

# Antimicrobial peptide LL-37 promotes YB-1 expression, and the viability, migration and invasion of malignant melanoma cells

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**Abstract.** The cathelicidin antimicrobial peptide, LL-37, is a multifunctional peptide with a broad spectrum of antimicrobial activities, such as chemotaxis and neutralizing endotoxins. Previous studies have demonstrated that LL-37 serves a functional role in the development of numerous types of cancer including ovarian, breast, prostate and lung cancer. However, its role in the development of malignant melanoma (MM) remains unclear. To determine the role of LL-37 and the potential interaction with Y-box binding protein 1 (YB-1) in MM, RNA interference, western blot, reverse transcription-quantitative polymerase chain reaction, MTT and Transwell assays were performed. The current study demonstrated that LL-37 induced YB-1 expression, and increased tumor cell proliferation, migration and invasion of A375 and A875 MM cell lines. In addition, inhibition of nuclear factor- $\kappa$ B (NF- $\kappa$ B) attenuated LL-37-induced YB-1 expression. These results demonstrate that, through the upregulation of YB-1 expression and the activation of the NF- $\kappa$ B signaling pathway, LL-37 may promote the malignant progression of MM cells *in vitro*.

## Introduction

Malignant melanoma (MM) originates from melanocytes and is a highly malignant skin tumor, which accounts for 1-2% of all skin cancer cases. MM has a high malignant potential, with a high risk of ulceration and metastasis. The global incidence rates of MM have increased rapidly over the past few decades, with an annual incidence rate of 3-8% (1-3).

The Y-box binding protein-1 (YB-1), which contains a 'cold shock' region, is a multifunctional protein that is located in the nucleus and the cytoplasm (4). YB-1 regulates the expression of target genes indirectly or by binding to target gene sequences directly, and the altering the splicing of target genes (5-8). YB-1 expression is upregulated in a number of human tumors, including ovarian cancer, breast cancer, glioblastoma, esophageal cancer, gastric cancer, colon cancer and lung cancer (9-11), and is therefore considered to be a prognostic marker. YB-1 overexpression is an indicator of increased tumor cell proliferation, migration and invasion (9-11). Additional studies have indicated that the expression levels of YB-1, the epidermal growth factor receptor (EGFR) and the human EGFR-2 (HER-2) are collectively increased in breast cancer tissues. In addition, HER-2 may regulate the nuclear translocation of YB-1 directly or indirectly (11). In contrast to its putative oncogenic role, inhibition of YB-1 expression has been demonstrated to increase the sensitivity of melanoma cells to chemotherapy; an effect that is closely associated with the phosphatidylinositol-4,5-bisphosphate 3-kinase/AKT serine/threonine kinase 1 signaling pathway (11,12). This demonstrates that the expression of YB-1 may serve a role in regulating tumor development and/or progression.

Advances in tumor biology have led to an increased focus on the potential antitumor activities of antimicrobial peptides. The human cathelicidin antimicrobial protein-18, also known as LL-37, is a key antimicrobial molecule that has been demonstrated to exhibit antitumor activities (13). It is the only member of the cathelin family in humans, and is composed of 37 amino acid residues in the C-terminal domain. LL-37 has been demonstrated to serve an important functional role in regulating innate immunity via neutrophils (13-15). In addition to its antibacterial and immune activities, LL-37 is reportedly involved in angiogenesis and cell proliferation (14,16). Studies have demonstrated that LL-37 expression is upregulated in ovarian, breast, prostate and lung cancer, which may promote tumor cell proliferation, migration and invasion (17-22). The oncogenic mechanisms by which LL-37 functions may be associated with the formyl peptide receptor (FPR)-like 1, EGFR and insulin-like growth factor 1 receptor; however, the specific mechanisms remain unclear (15,23-25). In addition,

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Thermo Fisher Scientific, Inc.). Reaction mixtures contained 5  $\mu$ l cDNA (diluted 1:5), 1  $\mu$ l forward primer, 1  $\mu$ l reverse primer, 10  $\mu$ l qPCR Master Mix 10 and 3  $\mu$ l nuclease-free water. Thermal cycling parameters consisted of 50 cycles of 95°C for 10 sec and 60°C for 30 sec. The primer sequences were as follows: Human *YB-1* forward, 5'-CAGAATAGTGAG AGTGGGG-3', and reverse, 5'-ATGTAGTAAGGTGGGAAC C-3'; Human  $\beta$ -actin forward, 5'-TTCCATATCGTCCCAGTT GGT-3', and reverse, 5'-CCAGGGCGTTATGGTAGGCA-3'. mRNA levels were calculated using the  $2^{-\Delta\Delta Cq}$  method (26).

