad-p27mt. Lung cancer cells (NCI H460) were transduced with 20 MOI of ad-p27wt and ad-p27mt. After 24 h incubation, cell cycle alterations were measured using the CycleTest plus kit protocol (Becton Dickinson, San Jose, CA).

Cdk2 Kinase Activity Assay. Twenty-four h after transduction with ad-p27wt and ad-p27mt, total cell extracts were precipitated with anti-Cdk2. Cdk2 kinase activities of the immunoprecipitates were determined in terms of their ability to phosphorylate histone H1. Reactions were performed for 30 min in 50 μl of H1 kinase buffer with 10 μg of histone H1 and 10 μCi of [γ-32P]ATP. Reaction products were resolved by 10% SDS-PAGE gel electrophoresis.

Annexin V Assays for Apoptosis. To confirm the presence of another p27 wt or ad-p27mt growth-suppressing mechanism, we observed the induction of apoptosis by Annexin V apoptosis kit (PharMingen). Briefly, lung cancer cells (NCI H460) were transduced with ad-p27wt or ad-p27mt at 20 MOI. Forty-eight and 96 h after transduction, cells were detached with a brief trypsin treatment. Cells were then stained with Annexin V and PI, according to the manufacturer’s instructions, and sorted using a FACScaliber flow cytometer (Becton Dickinson). Proportions showing early apoptosis (positive for Annexin V and negative for PI) were then measured.

Hoechst Staining. DNA fragmentation of apoptotic cells were measured by Hoechst 33258 staining. Lung cancer cells (NCI H460) were transduced with ad-p27wt and ad-p27mt. At 96 h, cells were stained with 1 μl of Hoechst 33258 for 10 min. At least 300 cells were counted under a fluorescence microscope with a 4',6-diamidino-2-phenylindole filter. Cells were defined to be apoptotic if their nuclei showed chromatin condensation, nuclear beading, or fragmentation.

PARP Cleavage Assay. To confirm apoptotic induction by ad-p27wt and ad-p27mt transduction, we measured the cleavage of PARP. Briefly, a lung cancer cell line (NCI H460) was transduced with ad-p27wt (20 MOI) or ad-p27mt (20 MOI) and incubated for 72 h. The expression and cleavage of PARP were assessed by Western blot assay using an anti-PARP monoclonal antibody (PharMingen).

Soft Agar Clonogenic Assay. To compare the effect of p27wt or p27mt gene transfer on in vitro tumorigenicity, soft agar clonogenic assay was performed using the method described previously (20). Briefly, tumor cells (NCI H358 and NCI H460) were transduced with 20 MOI of ad-p27wt or ad-p27mt and then were detached and plated in 0.2% agarose with a 1% agarose underlay (5 × 103 cells/plate).

Treatment of Established Lung Cancer Xenografts by Intratumoral Injection of ad-p27wt and ad-p27mt. s.c. xenografts of lung cancer were established in nude mice by injection of 2 × 106 cells (NCI H460) at day 14. After 2 weeks, day 0, when tumors were of moderate size (~350 mm3 in volume), intratumoral injections of ad-p27wt and ad-p27mt (1.0 × 109 plaque-forming units/injection) were initiated and repeated five times every day. Changes in tumor size were measured every other day using the following formula: tumor volume = 1/2 × (length)2 × width.

RESULTS

Transduction with ad-p27wt and ad-p27mt Produced p27 Protein and Induced the Dephosphorylation of pRb. Western blot assay for p27 in proteins extracted from ad-p27wt and ad-p27mt transduced lung cancer cells showed overexpression of p27 protein. p27wt and p27mt were indistinguishable by Western blot assay, but the amount of p27mt was slightly higher than that of p27wt. However, both p27wt and p27mt induced the dephosphorylation of pRb (Fig. 1).

Pulse Chase Experiment Showed that Mutant p27 Was More Resistant to Degradation Than p27wt. The amount of p27wt extracted from ad-p27wt transduced cells began to decrease after 15 min and markedly at 4 h. Almost no p27wt was found at 24 h. In contrast to p27wt, the amounts of p27mt remained stable up to 4 h and then decreased at 24 h (Fig. 2). This finding demonstrated the stability of p27mt, which would support the hypothesis of this experiment.

