

Inhibition of AIM2 expression enhance treatment effect of osimertinib in treatment of glioma

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Abstract

Introduction: Glioma is one of the most commonly tumours which occurs in the central nervous system and accounts for nearly 80% of brain tumours, with a significantly high mortality and morbidity. Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) are used as EGFR targeted therapy in various types of solid tumours; however, effective treatment for glioma is still limited. Osimertinib is an irreversible, oral third-generation TKI that targets the mutation at T790M, which causes cancer cells to acquire resistance to drugs. Osimertinib could be effective in the treatment of EGFR mutations with minimal effects on the activity of wild-type EGFR. Absent in melanoma 2 (AIM2) is highly expressed in glioma cells, promoting the maturation of pro-cancer cytokines and contributing to progression of glioma. However, the secretion of pro-cancer cytokines of tumour cells has been regarded as the resistance mechanism to EGFR-TKIs, including osimertinib. A high level of these cytokines also indicates a shorter progression-free survival (PFS). As AIM2 regulates the secretion of pro-cancer cytokines, we thought inhibition of AIM2 may contribute to the therapeutic effect of EGFR-TKIs.

Material and methods: We first established AIM2 inhibition and overexpression in cells. Then, the viability rate of cells was calculated by cell counting kit-8 (CCK-8) method, and apoptotic ratio of cells were measured by flow cytometry. The expression of inflammatory-related genes was detected using quantitative polymerase chain reaction (qPCR), concentrations of inflammatory-related factors were measured using enzyme-linked immunosorbent assay (ELISA). The expression of Wnt/ β -catenin and EGFR/Ras/Mitogen-activated protein kinase kinase 1 (MEK) signalling pathway components was detected using western blotting.

Results: We found that inhibition of AIM2 enlarged the effect of osimertinib on the upregulation of inflammatory gene expression and secretion of these genes, increasing apoptosis. In addition, we also found that AIM2 could enhance the effect of osimertinib on reducing the expression of the Wnt/ β -catenin and EGFR/Ras/MEK signalling pathways, resulting in the inhibition of cellular proliferation, and exerting an anti-tumour effect. These effects were also observed using in vivo experiments.

Conclusions: AIM2 presents a potential therapeutic target in treatment of glioma.

Key words: AIM2, osimertinib, glioma, Wnt/ β -catenin, EGFR/Ras/MEK, inflammatory response.

Introduction

Absent in melanoma 2 (AIM2) was first found in 1997 [15]. AIM2 initiates the formation of the AIM2

inflammasome by directly binding with double-stranded DNA in macrophages, leading to the inflammatory response and cell death mediated by caspase-1, performing a protective role against bacterial and viral

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pathogens [21,43]. Reduced AIM2 expression enhanced the hepatocellular carcinoma metastasis ability *via* the inflammasome pathway, while overexpression of AIM2 limits migration and invasion ability in renal carcinoma [8,26]. However, recent research found that expression of AIM2 leads to the tumorigenesis process of glioma. Glioma is one of the most commonly seen brain tumours in adults, and glioma is mostly found primarily in the brain, then develops into glial tissue [33].

Osimertinib was approved in 2015 for the treatment of non-small-cell lung cancer (NSCLC) patients with a T790M mutation in epidermal growth factor receptor (EGFR) [12,19]. Osimertinib could inhibit the expression of EGFR with T790M, de119 and L858R mutations with minimal inhibition of the expression of wild-type EGFR protein compared with other types of approved EGFR TKIs, reducing the severity of toxicities in dermatological and gastrointestinal [13]. In addition, penetration and efficacy of osimertinib to the central nervous system (CNS) were also increased compared with those of other first-line EGFR inhibitor therapies; the median progression-free survival (PFS) of patients with CNS progression who received osimertinib was 8.5 months, while it was 4.2 months with chemotherapy based on platinum [4,30]. AURA study (ClinicalTrials.gov identifier: NCT01802632.), includes 60 patients with advanced EGFR-mutant NSCLC without previous treatment and initiated therapy with osimertinib. The PFS was 20.5 months, nearly double that in the gefitinib, erlotinib, and afatinib treatment groups, in FLAURA study (NCT02296125), osimertinib therapeutic group significantly increased the PFS compared with erlotinib or gefitinib in patients with advanced NSCLC, and also significantly increased the median overall survival duration [11,36]. Another clinical trial observed that the median PFS was 8.7 months longer in the osimertinib therapy group than the other first-line EGFR inhibitor treatment group, including the CNS metastasis group. According to the study, 13% of patients who receive 16.2 months of osimertinib treatment experienced adverse events, while 18% of those patients received 11.5 months of standard treatment [42]. Therefore, we thought inhibition of AIM2 expression might enhance the therapeutic effect of osimertinib, and provide a long life for glioma patients.

Herein, we constructed an AIM2 inhibition and overexpression model in A172 and LN-18 cell lines, and apoptotic cells were also detected using flow cytometry. Osimertinib affects the expression and secretion of inflammatory genes, with a decrease in the secretion of angiogenesis-related molecules. Besides, we also found that the activation of the Wnt/ -catenin and EGFR/Ras/Mitogen-activated protein kinase kinase 1 (MEK) signalling pathways leads to inhibition proliferation of

cancer cells, thus exerting an antitumor effect. We further found that osimertinib function can be enhanced through inhibiting AIM2 expression. We found similar results using *in vivo* experiments. We thought inhibition of AIM2 expression might be a target for glioma treatment.

Material and methods

Material

H-DMEM (11965-092), FBS (10100), FastDigest BsmBI (ER0451) and Lipo 3000 (L3000015) were purchased from Thermo (Waltham, USA). Quick Ligase (M2200) and T4 PNK (M02015) were purchased from NEB (Beijing, China). A total RNA extraction kit (R1200), Cell Counting Kit-8 (CK04), puromycin (P8230) and G418 (G8160) were purchased from Solarbio (Beijing, China). UltraSYBR Mixture (CW0957) and a HiFiScript cDNA Synthesis Kit (CW2569) were obtained from CWBio (Beijing, China). Anti-Wnt (ab15251), β -catenin (ab32572), LEF1 (ab137872), Cyclin D1 (ab134175), Axin 2 (ab109307), GSK-3 β (ab32391), EGFR (ab52894), Ras (ab52939), Raf (ab200653), p-MEK1 (ab214445), MEK1 (ab96379), c-Fos (ab190289), c-Myc (ab32072), FGF (ab99979), VEGF (ab222510), TNF- α (ab181421), IL-6 (ab46027), IL-2 (ab174444) and IL-1 β (ab46052) kits were purchased from Abcam (Cambridge, UK).

Cell culture and grouping

A172 (CRL-1620), LN-18 (CRL-2610) and 293T (CRL-3216) cells were obtained from ATCC. Cells were cultured under H-DMEM supplied 10% FBS in a 37°C, 5% CO₂ humidified atmosphere. Cells were divided into four groups: the control group (NC), osimertinib treatment group (OG), AIM2 inhibition with osimertinib treatment group (AI) and AIM2 overexpression with osimertinib treatment group (AO). In the osimertinib treatment group, cells were first treated with 500 nM for 24 h before performing the following experiments.

Construction of vectors

AIM2 cDNA was obtained through the PCR method with this primer: Forward: 5'-AAAATGCTGGGGCGGTGTCT-3', Reverse: 5'-GTGTCCTCGTTTCTAACCCC-3'. The PCR product of AIM2 digested with BamHI and XhoI, then cloned into pCDNA3.1-3 \times Flag to construct the pCDNA3.1-3 \times Flag-AIM2 overexpression vector. Then, the AIM2 overexpression vector was transfected A172 and LN-18 cells with Lipo 3000 transfection reagent. Then, stable expressed A172/AIM2 and LN-18/AIM2 cells were screened using G418. The AIM2 knockdown vector was constructed according to the previous study [25]. Briefly, pairs of oligos were prepared with the

following primers: forward: 5'-CACCGCCTTATCCTACCTAACATG-3', reverse: 5'-AAACCATGTTAAGGTAGGATAAGGC-3', and the CRISPR plasmid was digested with BsmBI for 30 min at 37°C. Then, oligos were phosphorylated with T4 PNK using the following reaction sequence: incubated at 37°C for 30 min followed by incubation at 95°C for 5 min and ramp down to room temperature. The digested plasmid and oligos were ligated using Quick Ligase and then transfected into 293T cells to construct the AIM2-knockdown lentiviral vector. The stable A172/AIM2-knockdown and LN-18/AIM2-knockdown cells were screened using 2 µg/ml puromycin.

CCK-8 assay

Cells were seeded in a 96-well plate at the concentration 1×10^4 and grouped as described above. Then, cells were stimulated with CCK-8 for 4 h. After incubation, optical density was measured at 450 nm using a microplate reader (Varioskan LUX, Thermo).