**Immunofluorescence staining.** Following the transfer of a monolayer of cells ( $\sim 3 \times 10^5$  cells) to a cell climbing film (Medical Equipment Factory, Xi'an Jiaotong University, Xi'an, China), LL-37 was added at 0, 0.05, 0.5 or 5  $\mu$ g/ml and incubated for 48 h. Cells were subsequently fixed with 4% paraformaldehyde at room temperature for 10 min, and then treated with 5% Triton X-100 for 15 min followed by 1% goat serum for 30 min. Cells were incubated with the rabbit anti-human YB-1 monoclonal antibody (1:50 dilution) overnight at 4°C. Cells were subsequently incubated with a fluorescein isothiocyanate-labeled goat anti-rabbit antibody [from KIT-9710; UltraSensitive™ SP (Mouse/Rabbit) IHC Kit; Fuzhou Maixin Biotechnology Co., Ltd., Fuzhou, China] at 37°C for 1 h, and then stained with DAPI (Sigma-Aldrich; Merck Millipore) for 1 min. Staining intensities were visualized using an inverted fluorescence microscope (LSM 700; Zeiss AG, Oberkochen, Germany).

**Signal transduction pathway analysis.** Cells were seeded at  $1 \times 10^5$  cells/well in 6-well plates. They were initially treated with 10  $\mu$ M mitogen-activated protein kinase kinase (MEK) inhibitor (PD98059), 10  $\mu$ M p38/mitogen-activated protein kinase (p38/MAPK) inhibitor (SB203580) or 1  $\mu$ M NF- $\kappa$ B inhibitor (PDTC; all from Abcam), for 30 min. Cells were subsequently treated with 0.5  $\mu$ M LL-37 for a further 24 h. The protein expression levels of YB-1 and  $\beta$ -actin were then determined by western blot analysis using the aforementioned procedures.

**Statistical analysis.** Data are presented as the mean  $\pm$  standard deviation. Statistical significance between two groups was determined using the Student's *t*-test or one-way analysis of variance with least significant difference post-hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**YB-1 siRNA transfection inhibits YB-1 protein expression and reduces the viability and invasion of MM cells.** Total protein was extracted from A375 and A875 MM cells at 48 h following transfection with YB-1 siRNA, and YB-1 protein expression levels were determined by western blotting. Compared with the control group (transfected with non-targeting siRNA), the protein expression levels of YB-1 in YB-1 siRNA-transfected cells were significantly reduced (Fig. 1A). Initially, three siRNAs were tested and the most effective siRNA (siRNA1 in A375 cells and siRNA2 in A875 cells) were used in the following studies. In addition, the

viability of YB-1 siRNA-transfected A375 and A875 cells was significantly decreased at 24 h following transfection when compared with the control group ( $P = 0.016$  in A375 cells;  $P = 0.018$  in A875 cells; Fig. 1B), indicating that the YB-1 may regulate MM tumor cell viability. Results from the Transwell invasion assay demonstrated that the number of YB-1 siRNA-transfected cells that traversed the membrane following 24 h was significantly lower when compared with that in the control transfection group ( $P = 0.026$  in A375 cells;  $P = 0.021$  in A875 cells; Fig. 1C). This suggests that YB-1 may control tumor cell invasion. Therefore, YB-1 depletion may reduce the viability and invasiveness of A375 and A875 cells *in vitro*.

**LL-37 treatment increases the viability of MM cells.** In order to investigate the effect of LL-37 treatment on the viability of MM cells, A375 and A875 cells were exposed to increasing concentrations of LL-37 for 24, 48 and 72 h. Compared with the untreated group, exposure to 0.05, 0.5 and 5  $\mu$ g/ml LL-37 was associated with an increase in cell viability at all time points. A statistically significant increase was observed following exposure of the two cell lines to 0.5  $\mu$ g/ml at all time points, with the exception of A375 cells treated for 72 h ( $P = 0.003$  in A375 cells;  $P = 0.001$  in A875 cells; Fig. 1D).