Ad-p27mt Showed Stronger Growth Arrest on Human NSCLC Cell Lines Than ad-p27wt. In the four NSCLC cell lines tested, ad-p27mt induced stronger growth suppression than ad-p27wt, which suggests that p27mt is more potent at inducing growth arrest in lung cancer cell lines (Fig. 3).

Ad-p27mt Induced Stronger G1-S Arrest Than ad-p27wt on Flow Cytometric Analysis. Both ad-p27wt and ad-p27mt induced cell cycle progression inhibition in the lung cancer cell line (NCI H460) at the G1-S checkpoint. When comparing ad-p27wt and ad-p27mt, ad-p27mt transduction showed stronger G1-S arrest (G0/G1S: 25.18%/40.72% in control, 27.47%/41.53% in ad-null, 50.13%/49.78% in ad-p27wt).

PARP Cleavage Assay. To confirm apoptotic induction by ad-p27wt and ad-p27mt transduction, we measured the cleavage of PARP. Briefly, a lung cancer cell line (NCI H460) was transduced with ad-p27wt (20 MOI) or ad-p27mt (20 MOI) and incubated for 72 h. The expression and cleavage of PARP were assessed by Western blot assay using an anti-PARP monoclonal antibody (PharMingen).

Soft Agar Clonogenic Assay. To compare the effect of p27wt or p27mt gene transfer on in vitro tumorigenicity, soft agar clonogenic assay was performed using the method described previously (20). Briefly, tumor cells (NCI H358 and NCI H460) were transduced with 20 MOI of ad-p27wt or ad-p27mt and then were detached and plated in 0.2% agarose with a 1% agarose underlay (5 × 103 cells/plate).

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21.85% in ad-p27wt, and 81.26%/9.3% in ad-p27mt; Fig. 4). Similar findings were found in other lung cancer cell lines (data not shown). These findings suggest that p27mt protein is a more effective and powerful CDKI, although both p27wt and p27mt are effective.

Both ad-p27wt and ad-p27mt inhibited Cdk2 activity. Overexpression of both p27wt and p27mt almost completely inhibited the phosphorylation of histone H1 by immunoprecipitates with Cdk2 antibody (Fig. 5). Differences between p27wt and p27mt were not
observed in terms of their inhibition of Cdk2 activity. This finding suggests that both p27wt and p27mt are potent CDKIs.

**Ad-p27mt Was a More Potent Inducer of Apoptosis than ad-p27wt.** Both ad-p27mt and ad-p27wt induced apoptosis, as confirmed by Annexin V assay; however, ad-p27mt proved to be the more effective inducer of apoptosis. Apoptosis was not evident at 48 h. The proportions of early apoptotic cells (Annexin V, positive; PI, negative) were 4.46% of ad-lacZ transduced cells and 5.70% in ad-p27wt and 8.74% in ad-p27mt transduced cells. Induction of apoptosis became evident at 96 h. The proportion of early apoptotic cells was 11.14% in ad-lacZ, 36.20% in ad-p27wt, and 49.58% in ad-p27mt transduced cells. Although both ad-p27wt and ad-p27mt induced apoptosis, ad-p27mt was the more potent apoptosis inducer (Fig. 6).

**Hoechst Staining Demonstrated That ad-p27mt Induced More Apoptosis by DNA Fragmentation Than ad-p27wt.** Transduction with ad-p27wt and ad-p27mt effectively induced apoptosis, as manifested by nuclear fragmentation (Fig. 7). Forty-six % of ad-p27mt transduced cells and 31% of ad-p27wt transduced cells showed apoptotic changes (4% in ad-lacZ transduced cells).

**PARP Cleavage by ad-p27wt and ad-p27mt.** Compared with the control and ad-lacZ transduced cells, transduction with ad-p27wt and ad-p27mt demonstrated cleavage of PARP by reduction of the M, 116,000 band and the appearance of a M, 85,000 band (Fig. 8), which could also provide evidence of apoptosis induction by p27wt and p27mt.