RNA extraction

RNA extraction was performed according to the manufacturer's protocol of the RNA extraction kit. Cells were grouped and treated as described above. Then, cells were lysed when confluence of cells reached 80-90%. After incubation for 5 min at room temperature, chloroform was added to the mixture, and incubated for 5 min at room temperature. After centrifugation at 12000 rpm for 10 min, the RNA in the water phase was transferred into a collection tube. RNA was eluted into the collection tube by elution buffer. Concentration of RNA was measured using NanoDrop 2000.

Reverse transcription and real-time quantitative polymerase chain reaction (qPCR)

Reverse transcription was performed under the guideline of the manufacture's protocol of the HiFiScript cDNA Synthesis Kit. Briefly, the reaction mixture of reverse transcription was prepared according to the protocol. The reaction was performed with the following procedure: 42°C for 15 min, 85°C for 5 min. The product of reverse transcription was used to perform qPCR assays. Then, the reaction mixture of qPCR was prepared as recommended, and the reaction was performed using the following procedure: pre-degeneration at 95°C for 10 min, degeneration at 95°C for 15 s, annealing at 60°C for 1 min, degeneration and annealing were repeated for 40 cycles. Primers used in the qPCR experiment were listed: EGFR: forward: 5'-CAGAAGCCATCTCTGACTCCC-3', reverse: 5'-GTCCAGTGGTCAACAAGGTG-3'; AIM2: for-

ward: 5'-CTGCAGTGATGAAGACCATTGTA-3', reverse: 5'-GGTGACGACGTTGCTTTG-3'; IL-1β: forward: 5'-CGGCCACATTTGGTTCTAAGA-3', reverse: 5'-AGGGAAGCGTTGCTCATC-3', IL-2: forward: 5'-GTCCAAGGACACAGGCTTCTT-3', reverse: 5'-AAATTTGGCTGGTCCAAGG-3'; IL-6: forward: 5'-TCGAGCCACCGGAACGAA-3', reverse: 5'-GTGGCTGTCTGTGTGGGCG-3'; TNF-α: forward: 5'-TGGTGGTCTTGTGCTTAAAGTTC-3', reverse: 5'-CGAATCCAACCTCCCAAAC-3'. The expression of each target gene was analyzed using the $2^{-\Delta\Delta Cq}$ method [23]. GAPDH was used as an internal reference.

Western blotting analysis

Cells were divided into four groups as described above, and then lysed with RIPA buffer with a proteinase inhibitor cocktail. After centrifugation for 10 min at 12000 rpm, the supernatant was transferred into a collection tube. Concentration of proteins was determined using BCA assay. Then, 60 µg samples were separated by electrophoresis. Protein samples were transferred onto a 0.22 µm nitrocellulose membrane by semidry method. The membranes were incubated with skim milk followed by incubation with primary antibodies (1 : 1000) overnight at 4°C. Then, the membranes were incubated with secondary antibodies. The gray value of each target protein was detected by chemiluminescence.

ELISA

ELISA was performed according to the manufacturer's protocol of each kit. Briefly, standards and samples with antibodies were added and incubated at room temperature. After washing, TMB substrate was added and incubated at room temperature away from light. The OD value was measured at 450 nm after incubation with stop solution.

Flow cytometry

Flow cytometry was performed under the guideline of the manufacture's protocol of the apoptosis detection kit (CA1020, Solarbio). Briefly, cells were first digested with trypsin and then dissolved in binding buffer to a final concentration at 1×10^6 /ml. Then, 100 µl cell supernatants were incubated with Annexin V and PI away from light. The apoptotic cells were detected using a flow cytometry system (Navios, Beckman).

Statistical analysis

Data were shown as the mean \pm SD. One-way ANOVA was used to compare the significance of differences between groups. Each experiment was repeated three times independently. $P < 0.05$ was set as a significant difference.

Results

Effect of osimertinib on cell proliferation

In Figure 1A, C, the viability rates of A172 cells were 76.4 ± 6.6 , 58.6 ± 5.2 , and 94.1 ± 6.8 , respectively. Viability rates of LN-18 cells were 78.3 ± 6.5 , 60.2 ± 5.3 , and 96.8 ± 7.2 , respectively. In addition, in Figure 1E, F, the number of apoptotic A172 and LN-18 cells was increased after osimertinib treatment and higher after AIM2 inhibition. However, the number of apoptotic cells was decreased after AIM2 overexpression.

Detection of AIM2 expression in each group without osimertinib

In Figure 1B, D, the expression of AIM2 in A172 cells was 1.45 ± 0.10 , 1.80 ± 0.12 and 0.48 ± 0.03 in the NC, AO and AI groups, respectively. The expression of AIM2 in LN-18 cells was 1.40 ± 0.09 , 1.83 ± 0.12 and 0.59 ± 0.04 , respectively, indicating the successful establishment of AIM2 overexpression and knockdown cell models.

The expression of inflammation-related gene under osimertinib treatment in A172 cells

In Figure 2, the expression of the AIM2 gene was 1.05 ± 0.14 , 0.97 ± 0.11 , 0.73 ± 0.08 and 1.28 ± 0.16 , respectively. The expression of EGFR was 0.82 ± 0.08 , 0.71 ± 0.05 , 0.52 ± 0.03 and 1.01 ± 0.12 , respectively. The expression of IL-1 β was 1.23 ± 0.14 , 0.92 ± 0.10 , 0.71 ± 0.06 and 1.45 ± 0.13 , respectively. The expression of IL-2 was 0.96 ± 0.07 , 1.16 ± 0.09 , 1.34 ± 0.12 and 0.72 ± 0.05 , respectively. The expression of IL-6 was 1.43 ± 0.17 , 1.02 ± 0.12 , 0.75 ± 0.09 and 1.76 ± 0.15 , respectively. The expression of TNF- α was 1.10 ± 0.14 , 1.26 ± 0.19 , 1.54 ± 0.13 and 0.83 ± 0.09 , respectively.

Expression of inflammation-related genes under osimertinib treatment in LN-18 cells

In Figure 3, the expression of AIM2 in the NC, OT, AI and AO groups was 1.32 ± 0.12 , 1.02 ± 0.09 , 0.84 ± 0.06 and 1.26 ± 0.10 , respectively. The expression of EGFR was 1.02 ± 0.12 , 0.81 ± 0.09 , 0.57 ± 0.04 and 1.20 ± 0.16 , respectively. The expression of IL-1 β was 0.98 ± 0.08 , 0.76 ± 0.06 , 0.61 ± 0.04 and 1.25 ± 0.13 , respectively. The expression of IL-2 was 0.85 ± 0.09 , 1.03 ± 0.10 , 1.38 ± 0.15 and 0.92 ± 0.08 , respectively. The expression of IL-6 was 1.15 ± 0.14 , 1.07 ± 0.10 , 0.83 ± 0.08 and 1.38 ± 0.16 , respectively. The expression of TNF- α was 0.73 ± 0.06 , 0.92 ± 0.08 , 1.25 ± 0.14 and 0.86 ± 0.09 , respectively.

Secretion of inflammation-related molecules under osimertinib stimulation in A172 cells

In Figure 4, concentrations of TNF- α in the medium of A172 cells were 486.5 ± 19.8 , 563.2 ± 20.4 , 640.1 ± 23.4 and 528.5 ± 19.4 pg/ml in the NC, OT, AI and AO groups, respectively. Concentrations of IL-1 β were 211.5 ± 17.5 , 171.7 ± 14.5 , 156.3 ± 14.7 and 262.4 ± 18.6 pg/ml, respectively. Concentrations of IL-2 were 136.7 ± 12.8 , 164.3 ± 13.5 , 196.2 ± 16.4 and 146.8 ± 14.7 pg/ml, respectively. Concentrations of IL-6 were 155.3 ± 11.6 , 138.3 ± 11.2 , 105.6 ± 9.5 , and 170.4 ± 15.8 pg/ml, respectively. Concentrations of VEGF were 432.1 ± 20.6 , 378.4 ± 16.7 , 330.2 ± 14.5 and 416.3 ± 18.5 pg/ml, respectively. Concentrations of FGF were 360.2 ± 19.2 , 305.8 ± 17.6 , 265.1 ± 14.2 and 330.2 ± 17.8 pg/ml, respectively.