**LL-37 promotes the migration and invasion of MM cells.** Compared with the untreated control group, exposure of A375 and A875 cells to 0.05, 0.5 and 5  $\mu$ g/ml LL-37 was associated with an increase in cell migration after 12 h ( $P = 0.018$ , Fig. 2A; and  $P = 0.001$ , Fig. 2B) and invasion after 24 h ( $P = 0.011$ , Fig. 2C; and  $P = 0.015$ , Fig. 2D). A statistically significant increase in A375 and A875 cell migration and invasion was observed following exposure to 0.5  $\mu$ g/ml LL-37 at both time points.

**LL-37 promotes YB-1 expression in MM cells.** Compared with the untreated control, exposure of A375 and A875 cells to 0.5  $\mu$ g/ml LL-37 for 24 h, was associated with a significant increase in YB-1 mRNA expression levels [ $P = 0.048$  (time) and  $P = 0.031$  (dose) in A375 cells;  $P = 0.012$  (time) and  $P = 0.023$  (dose) in A875 cells; Fig. 3A]. Western blot analysis of YB-1 protein expression demonstrated that exposure to LL-37 for 48 h led to a marked increase in YB-1 protein expression levels within A375 and A875 cells, with the most notable increase observed at a concentration of 0.5  $\mu$ g/ml (Fig. 3B and C). These results were confirmed by immunofluorescence staining of YB-1 in cells following exposure to increasing concentrations of LL-37, which demonstrated increased fluorescence intensities (Fig. 4A). The most notable increase in YB-1 expression was observed following exposure of A375 and A875 cells to 0.5  $\mu$ g/ml LL-37 (Fig. 4A). These results suggest that LL-37 promoted the expression of YB-1 in A375 and A875 cells.

**Inhibition of the NF- $\kappa$ B signalling pathway prevents LL-37-mediated induction of YB-1 expression.** Based on earlier results demonstrating that YB-1 expression induced by LL-37 was mediated by the NF- $\kappa$ B signalling pathway in ovarian cancer cells (27), the role of the NF- $\kappa$ B signalling pathway in mediating this effect in MM cells was investigated.