**Transduction with ad-p27mt Induced Stronger Suppression of the in Vitro Tumorigenicity of Lung Cancer Cell Lines.** Consistent with a stronger antitumor effect on G1-S arrest and apoptosis induction, the in vitro tumorigenicity of the lung cancer cell line (NCI H460) transduced with ad-p27mt was strongly suppressed (Fig. 9).

**Intratumoral Injection of ad-p27mt Resulted in the Partial Regression of Established Lung Cancer Xenografts.** We began intratumoral injections into relatively large (350 mm³ on average) tumors 14 days after tumor cell inoculation. Injection of ad-p27wt induced partial tumor growth suppression; however, ad-p27mt injection induced an initial partial regression that was followed by delayed growth. Furthermore, 40% of mice showed complete tumor regression (Fig. 10).

**DISCUSSION**

Cancer gene therapies can be broadly classified into two groups: (a) those that modify the host responses to a tumor; or (b) those that induce direct antitumor action by introducing genetic material that directly affects the cancer cell and halts its growth (21). Replacement therapies involving tumor suppressor genes are the prototypes of gene therapies with direct antitumor action. p27 is a member of the multifunctional universal CDKI family (22). In addition to its role as a CDKI, p27 is known as a putative tumor suppressor gene (14), as a regulator of drug resistance in solid tumors (23), as a promoter of apoptosis (24, 25), and as a safeguard against inflammatory injury (26). During tumor development, the level of p27 protein decreases, which is mainly caused by posttranslational regulation by ubiquitin-proteosome-mediated degradation (27). Low levels of p27 have been strongly and independently associated with poor prognosis in many cancers including lung (28), breast (29, 30), and colorectal (31).
cancer. Overexpression of p27 via adenoviral gene transfer could suppress cancer cell growth regardless of p27 mutation (15). These findings suggest the tumor-suppressive nature of p27.

In this study, we attempted to develop a more potent p27 gene therapy by manipulating the p27 metabolic pathway. As we described, p27 metabolism depends on phosphorylation of Thr-187 and a subsequent ubiquitination. Montagnoli et al. (10) showed that the ubiquitination of p27 did not occur in p27mt with Thr-187 to Ala [p27(T187A)]. Sheaff et al. (11) showed that the transfection of p27(T187A) plasmid causes a G1 block that is both resistant to and not modulated by cyclin E/Cdk2.

On the basis of these observations of p27 metabolism and the nature of the p27 tumor suppressor gene, we tried to use p27mt at Thr-187 for cancer gene therapy, expecting a super-repressor effect on cell cycle arrest. Adenovirus expressing p27mt (Thr-187/Pro-188 to Met-187/Ile-188) was constructed to block cyclin E/CDK2 more stably. 187TPKK190 is a conserved COOH-terminal Cdk target site for phosphorylation (8). Two amino acids (Thr-187 and Pro-188) were mutated to completely disrupt the conserved COOH-terminal CDK target site (TPKK).

Western blot assay for p27 showed that the transduction of both adenoviruses induced p27 protein overexpression and the dephosphorylation of pRb. The p27 monoclonal antibody was specific for the NH2 portion of p27 that was unchanged in p27mt. The [35S]methionine/cysteine-pulse chase experiment demonstrated that p27mt was resistant to degradation and more stable than p27wt. Contradictory results have been reported of the stability of p27mt. Vlach et al. (8) reported that mutation of Thr-187 to Val of p27k− (p27 deficient in interaction with Cdns) could increase the stability; however, the same mutation in p27wt failed to increase the stability. However, Nguyen et al. (32) reported that mutation of Thr-187 to Ala stabilized p27 in S-phase extracts. Recently, the degradation-resistant novel p27 isoform (p27Kip1R) was cloned. This p27 isoform has no conserved COOH-terminal, Cdk-phosphorylation site (TPKK) and is part of a putative bipartite nuclear-localization signal (residues 159–169). This p27Kip1R was resistant by in vitro degradation assay and was more effective at inducing apoptosis than p27wt. However, they reported that p27T187A was degraded in a manner similar to p27wt in an in vitro degradation assay (33). In the present study, we demonstrated that p27mt with double mutation of Thr-187/Pro to Met-187/Ile was resistant to degradation in a pulse chase experiment, in which Western blot assay of whole lysates from ad-p27wt/mt transduced cells (NCI H460) with PARP antibody was performed. Compared with the control and ad-lacZ transduction, transduction with ad-p27wt and ad-p27mt induced cleavage of PARP, which was manifested by the reduction of the M, 116,000 (116 kD) band and appearance of a M, 85,000 (85 kD) band.