Secretion of inflammation-related molecules under osimertinib stimulation in LN-18 cells

In Figure 5, concentrations of TNF- α in the culture medium of LN-18 cells in the NC, OT, AI and AO groups were 368.4 ± 18.2 , 410.2 ± 20.1 , 532.1 ± 23.2 and 383.5 ± 17.9 pg/ml, respectively. Concentrations of IL-1 β were 184.3 ± 11.6 , 150.3 ± 10.5 , 122.6 ± 9.8 and 252.1 ± 13.7 pg/ml, respectively. Concentrations of IL-2 were 115.2 ± 8.3 , 163.1 ± 10.8 , 220.4 ± 14.2 and 135.2 ± 8.7 pg/ml, respectively. Concentrations of IL-6 were 140.2 ± 9.6 , 120.4 ± 8.7 , 93.6 ± 7.7 and 188.5 ± 12.7 pg/ml, respectively. Concentrations of VEGF were 396.2 ± 28.4 , 340.3 ± 22.3 , 285.1 ± 16.2 and 364.5 ± 24.1 pg/ml, respectively. Concentrations of FGF were 343.2 ± 21.8 , 316.4 ± 18.5 , 276.2 ± 16.3 and 325.4 ± 19.6 pg/ml, respectively.

Expression of the Wnt/ β -catenin pathway in each group of A172 cells

In Figure 6, the expression of Wnt in the NC, TO, AI and AO groups of A172 cells was 1.05 ± 0.07 , 0.71 ± 0.05 , 0.21 ± 0.01 and 0.99 ± 0.07 , respectively. Expression of β -catenin was 1.46 ± 0.10 , 0.80 ± 0.05 , 0.51 ± 0.03 and 1.19 ± 0.08 , respectively. The expression of LEF1 in these groups was 1.34 ± 0.09 , 1.14 ± 0.08 , 1.10 ± 0.07 and 1.69 ± 0.11 , respectively. The expression of Axin in these groups was 0.51 ± 0.03 , 0.65 ± 0.04 , 1.13 ± 0.08 and 0.72 ± 0.05 , respectively. The expression of GSK-3 β was 0.92 ± 0.06 , 1.07 ± 0.07 , 1.12 ± 0.07 and 0.85 ± 0.06 , respectively in these groups. The expression of cyclin D1 in these groups was 1.48 ± 0.10 , 0.62 ± 0.04 , 0.64 ± 0.04 and 1.02 ± 0.07 , respectively.

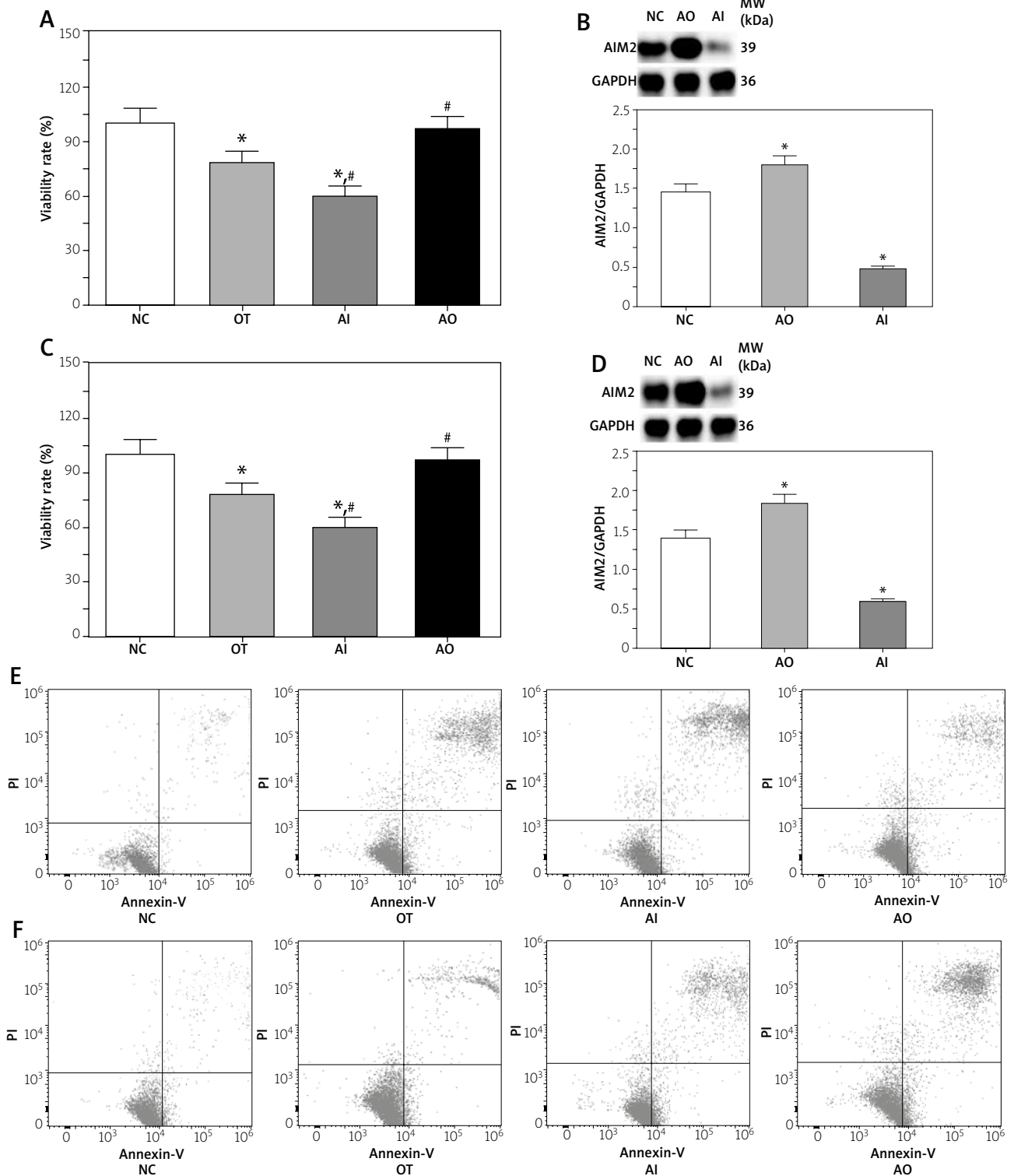


Fig. 1. Detection of the effect of osimertinib on proliferation of A172 and LN-18 cells in each group using the MTT assay. **A)** Effect of osimertinib on proliferation of A172 cells. **B)** Expression of AIM2 in NC, AO and AI groups of A172 cells. **C)** Effect of osimertinib on proliferation of LN-18 cells. **D)** Expression of AIM2 in NC, AO and AI groups of LN-18 cells. **E)** Apoptotic cells in each group of A172 cells. **F)** Apoptotic cells in each group of LN-18 cells. * $p < 0.05$ vs. the NC group, # $p < 0.05$ vs. the OT group. Data were presented as mean \pm SD. Each experiment was repeated three times independently.