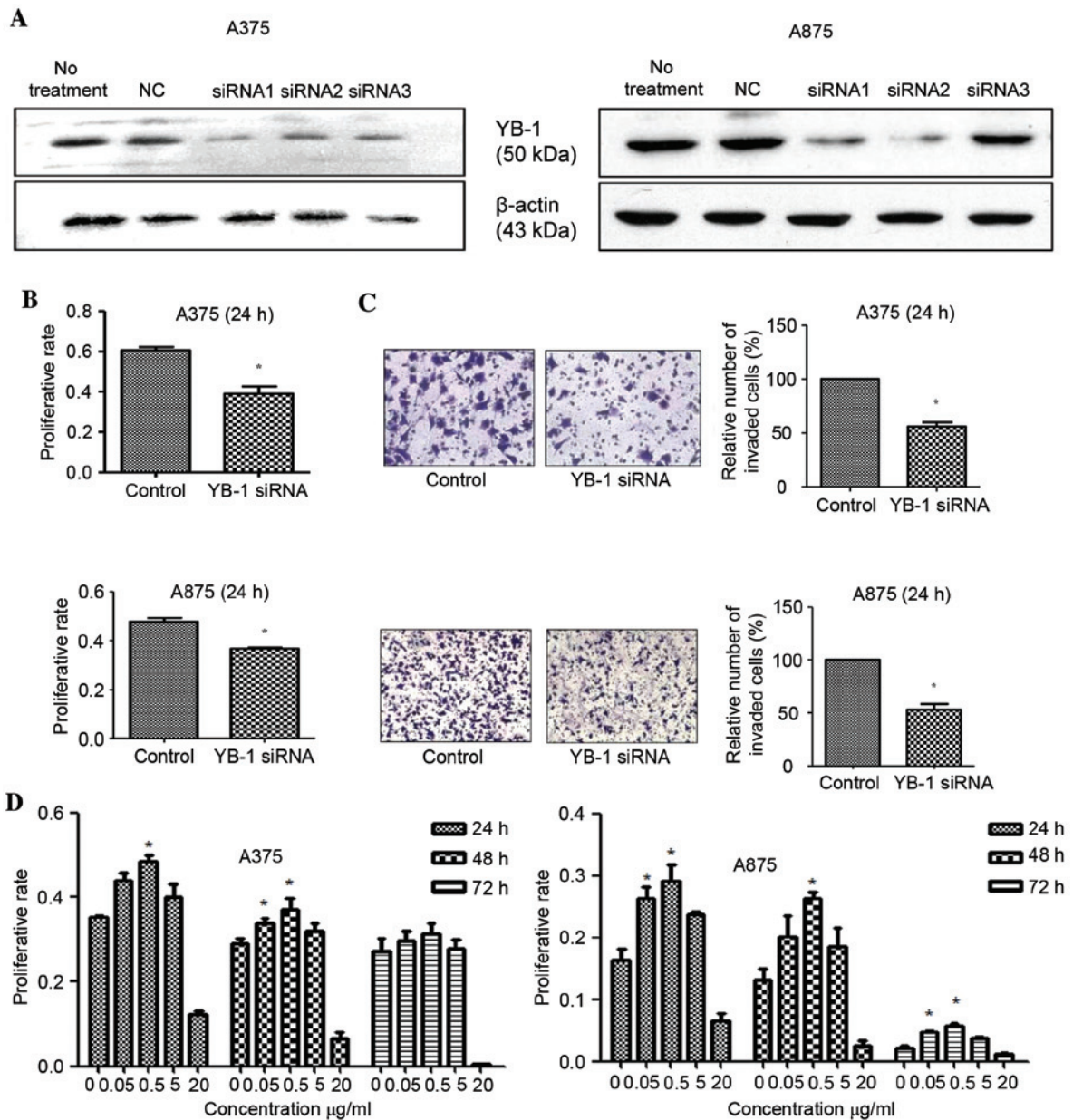


Figure 1. Effect of YB-1 knockdown on MM cell viability and invasion, and the effect of LL-37 exposure on MM cell viability. (A) Western blot analysis showed that siRNA1 in A375 cells and siRNA2 in A875 cells successfully reduced YB-1 protein expression. (B) Cell viability was reduced in A375 and A875 cells following YB-1 siRNA transfection, as demonstrated by MTT assay analysis. (C) The Transwell invasion assay demonstrated that the number of invaded cells was reduced at 24 h following transfection with YB-1 siRNA (magnification,  $\times 200$ ). (D) The viability of A375 and A875 cells following exposure to 0, 0.05, 0.5, 5 and 20  $\mu$ g/ml LL-37 for 24, 48 and 72 h as determined by MTT assay. Data are presented as mean  $\pm$  standard deviation of three independent experiments, with five samples in each treatment group. \* $P < 0.05$  vs. control (non-targeting siRNA) or 0  $\mu$ g/ml LL-37. YB-1, Y-box binding protein 1; MM, malignant melanoma.

To do this, A375 and A875 cells were treated with a MEK inhibitor (PD98059), a p38/MAPK inhibitor (SB203580), or a NF- $\kappa$ B inhibitor (PDTC) prior to treatment with LL-37. The p38/MAPK and NF- $\kappa$ B inhibitors significantly reduced LL-37-induced YB-1 protein expression levels in A375 cells, whereas the MEK and NF- $\kappa$ B inhibitors significantly reduced the expression in A875 cells. Thus, the NF- $\kappa$ B inhibitor PDTC significantly decreased the LL-37-induced expression of YB-1 in both cell lines ( $P = 0.001$  in A375 cells;  $P = 0.002$  in A875 cells; Fig. 4B). These results suggest that the NF- $\kappa$ B signaling pathway may serve a role in mediating the induction of YB-1 expression by LL-37 in MM cells.

## Discussion

LL-37 is a member of the antimicrobial peptide family, and its expression is correlated with the proliferation of epidermal cells (13,14). Previous studies have demonstrated that LL-37 may promote the progression of numerous malignant tumors, including lung, ovarian and prostate cancer, as well as oral squamous cell carcinoma. LL-37-mediated tumor progression is suggested to be associated with the upregulation of EGFR and the HER-2 receptor tyrosine kinase (15,23-25,28). LL-37 transactivates the EGFR via matrix metalloproteinase (MMP)-mediated cleavage of membrane-anchored

