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Original Article

Nuclear translocation of Axl contributes to the malignancy of oral cancer cells

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KEYWORDS

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Abstract *Background/purpose:* Dysregulation of receptor tyrosine kinases is implicated in cancer development. This study aimed to investigate the nuclear translocation of Axl, a membrane protein and receptor tyrosine kinase in cancer malignancy.

Materials and methods: We examined Axl's entry into the cell nucleus and validated it with the nuclear export inhibitor leptomycin. Transfection experiments with mutated nuclear localization signals were conducted to assess the impact of reduced nuclear Axl levels on cancer cell malignancy. Additionally, we evaluated the effects of decreased nuclear Axl on sensitivity to radiation and cisplatin, a chemotherapeutic drug.

Results: In the present study, we observed nuclear translocation of Axl in cancer cells. Reducing nuclear Axl levels led to a decrease in cancer cell malignancy. This nuclear translocation was further validated using a nuclear export inhibitor, leptomycin. Additionally, transfection experiments with mutated nuclear localization signals confirmed the functional significance of Axl's nuclear localization. Notably, decreased nuclear Axl levels also increased the sensitivity of cancer cells to radiation and cisplatin treatment.

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Conclusion: This study suggests that Axl's nuclear translocation plays a significant role in cancer malignancy. Targeting Axl's nuclear localization could offer a potential strategy to inhibit cancer progression and improve the efficacy of radiation and chemotherapy treatments.

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Introduction

Dysregulation of receptor tyrosine kinases is a major contributor to cancer development.¹ Axl is a receptor tyrosine kinase that belongs to the Tyro3, Axl, and Mer (TAM) family. Upon binding to its ligand growth arrest-specific 6 (Gas6), Axl activates cell signaling pathways that promote proliferation.² Axl has been identified as an oncogene, and its over-expression has been associated with poor prognosis in various cancers, including oral cancer,³ lung cancer,⁴ breast cancer,⁵ and prostate cancer.⁶ Inhibitors targeting Axl show promise as therapeutic agents for addressing dysregulated Axl signaling pathways and inhibiting cancer cell growth.²

Membrane proteins are primarily located within the cellular membrane, while the cell nucleus is enclosed by the nuclear membrane. Therefore, the translocation of membrane proteins into the nucleus necessitates a series of transport processes. Many membrane proteins possess specific nuclear localization signals (NLS) embedded within their amino acid sequences.⁷ These signals are recognized by nuclear import receptors. NLS often comprise sequences that are rich in positively charged amino acids, such as arginine and lysine, which facilitate the translocation of membrane proteins into the cell nucleus.⁸

In our previous study, Axl was identified as a prognostic marker in oral cancer.⁹ Moreover, the activation of the Axl signaling pathway by tumor-associated macrophages was observed to promote oral cancer progression.¹⁰ Given the reports of nuclear trafficking and treatment resistance associated with specific receptor tyrosine kinases,¹¹ it is hypothesized that Axl may undergo nuclear transport, potentially contributing to the increased malignant potential of oral cancer.

Materials and methods

Cell lines and cell culture

Seven human oral cancer cell lines, namely YD-8, YD-10B, YD-15, YD-38, OEC-M1, SAS, and SCC-25, were employed in this study. These cells were cultured in Roswell Park Memorial Institute 1640 medium containing 10% fetal bovine serum (GIBCO, Eggenstein, Germany) and 1% Penicillin-Streptomycin-Amphotericin B (Biological Industries, Cromwell, CT, USA) at 37 °C and 5% CO₂. All cancer cell lines were confirmed to be free of mycoplasma.

Reagents and antibodies

Leptomycin B was purchased from Cayman Chemical (Ann Arbor, MI, USA). Cisplatin was purchased from Selleckchem (Houston, TX, USA). Antibodies against GAPDH, slug, and P-glycoprotein (P-gp) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against matrix metalloproteinase 9 (MMP9) and Axl were acquired from Abcam (Cambridge, UK), while E-cadherin antibody was obtained from BD Biosciences (San Jose, CA, USA). α -tubulin and Lamin B1 antibodies were obtained from Proteintech (Wuhan, China). Ataxia telangiectasia mutated (ATM) antibody was obtained from GeneTex (Irvine, CA, USA). Anti-rabbit or anti-mouse Horseradish Peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Alexa Fluor 488 anti-rabbit immunoglobulin G (IgG) was purchased from Invitrogen (Carlsbad, CA, USA).