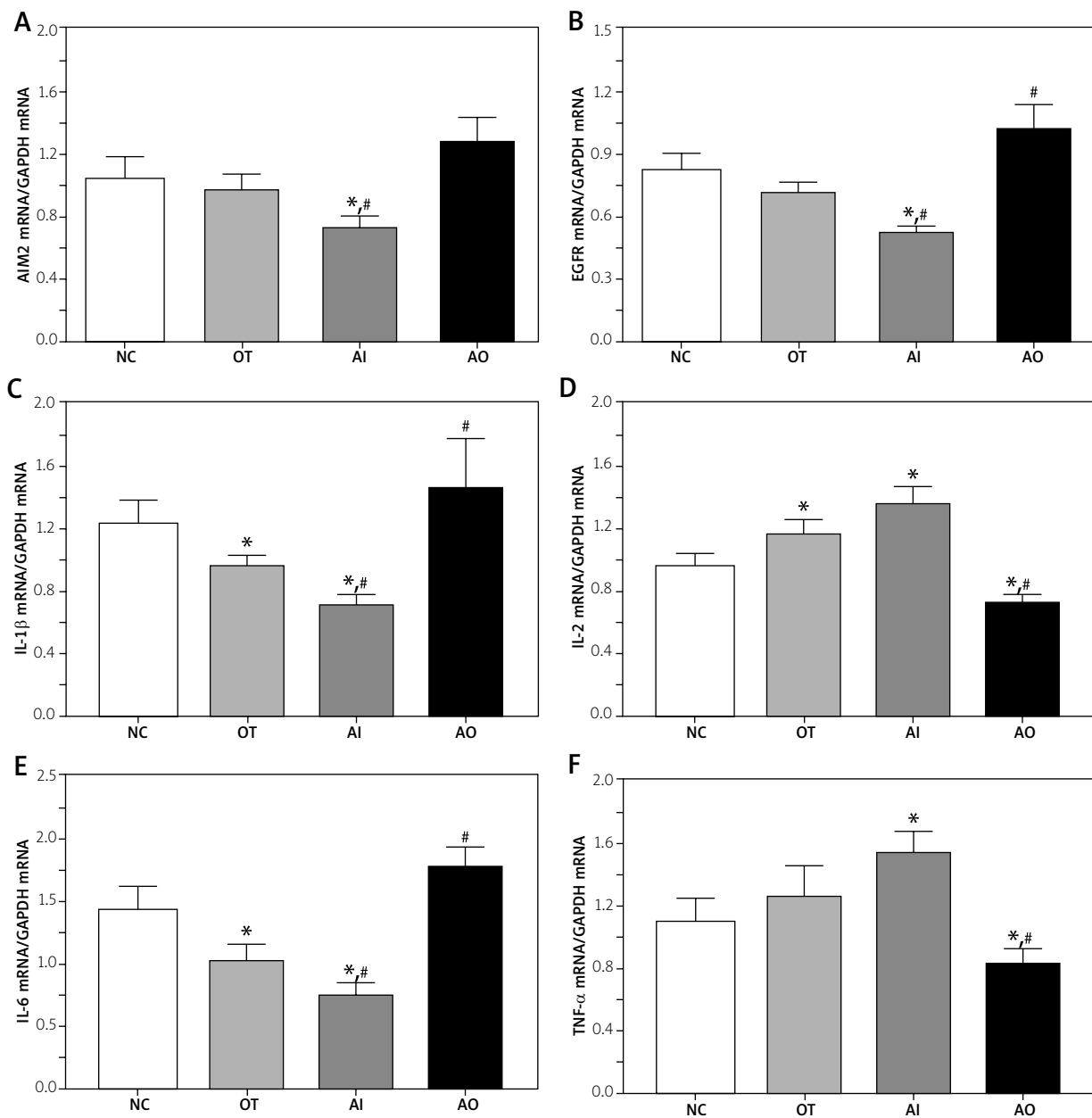


Fig. 2. Detection of the effect of osimertinib on expression of inflammation-related genes in A172 cells using qPCR method. **A)** Expression of AIM2 in each group. **B)** Expression of EGFR in each group. **C)** Expression of IL-1 β in each group. **D)** Expression of IL-2 in each group. **E)** Expression of IL-6 in each group. * $p < 0.05$ vs. the NC group, # $p < 0.05$ vs. the OT group. Data were presented as mean \pm SD. Each experiment was repeated three times independently. GAPDH was used as an internal control.

Expression of the Ras/MAPK pathway in each group of A172 cells

In Figure 7, the expression of EGFR in the NC, TO, AI and AO groups of LN-18 cells was 0.97 ± 0.06 , 0.87 ± 0.06 , 0.62 ± 0.04 and 1.09 ± 0.07 , respectively. The expression of Ras in these groups was 1.14 ± 0.08 , 1.02 ± 0.07 , 0.91 ± 0.06 and 1.22 ± 0.08 , respectively.

The expression of Raf in these groups was 1.50 ± 0.10 , 1.16 ± 0.08 , 0.81 ± 0.05 and 1.25 ± 0.08 , respectively. Ratios of p-MEK/MEK in these groups were 1.28 ± 0.09 , 1.13 ± 0.08 , 0.93 ± 0.06 and 1.59 ± 0.11 , respectively. The expression of c-Fos was 1.21 ± 0.08 , 1.11 ± 0.07 , 0.89 ± 0.06 and 1.47 ± 0.10 , respectively. The expression of c-Myc in these groups was 1.02 ± 0.07 , 0.95 ± 0.06 , 0.76 ± 0.05 and 1.24 ± 0.08 , respectively.

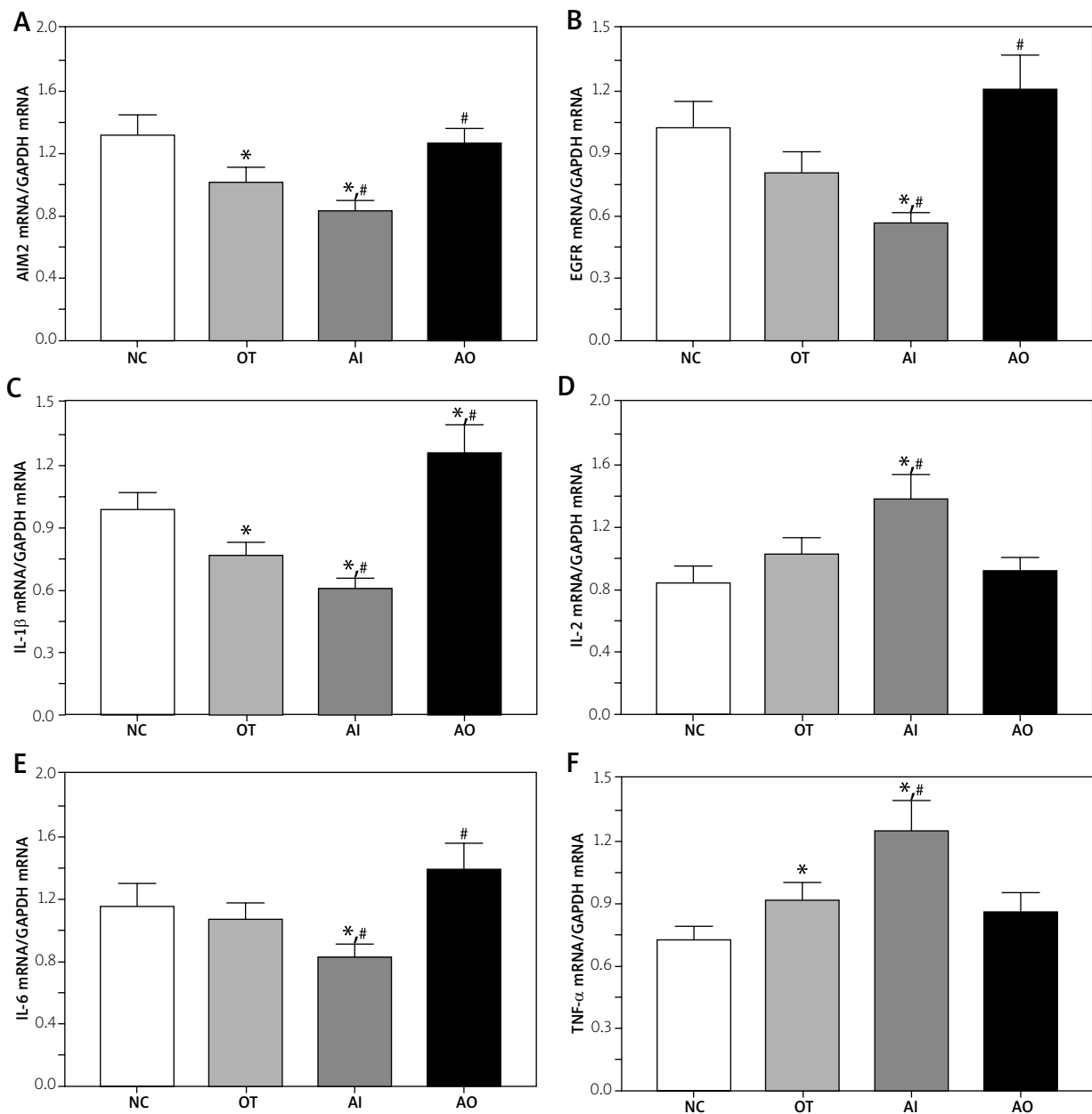


Fig. 3. Detection of the effect of osimertinib on expression of inflammation-related genes of LN-18 cells using the qPCR method. **A)** Expression of AIM2 in each group. **B)** Expression of EGFR in each group. **C)** Expression of IL-1β in each group. **D)** Expression of IL-2 in each group. **E)** Expression of IL-6 in each group. **p* < 0.05 vs. the NC group, #*p* < 0.05 vs. the OT group. Data were presented as mean ± SD. Each experiment was repeated three times independently. GAPDH was used as an internal control.

Expression of the Wnt/β-catenin pathway in each group of LN-18 cells

In Figure 8, the expression of Wnt in the NC, TO, AI and AO groups of A172 cells was 0.84 ± 0.06, 0.68 ± 0.05, 0.17 ± 0.01 and 0.62 ± 0.04, respectively. The expression of β-catenin in these groups was 0.80 ± 0.05, 0.67 ± 0.04, 0.45 ± 0.03 and 1.01 ± 0.07, respectively. The

expression of Axin in these groups was 0.17 ± 0.01, 1.26 ± 0.08, 1.50 ± 0.10 and 0.98 ± 0.07, respectively. The expression of LEF1 in these groups was 0.69 ± 0.05, 0.57 ± 0.04, 0.48 ± 0.03 and 0.54 ± 0.04, respectively. The expression of GSK-3β was 0.74 ± 0.05, 0.98 ± 0.07, 1.21 ± 0.08 and 0.59 ± 0.04, respectively in these groups. The expression of cyclin D1 in these groups was 0.91 ± 0.06, 0.75 ± 0.05, 0.60 ± 0.04 and 0.79 ± 0.05, respectively.