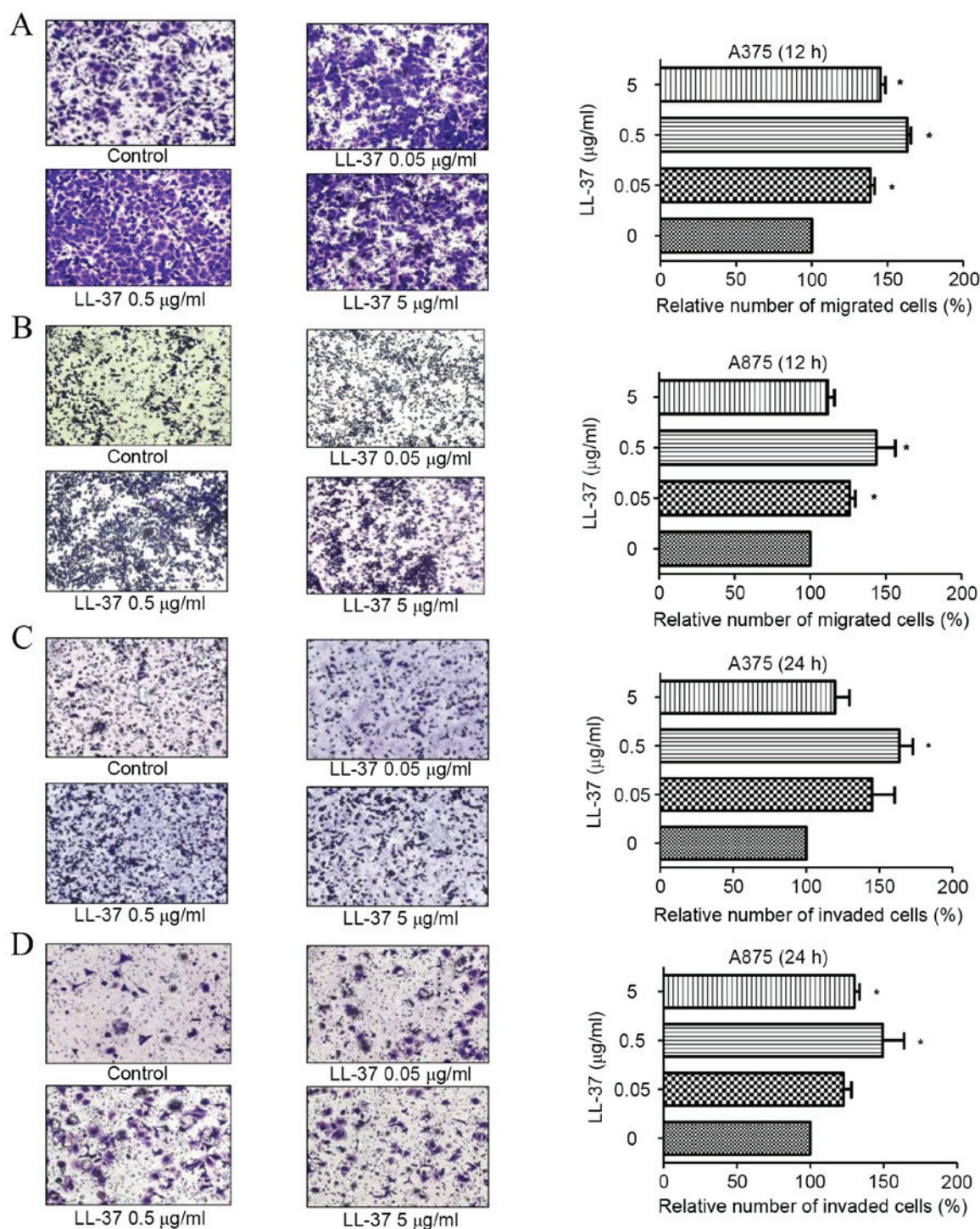


Figure 2. LL-37 increases the migration and invasion of MM cells. The relative migration of (A) A375 and (B) A875 cells, and the relative invasion of (C) A375 and (D) A875 cells following exposure to 0, 0.05, 0.5 and 5 µg/ml LL-37 for 12 and 24 h, respectively, as determined using Transwell assays (magnification, x200). Data represent the mean ± standard deviation of three randomly selected fields of view. \*P<0.05 vs. 0 µg/ml LL-37. LL-37, cathelicidin antimicrobial peptide; MM, malignant melanoma.

EGFR-ligands (29), which may depend on G-protein-coupled receptors (15,23-25). In lung squamous cell carcinoma, LL-37 stimulates the proliferation and invasion of tumor cells via its mitogenic effect on EGFR phosphorylation, and the subsequent activation of the Ras/MAPK signaling cascade (22). EGFR signaling in lung cancer cells serves a direct role in promoting cell proliferation, anti-apoptotic signaling pathways, angiogenesis, invasion and metastasis (15,22). The majority

of EGFR ligands, such as transforming growth factor and heparin-binding epidermal growth factor, are soluble transmembrane precursors, which are released following proteolytic cleavage (24,30,31). These precursors diffuse freely, and bind to and activate the EGFR. Thus, the oncogenic effects of LL-37 in specific tissues may be mediated by the activation of EGFR (24,30,31). In breast cancer, LL-37 promotes tumor progression via activation of the HER-2-mediated signaling