Western blot analysis and cellular fractionation

For Western blot analysis, cell lysates were prepared, following previously described procedures.^{12–16} For cellular fractionation, the cells were washed with phosphate-buffered saline and detached using accutase. The NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, Waltham, MA, USA) were used for cellular fractionation according to the manufacturer's protocol.

Confocal imaging

The cells were incubated overnight at 4 °C with an anti-Axl antibody, then washed. They were subsequently incubated with the secondary antibody, Alexa Fluor 488 anti-rabbit IgG, conjugated with a green fluorescent dye, at dilutions of 1:1000. Next, the cells were treated with the red fluorescent dye Nuclear red (AAT Bioquest, Gaithersburg, MD, USA) at a 1:600 dilution for 10 min. Confocal fluorescence images were captured using a Zeiss LSM 510 Inverted Confocal Microscope (Oberkochen, Germany). The number of overlapping nuclei was counted, and the percentage of overlap was determined.

Construction of plasmid DNA and establishment of stable transfectants cell lines and cell culture

Human Axl cDNA was cloned into the mammalian expression vector with the cytomegalovirus promoter (pCMV)-Tag 2A as

previously described.¹⁷ To generate the NLS construct, the Axl mutant sequence was amplified using a polymerase chain reaction (PCR) system (Thermo Fisher Scientific) machine with the following primers: Forward primer mutant: GCGGCAGCGGCGGAGACCCGTTATGGAGAAGTGT and reverse primer mutant: CGCCGCTGCCGCGTGGA-CAAGGAAGAGAGCCAA. The PCR primers annealed and complemented the target sequence. The amplified product was ligated into the pCMV vector to produce the pCMV-Axl NLS mutant plasmid. After transforming this plasmid into competent cells, clones were selected using Kanamycin/Neomycin. The plasmid's accuracy was first verified by DNA PCR, then confirmed by sequencing. Upon verification, the plasmid was transfected into YD-10B cells for further experiments. Post-transfection, cells were cultured in medium supplemented with Geneticin according to the

manufacturer's recommendations. Stable clones were maintained in medium with a reduced concentration of Geneticin.

(3-4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and colony formation assay

Cell proliferation was assessed using MTT assay. The resulting purple crystals were solubilized in dimethyl sulfoxide (DMSO), and their absorbance was measured at 570 nm using an enzyme-linked immunosorbent assay reader. For the colony formation assay, oral cancer cells were seeded at a density of 1000 cells per well in 6-well plates and incubated in a cell culture incubator for 7 days.