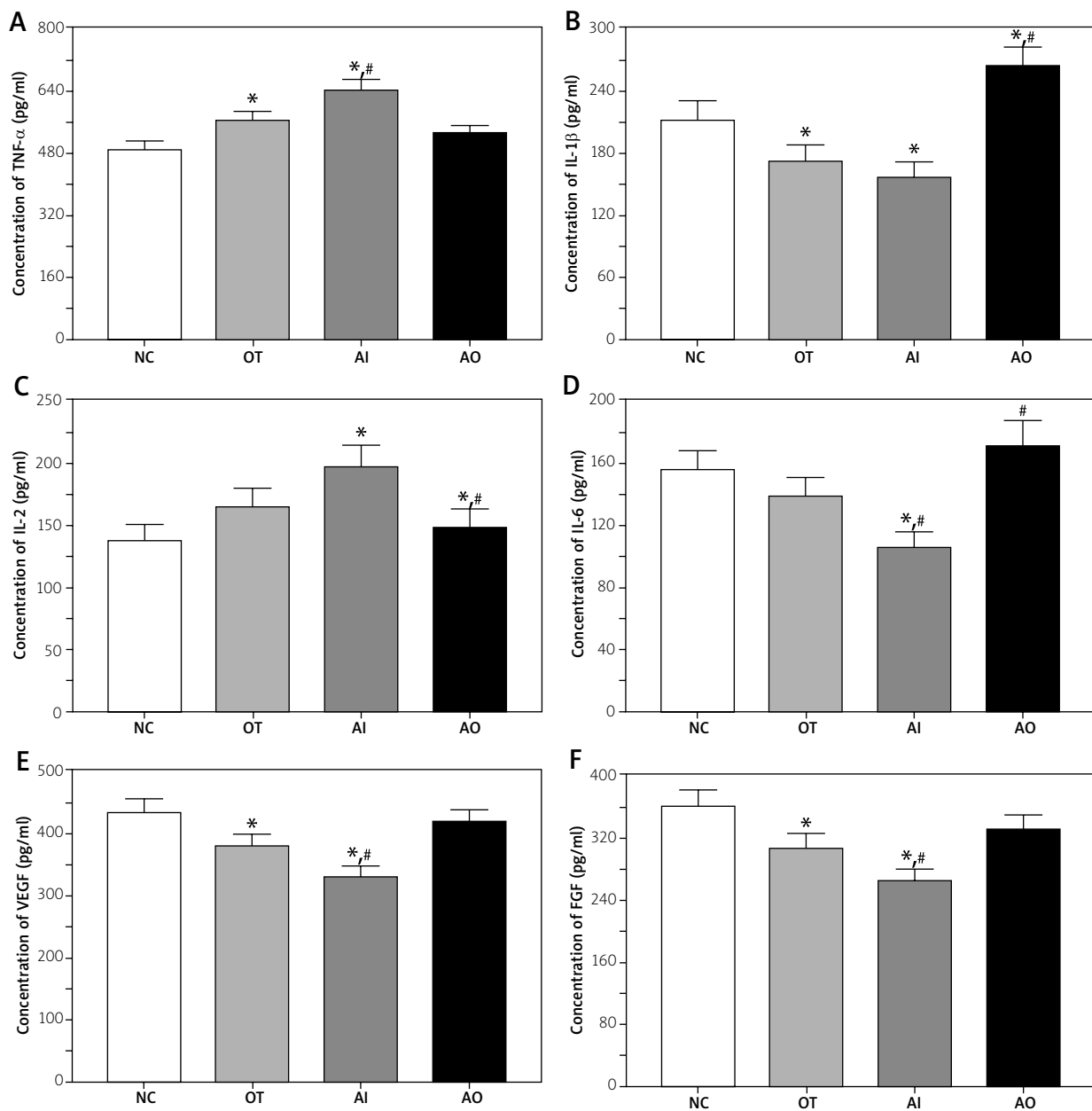


Fig. 4. Detection of concentration of inflammation- and angiogenesis-related factors in the culture medium of A172 cells under osimertinib stimulation using the ELISA method. **A)** Concentration of TNF- α in the culture medium. **B)** Concentration of IL-1 β in the culture medium. **C)** Concentration of IL-2 in the culture medium. **D)** Concentration of IL-6 in the culture medium. **E)** Concentration of VEGF in the culture medium. **F)** Concentration of FGF in the culture medium. * $p < 0.05$ vs. the NC group, # $p < 0.05$ vs. the OT group. Data were presented as mean \pm SD. Each experiment was repeated three times independently.

Expression of the Ras/MAPK pathway in each group of LN-18 cells

In Figure 9, the expression of EGFR in the NC, TO, AI and AO groups of LN-18 cells was 0.93 ± 0.06 , 0.79 ± 0.05 , 0.21 ± 0.01 , 1.00 ± 0.07 , respectively. The expression of Ras in these groups was 0.95 ± 0.06 , 0.75 ± 0.05 , 0.41 ± 0.03 and 1.06 ± 0.07 , respectively. The expression

of Raf in these groups was 0.93 ± 0.06 , 0.83 ± 0.06 , 0.46 ± 0.03 and 1.06 ± 0.07 , respectively. Ratios of p-MEK/MEK in these groups were 0.86 ± 0.06 , 0.78 ± 0.05 , 0.62 ± 0.04 and 0.94 ± 0.06 , respectively. The expression of c-Fos was 1.27 ± 0.08 , 0.76 ± 0.05 , 0.70 ± 0.05 and 1.04 ± 0.07 , respectively. The expression of c-Myc in these groups was 1.01 ± 0.07 , 0.88 ± 0.06 , 0.61 ± 0.04 and 1.40 ± 0.09 , respectively.