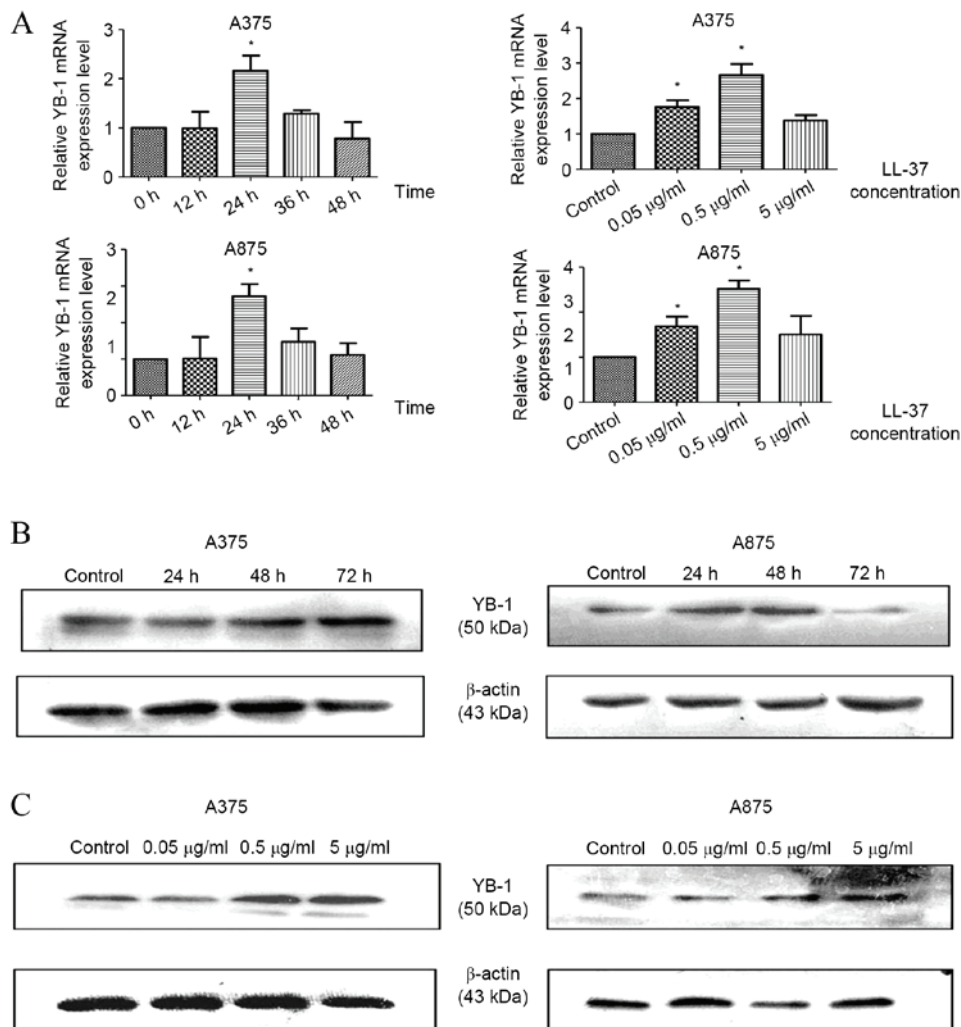


Figure 3. LL-37 promotes YB-1 expression in A375 and A875 cells. (A) Reverse transcription-quantitative polymerase chain reaction analysis of YB-1 mRNA expression in cells exposed to 0.5 µg/ml LL-37 for 0, 12, 24, 36 or 48 h or exposed to 0, 0.05, 0.5 and 5 µg/ml LL-37 for 24 h. The mRNA levels of YB-1 are shown relative to β-actin mRNA. Western blot analysis of YB-1 protein expression levels in A375 and A875 cells following exposure to (B) 0.5 µg/ml LL-37 for 0, 24, 48 and 72 h, and (C) 0, 0.05, 0.5 and 5 µg/ml LL-37 for 48 h. Data are presented as the mean ± standard deviation of three independent experiments. \*P<0.05 vs. control. LL-37, cathelicidin antimicrobial peptide; YB-1, Y box binding protein 1; MM, malignant melanoma.

pathway, which may also be mediated by FPR2 (20). LL-37 triggers the activation of MAPK and the Janus kinase signal transducer in a biochemical cascade that leads to the activation of multiple transcription factors. This process may be dependent or independent of FPR2 (23,25,32). LL-37 enhances the invasiveness of ovarian cancer cells by increasing the activity of tissue remodelling enzymes, such as the MMP-2 (21,24,30,31). FPR2 in ovarian cancer cells increases the expression of MMP-2, thus inhibiting or blocking LL-37 receptors and promoting tumor cell invasiveness (21,24,30,31).