Fig. 8. ad-p27wt and ad-p27mt induced the cleavage of PARP. Western blot assay of whole lysates from ad-p27wt/mt transduced cells (NCI H460) with PARP antibody was performed. Compared with the control and ad-lacZ transduction, transduction with ad-p27wt and ad-p27mt induced cleavage of PARP, which was manifested by the reduction of the M, 116,000 (116 kD) band and appearance of a M, 85,000 (85 kD) band.

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Fig. 9. Suppression of in vitro tumorigenicity by transduction with ad-p27wt and ad-p27mt. Soft agar clonogenic assay showed that ad-p27mt (20 MOI) suppressed in vitro tumorigenicity more strongly than ad-p27wt in lung cancer cell lines (NCI H358 and NCI H460) tested.
suggested an indirect role for p27Kip1. However, some studies have showed that ad-p27 induced apoptosis in Rb-positive cells after day 3, reported the induction of apoptosis by demonstrating DNA degradation. Katayose et al. (23) demonstrated that p27 is a regulator of chemoresistance and that apoptotic stimuli, such as those induced by cytotoxic drugs, induced caspase-mediated p27 cleavage (38). St. Croix et al. (24) reported that the overexpression of endogenous p27 shows antiapoptotic properties. This growth inhibition in this study could be explained by two mechanisms, cell cycle arrest and induction of apoptosis. Because of the known role of CDK1, we had already expected G1-S arrest by p27mt and p27wt. In four cell lines tested, ad-p27mt induced a more profound G1-S arrest than ad-p27wt. The role of the CDK1 of p27wt and p27mt was confirmed again by its suppression of Cdk2 kinase activity.

Conflicting results have been reported of the effect of p27 upon cell susceptibility. Several reports on adenoviral gene transfer of p27 have revealed apoptotic induction by p27 overexpression in several cancer cell lines (24, 35–37). Katayose et al. (24) reported that the overexpression of p27 triggers apoptosis in several cancer cell lines. They demonstrated the induction of apoptosis by terminal deoxynucleotidyltransferase-mediated nick end labeling assay and found that the overexpression of p27 was followed by cleavage of PARP and the degradation of cyclin B1. Naruse et al. (35) also showed that ad-p27 induced apoptosis by terminal deoxynucleotidyltransferase-mediated nick end labeling assay and PI flow cytometry. Wang et al. (36) also reported the induction of apoptosis by ad-p27 by demonstrating DNA fragmentation and PARP cleavage. Schreiber et al. (37) compared several adenoviral vectors expressing CDKIs including p27 and also showed that ad-p27 induced apoptosis in Rb-positive cells after day 3, suggesting an indirect role for p27Kip1. However, some studies have shown that apoptotic stimuli, such as those induced by cytotoxic drugs, induced caspase-mediated p27 cleavage (38). St. Croix et al. (23) demonstrated that p27 is a regulator of chemoresistance and that tumor-targeted p27 antagonists act as chemosensitizers.

In this report, we clearly confirmed again that ad-p27wt/mt induced apoptosis in lung cancer cell lines by Annexin V assay, Hoechst staining, and PARP cleavage. More interestingly, ad-p27mt is a more potent apoptosis-inducer than ad-p27wt, as confirmed by Annexin V assay and Hoechst staining.

Consistent with previous reports (35, 37), apoptosis was not evident at 48 h but became evident at 96 h. From these reports, we think that ectopic overexpression of p27 can induce apoptosis, and high expression of endogenous p27 shows antiapoptotic properties. This stronger antitumor effect of p27mt was reconfirmed by an adenovirus expressing antisense insulin-like growth factor-I receptor on human lung cancer cell lines. Cancer Res., 56: 1836–1838, 1996.


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