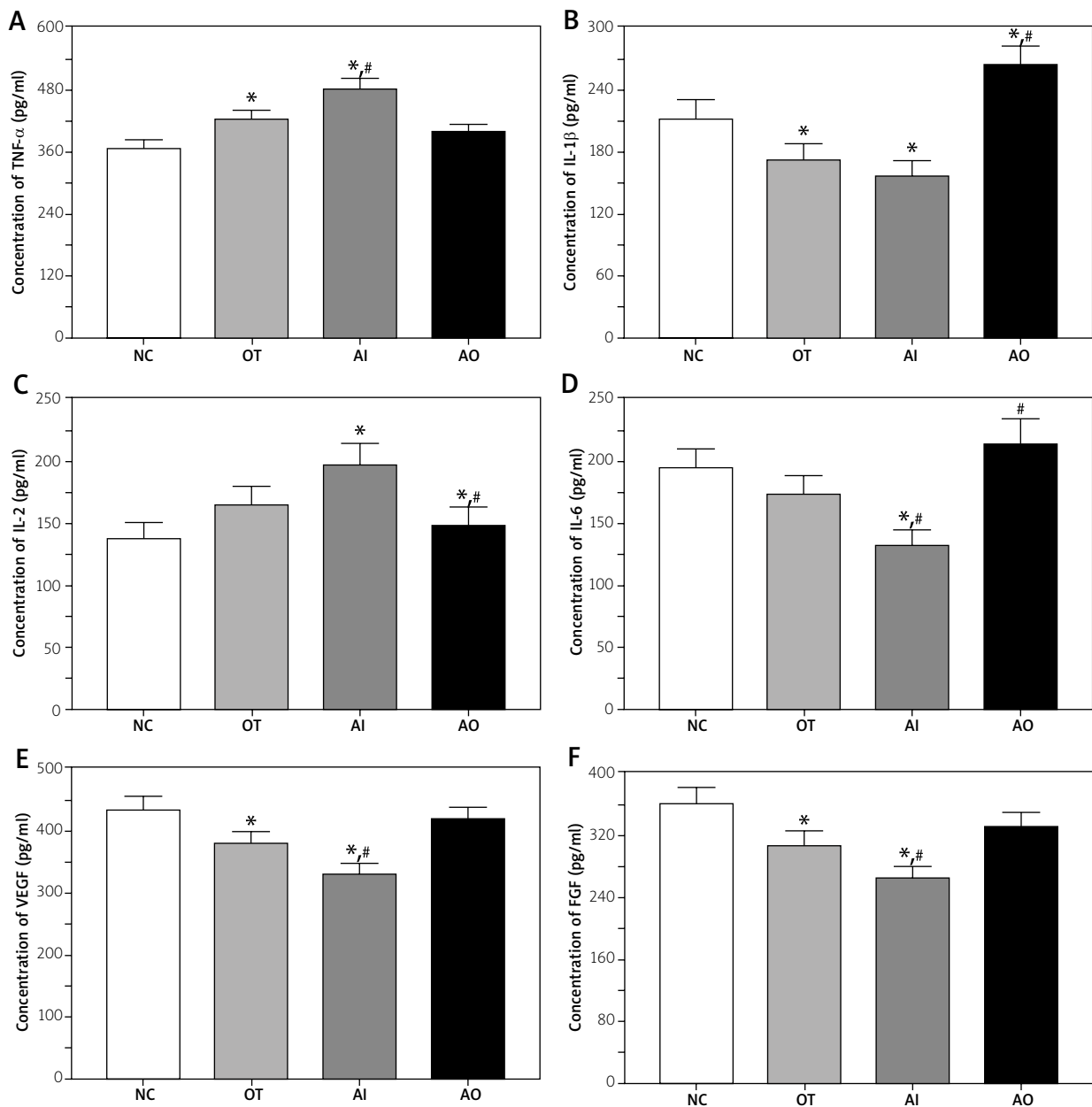


Fig. 5. Detection of concentration of inflammation- and angiogenesis-related factors in the culture medium of LN-18 cells under osimertinib stimulation using the ELISA method. **A)** Concentration of TNF- α in the culture medium. **B)** Concentration of IL-1 β in the culture medium. **C)** Concentration of IL-2 in the culture medium. **D)** Concentration of IL-6 in the culture medium. **E)** Concentration of VEGF in the culture medium. **F)** Concentration of FGF in the culture medium. * $p < 0.05$ vs. the NC group, # $p < 0.05$ vs. the OT group. Data were presented as mean \pm SD. Each experiment was repeated three times independently.

Discussion

Glioma is one of the most commonly seen primary tumours in brain tissue, and nearly 80% of malignancies of the central nervous system are gliomas. However, effective treatment for glioma is still lacking [50].

The expression of IL-1 β is commonly observed in a variety of cancer types, including breast, prostate,

colon and lung cancer. Patients with a high expression of IL-1 β always have a worse prognosis [22]. IL-1 β is a key molecule in carcinogenesis and tumour growth [27]. The key mechanism is that IL-1 β drives chronic unresolved inflammation, endothelial cell activation, tumour angiogenesis and the induction of immunosuppressive cells [3,44], leading to the inhibition of adaptive immunity, promotion and metastasis. IL-2 is mainly

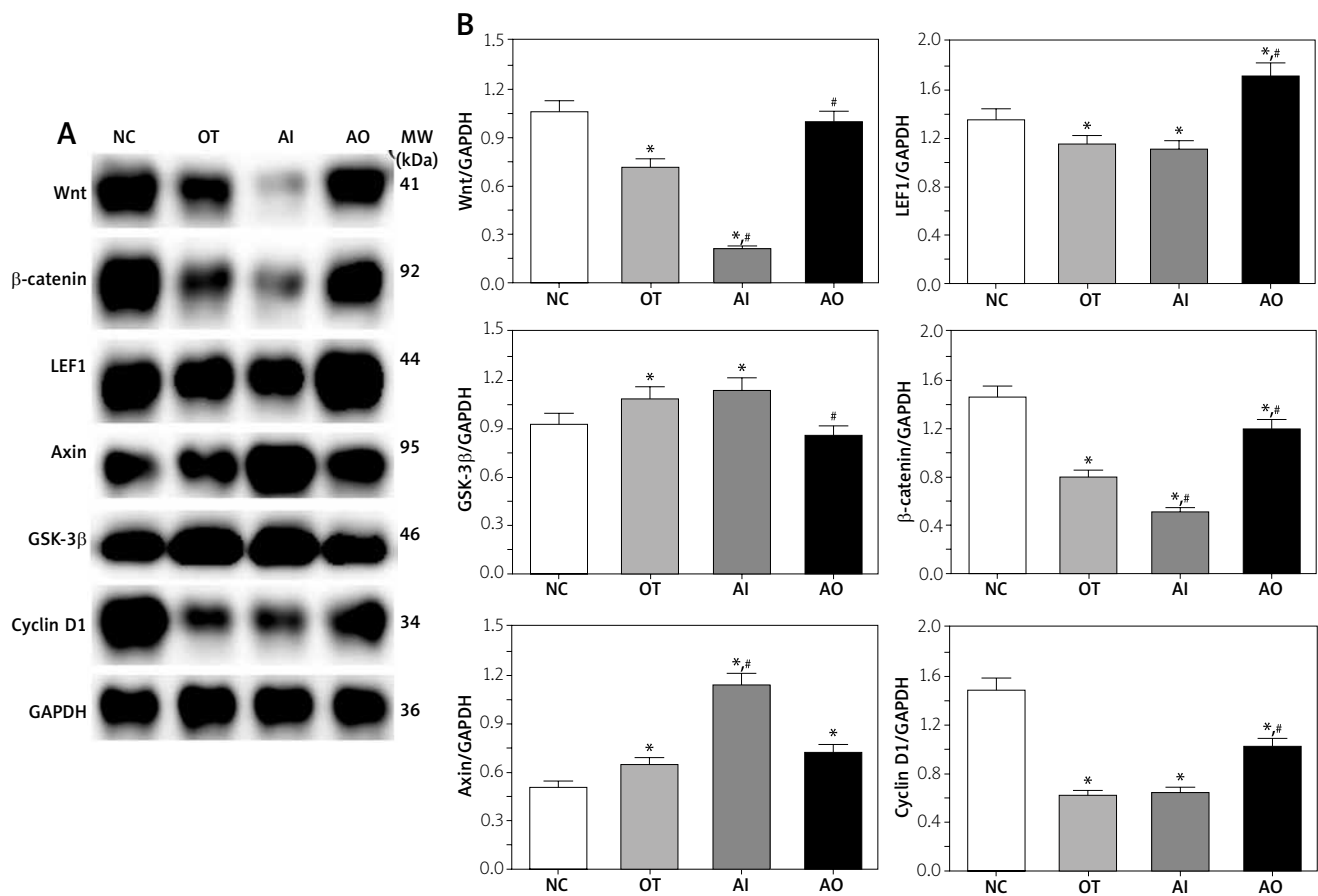


Fig. 6. Detection of the effect of osimertinib on activation of the Wnt/ β -catenin signalling pathway in A172 cells using Western blotting analysis. * $p < 0.05$ vs. the NC group, # $p < 0.05$ vs. the OT group. Data were presented as mean \pm SD. Each experiment was repeated three times independently. GAPDH was used as an internal control.

secreted by CD4⁺ T cells, secretion of IL-2 in tumour models could lead to the administration of T-cell growth factor to patients with cancer and thus might be a treatment in a variety of cancer types [38]. A previous study found that after IL-2 was applied to tumour-bearing mice, pulmonary metastases regressed [39], exerting an anti-tumour effect. IL-6 is a pleiotropic cytokine that performs multiple functions in various biological systems and organs. IL-6 is synthesized by macrophages and dendritic cells and recognizes pathogens at the site of infection or injury *via* toll-like receptors. An increase in IL-6 in circulation blood was observed in a mouse model and inhibited hepatocarcinogenesis, reducing the development of HCC [31]. Overexpression of IL-6 is commonly encountered in multiple cancer cells, and an increase in IL-6 serum levels is correlated with poor outcomes in cancer patients [40]. Osimertinib could affect the expression and secretion of inflammatory factors, resulting in the inhibition of cancer formation, and exerting an anti-tumour effect. Inhibition of AIM2

expression increased the function of osimertinib, while overexpression of AIM2 reduced it. VEGF was regarded as a regulator of vascular permeability because VEGF can induce vascular leakage thereby plays an important role in the angiogenesis process [7,41]. Inhibition of the VEGF signalling pathway expression showed therapeutic efficacy in patients with cancer. FGF is also an angiogenic factor produced, and also critical for vascular development and progression [17,46]. Blood vessels induced by VEGF always with high permeability, while FGF-induced blood vessels with low permeability [5]. Recent studies found that the VEGF and FGF signalling pathways both regulate the angiogenesis and cell proliferation, even the resistance ability of cancer to chemotherapy in preclinical and clinical trials [45,47]. Osimertinib could also inhibit the secretion of VEGF and FGF, further inhibiting the formation of vascular and angiogenesis processes in tumour cells, thus exerting an antitumor effect. These effects were enhanced after AIM2 was inhibited.