Previous studies have demonstrated that YB-1 regulates epithelial cell proliferation and is overexpressed in numerous tumors (33-36). In transgenic mice, an increase in FPR2 expression promotes the transcription of YB-1 (12,37). Previous studies have demonstrated that Y-box family proteins may mediate the regulation of EGFR and HER-2 expression by binding to the enhancer and promoter regions of these genes, respectively (7,9). An additional study demonstrated that upregulation of YB-1 is mediated by the upregulation of extracellular signal-regulated kinase 2 (ERK2) and glycogen synthase

kinase-3 (GSK-3β) activity. ERK2 and GSK-3β activity is critical for cell proliferation and apoptosis. Therefore, YB-1 may be a downstream target of ERK2 and GSK-3β (11,38).

To the best of our knowledge, no previous report has demonstrated the interaction between LL-37 and YB-1. However, the current study demonstrated that LL-37, which shares certain common features with YB-1, may promote the progression and malignant biological behavior of numerous malignant tumors. The results of the present study demonstrated that knockdown of YB-1 expression inhibits the viability and invasion of A375 and A875 MM cells *in vitro*. Following exposure to 0.05-5 µg/ml LL-37, A375 and A875 cells exhibited an increase in YB-1 expression. These results suggest that LL-37 may function to upregulate YB-1 expression MM cells. Previous studies have reported that LL-37 increases the malignant potential of tumor cells, which may be associated with activation of the Ras/MAPK and NF-κB signaling pathways (39,40). Therefore, ERK, MAPK and NF-κB inhibitors were employed in the present study to investigate the role of the NF-κB signaling pathway in mediating

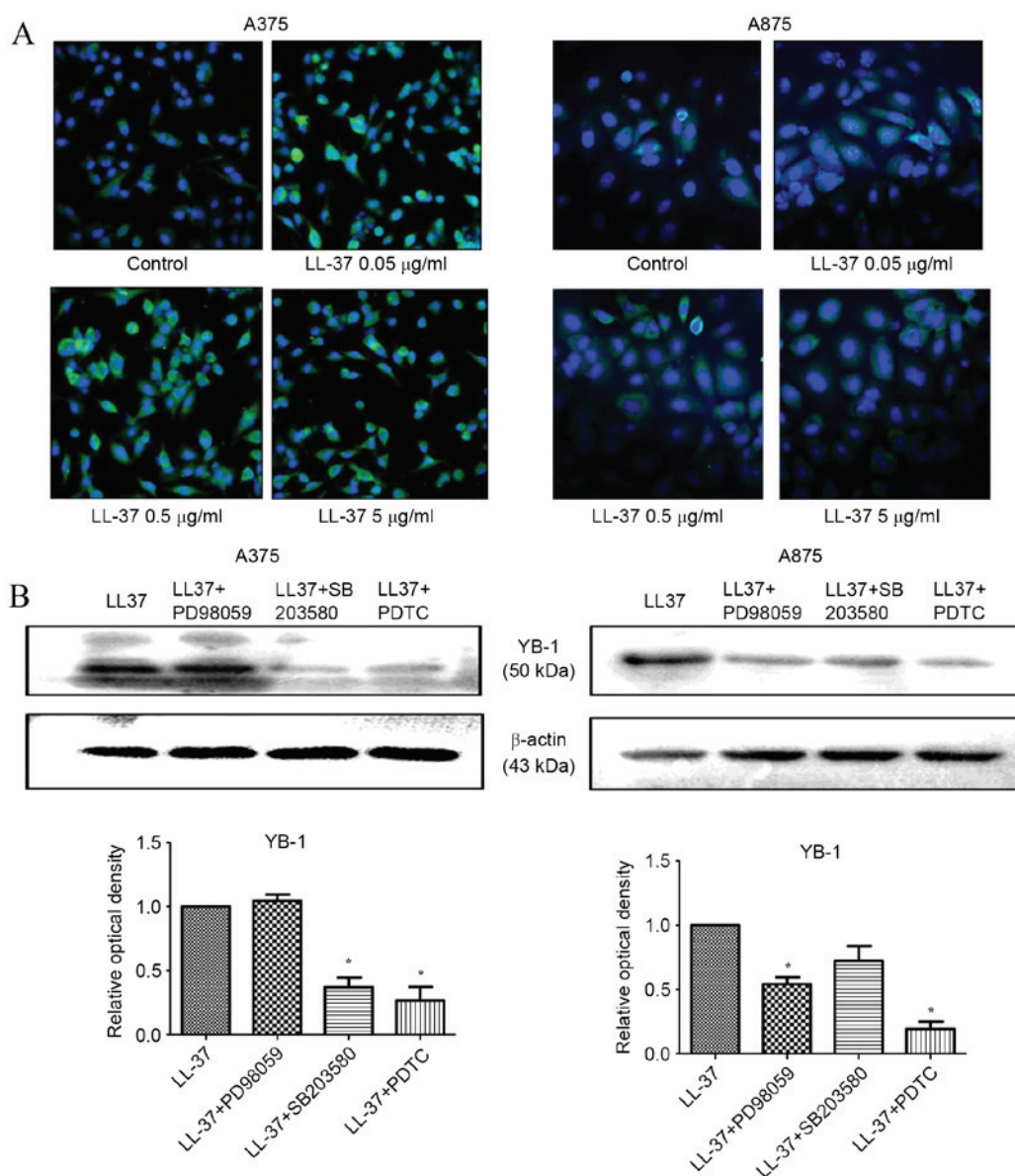


Figure 4. LL-37-induced YB-1 expression in MM cells is attenuated by inhibition of members of the NF- $\kappa$ B signaling pathway. (A) Immunofluorescence staining of YB-1 expression in A375 and A875 cells following exposure to various concentrations of LL-37 for 48 h (magnification,  $\times 400$ ). (B) Western blot analysis of YB-1 protein expression levels following treatment of A375 and A875 cells with LL-37, LL-37 + 10  $\mu\text{M}$  mitogen-activated protein kinase kinase inhibitor (PD98059), LL-37 + 10  $\mu\text{M}$  p38/mitogen-activated protein kinase inhibitor (SB203580) or LL-37 + 1  $\mu\text{M}$  NF- $\kappa$ B inhibitor (PDTTC). The NF- $\kappa$ B signaling pathway was involved in the upregulation of YB-1 stimulated by LL-37. Protein expression levels are shown relative to  $\beta$ -actin. Data are presented as the mean  $\pm$  standard deviation of three independent experiments. \* $P < 0.05$  vs. LL-37. LL-37, cathelicidin antimicrobial peptide; YB-1, Y box binding protein 1; MM, malignant melanoma; NF- $\kappa$ B, nuclear factor- $\kappa$ B.

LL-37-induced YB-1 expression. Inhibition of NF- $\kappa$ B in A375 and A875 cell lines significantly reduced the LL-37-induced YB-1 protein expression. These results suggest that LL-37 may upregulate YB-1 expression via the NF- $\kappa$ B signaling pathway. LL-37-mediated activation of EGFR and HER-2 is accompanied by the phosphorylation of EGFR and activation of the subsequent Ras/MAPK cascade, thereby promoting the expression of MMP-2 via FPR2. In addition, EGFR, HER-2 and FPR2 may regulate YB-1 expression (22,23), and the overexpression of these factors may enhance tumor cell proliferation and invasion. This suggests that, LL-37 may promote the proliferation and invasion of A375 and A875 cells through the upregulation of YB-1 expression. NF- $\kappa$ B is a transcription factor that is known to regulate the expression

of multiple genes, and is involved in a wide range of cellular responses (41,42). NF- $\kappa$ B activation serves a significant role in promoting tumor metastasis, and inhibition of NF- $\kappa$ B can prevent the induction of apoptosis (41,42).

In conclusion, the results of the present study are consistent with the reported role of YB-1 as a marker of malignancy in MM. In addition, the antimicrobial peptide LL-37 was demonstrated to upregulate YB-1 expression and promote the viability, migration and invasion of A375 and A875 cells *in vitro*. Furthermore, the NF- $\kappa$ B signaling pathway may mediate LL-37-induced YB-1 expression. This study provides a novel insight into the association between LL-37 and YB-1 in MM, and provides potential therapeutic targets for the treatment of MM.

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