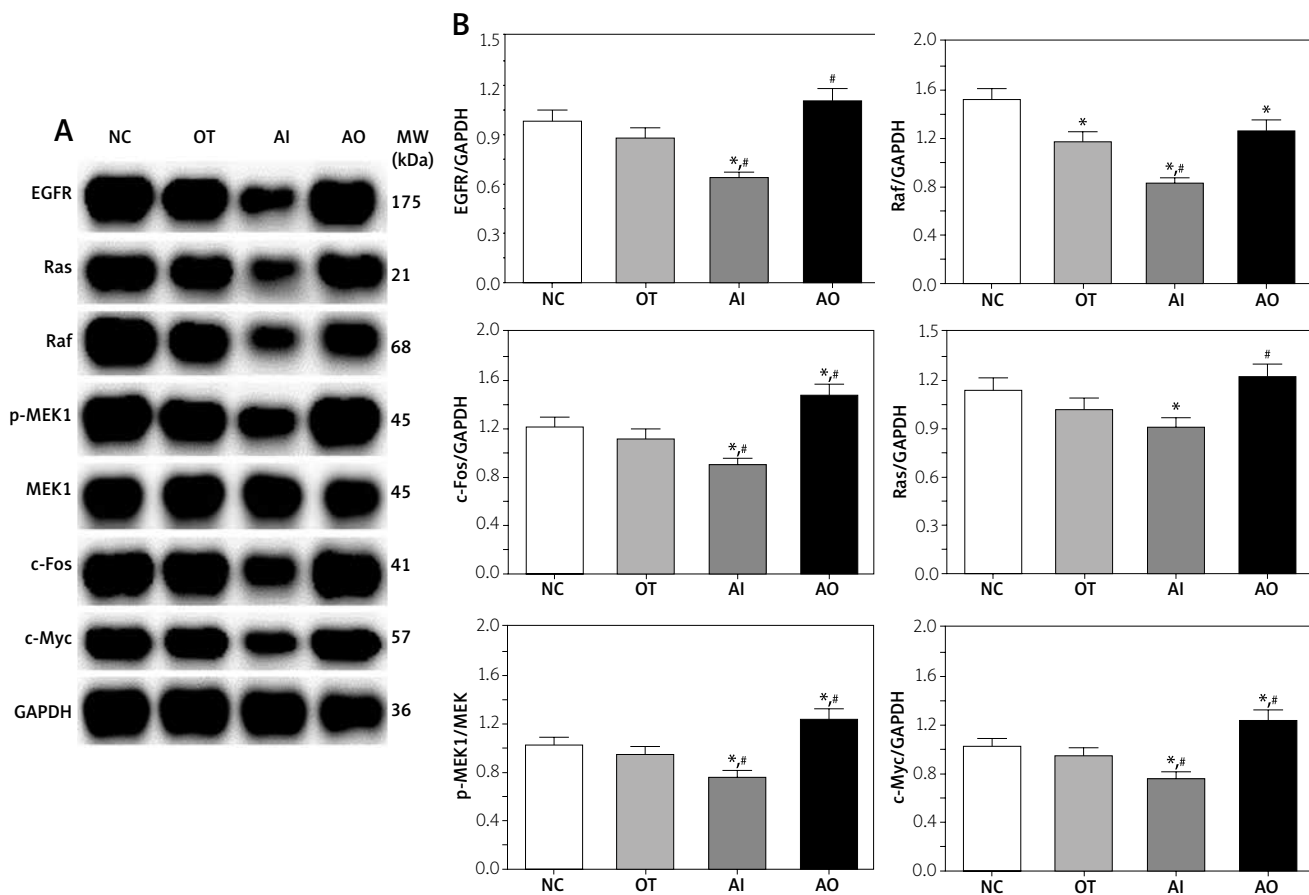


Fig. 7. Detection of the effect of osimertinib on activation of the Wnt/ β -catenin signalling pathway in LN-18 cells using Western blotting analysis. * $p < 0.05$ vs. the NC group, # $p < 0.05$ vs. the OT group. Data were presented as mean \pm SD. Each experiment was repeated three times independently. GAPDH was used as an internal control.

We further detected activation of the Wnt/ β -catenin and EGFR/Ras/MEK pathways in each group. Wingless-related integration site (Wnt)/ β -catenin is an important signalling pathway that regulates cellular proliferation, polarity and migration [24]. In the absence of extracellular Wnt ligands, β -catenin is phosphorylated by glycogen synthase kinase 3 (GSK3), which consists of casein kinase 1 (CK1), the tumour suppressor adenomatous polyposis coli gene product (APC) and Axin protein [35], degrades of β -catenin expression through the ubiquitin system. β -catenin is the regulator of cell-cell adhesion via interaction with the E-cadherin [28]. Activation of Wnt ligands would lead to the sequestration of Axin, followed by inhibition of GSK-3 β [32], inducing accumulation and translocation of β -catenin into nucleus. After translocation, β -catenin could bind with transcription factors and further regulate downstream target genes [34]. Thus, the Wnt/ β -catenin pathway is important for the proliferation of cancer cells, leading to the increasing ability

of aggression and invasion [20]. Osimertinib inhibited the expression of Wnt, increases the axin and GSK-3 β expression, resulting in the expression of β -catenin, as well as LEF1 and cyclin D1. These results indicated that osimertinib exerted an antitumor effect in NSCLC via inhibition of Wnt/ β -catenin. In addition, we also found that inhibiting the expression of AIM2 enhanced osimertinib function in treatment of NSCLC, and inhibiting the expression of AIM2 further decreased activation of the Wnt/ β -catenin pathway.

Ras is a member of the small GTPase family, which functions as a molecular switch that mediates external signals into nucleus by GDP-bound and GTP-bound cycling [37]. Ras is a member of mitogen-activated protein kinase (MAPK) signalling pathway, can be activated when binding with receptor tyrosine kinases (RTKs) such as epidermal growth factor receptor (EGFR), further inducing activation of downstream molecules, including extracellular signal-regulated kinase (ERK), MAPK/ERK kinase (MEK) and rapidly accelerated fibro-

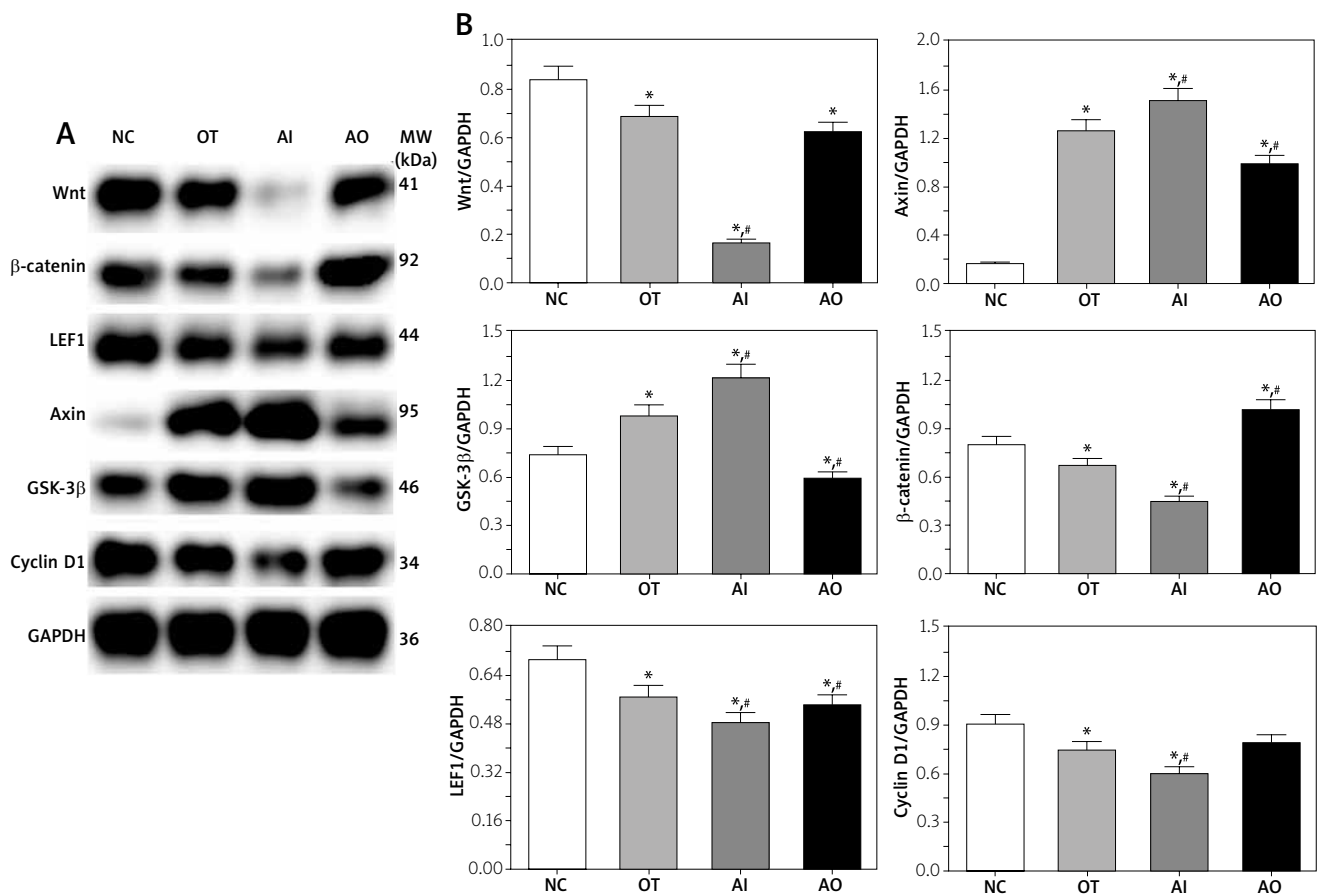


Fig. 8. Detection of the effect of osimertinib on activation of the Ras/MEK signalling pathway in A172 cells using Western blotting analysis. * $p < 0.05$ vs. the NC group, # $p < 0.05$ vs. the OT group. Data were presented as mean \pm SD. Each experiment was repeated three times independently. GAPDH was used as an internal control.

sarcoma (RAF). In an active state, Ras can recruit and activate the downstream molecule Raf at the plasma membrane, followed by phosphorylation of MEK1/2. Once activated, MEK1/2 further regulates transcription target genes participating in the regulation of cell growth, migration and proliferation [6]. Osimertinib significantly decreased the activation of the Ras/MEK pathway, exerting an antitumor effect by regulation of downstream molecules. Inhibition of AIM2 expression would enlarge this trend.

c-Fos is a member of the activating protein 1 (AP-1) family, highly expressed in different types of tumours [16]. c-Fos is critical for invasion, metastasis and growth of cancer with the ability to regulate neoplastic transformation and EMT. The function of c-Fos can be augmented by growth factors, membrane-bound and cytoplasmic oncogenes and tumour promoting agents [2,9]. Thus, c-Fos is regarded as an oncogene. c-Fos encodes a nuclear DNA binding protein domain, regulates the transcription of downstream targets, and

participates in signalling transduction, cellular proliferation and differentiation processes of tumour cells [29]. c-Myc regulates various cellular functions under physiological conditions, including cellular proliferation, differentiation, cell cycle progression and angiogenesis [18]. The expression of c-Myc is normally regulated by extracellular matrix contacts and growth factors. In quiescent cells, the expression of c-Myc remains at a low level; once the cell enters the cell cycle, the expression of c-Myc is immediately upregulated, and remains expressed in cycling cells [1]. A recent study found that amplification of c-Myc was observed in nearly 50% of breast cancer susceptibility gene (BRCA) 1-mutated breast cancer patients [48], and according to The Cancer Genome Atlas (TCGA) database, genetic abnormalities of c-Myc were found in 28% of all cancer patients [49]. Dysregulation of c-Myc is commonly observed in pathological conditions, including genomic instability, immune escape, uncontrolled proliferation, angiogenesis and metastasis [14], indicating that c-Myc is critical

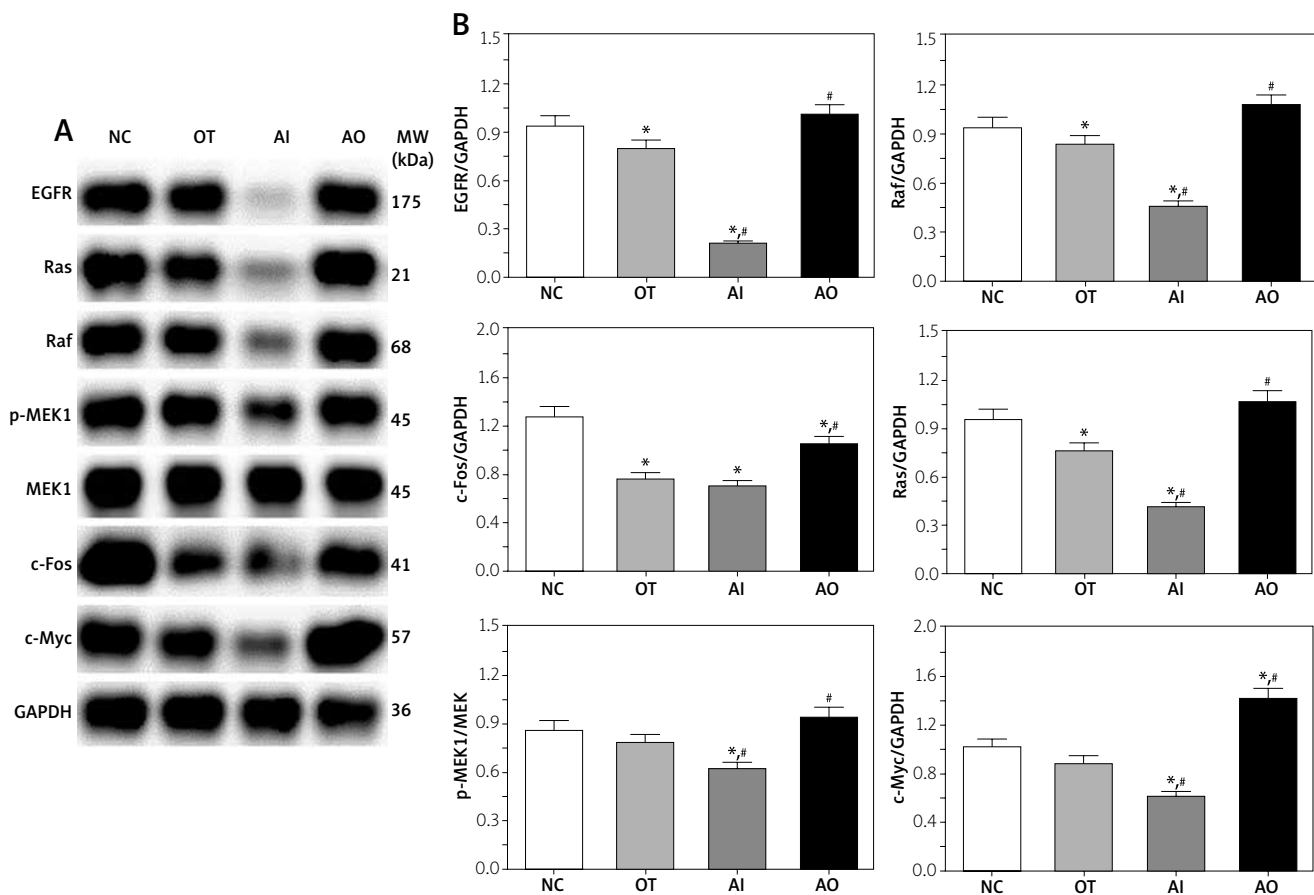


Fig. 9. Detection of the effect of osimertinib on activation of the Ras/MEK signalling pathway in LN-18 cells using Western blotting analysis. * $p < 0.05$ vs. the NC group, # $p < 0.05$ vs. the OT group. Data were presented as mean \pm SD. Each experiment was repeated three times independently. GAPDH was used as an internal control.

for the growth and proliferation of tumour cells, which could also increase the chance of secondary mutations, resulting in resistance to therapy and poor prognosis [10]. As a result of the inhibition of Wnt/ β -catenin and EGFR/Ras/MEK signalling pathway activation in A172 and LN-18 cells by osimertinib treatment, the expression of c-Fos and c-Myc were both downregulated, indicating that the proliferation and metastasis ability of tumour cells might be inhibited, and has an antitumor effect. We also noticed that the effect of osimertinib could be enhanced by inhibiting AIM2 expression.

In this study, we established AIM2 overexpression and inhibition cell models in A172 and LN-18 cells, and detected the effect of osimertinib on the expression and secretion of inflammation-related genes. Cellular apoptosis was also detected by FACS. We found that osimertinib treatment reduced the expression of pro-tumor factors, also increased tumour inhibition of related factors. These effects might be mediated by the inhibition of

Wnt/ β -catenin and EGFR/Ras/MEK signalling pathways. In addition, the antitumor effect of osimertinib could be enhanced by the inhibition of AIM2, while overexpression of AIM2 reduced these effects.

Conclusions

Inhibition of AIM2 expression inhibited the activation of the Wnt/ β -catenin and EGFR/Ras/MEK signalling pathways and induced the activation of the inflammatory response process in cancer cells, resulting in inhibition of cellular proliferation and promotion of the cellular apoptosis process. Hence, we speculated that inhibition of the expression of AIM2 might contribute to the therapeutic effect of glioma, and contributes to improve the quality of life of patients and reduces the cost of public health.

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Disclosures

Experiments on animal models were performed according to the principles of the National Institutes of Health Guide for the Care and Use of Animals, and the experiments were approved by the Health Animal Care and Use Committee of Fujian Provincial Hospital.

The authors report no conflict of interest.

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