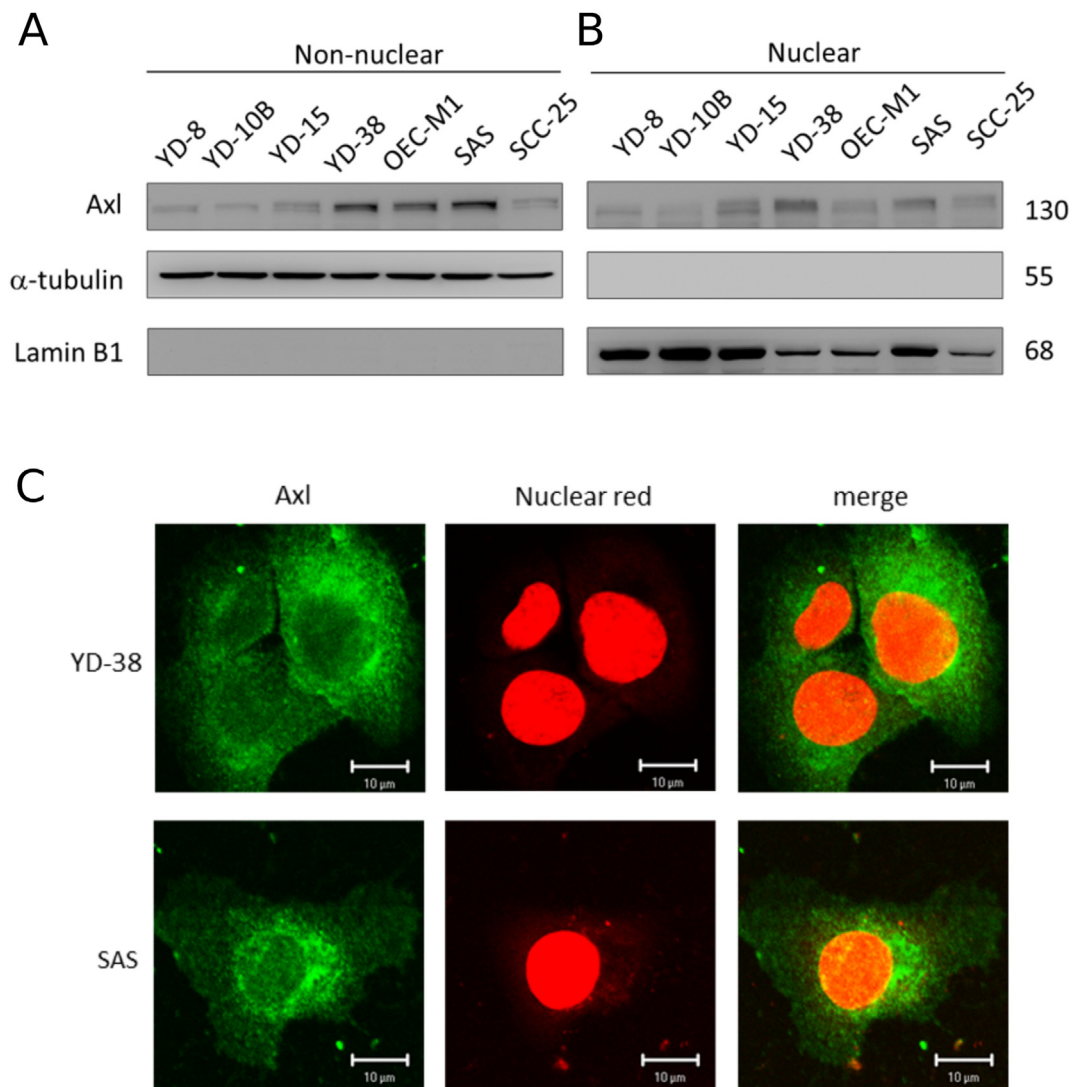


Figure 1 Axl expression patterns in oral cancer cells. (A) Non-nuclear and (B) nuclear extracts from seven oral cancer cell lines were analyzed by immunoblot with antibodies against Axl, α-tubulin and lamin B1. α-tubulin and lamin B1 were used as cytoplasmic and nuclear controls, respectively. (C) Confocal microscopic images of immunofluorescence staining for Axl (green), and Nuclear red (red) in YD-38 and SAS cells show co-localization of Axl and the nucleus. Scale bars: 10 μm.

After incubation, the cells were stained with a 1% crystal violet solution for 30 min on a shaker, followed by washing off the excess dye with water. The number of colonies was then quantified using ImageJ software.

Cell migration and invasion assay

Cell migration was evaluated through a wound-healing assay. A small wound was created in the cell monolayer using a 1000 μ L pipette tip. The area of the wound was documented by capturing images under a microscope. Cell invasion was measured using a transwell chamber. The insert's membrane was coated with diluted Matrigel. The cells that invaded into the lower chambers were fixed with methanol for 40 min, stained with propidium iodide (PI) for 30 min, and then the cells were counted in 5 random fields. The number of invasive cells was quantified using ImageJ software.

Statistical analysis

Statistical comparisons were conducted using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test to compare multiple groups, while Student's *t*-test was employed to compare two groups.

GraphPad Prism (GraphPad Software, San Diego, CA, USA) was utilized for statistical calculations and graphical representations. A *P*-value less than 0.05 was considered indicative of a significant difference. All data were expressed as the mean $\pm$ standard error of the mean (SEM).

Results

Presence of nuclear Axl confirmed in oral squamous cell carcinoma (OSCC) cell lines

First, we investigated the expression of nuclear Axl through Western blot analysis in various OSCC cell lines, including YD-8, YD-10B, YD-15, YD-38, OEC-M1, SAS, and SCC-25. The nuclear lysates obtained were confirmed to be free from cytoplasmic protein contamination, as demonstrated by the absence of α -tubulin. The nuclear protein lamin B1 was employed as a loading control. Notably, YD-15, YD-38, OEC-M1, and SAS cells exhibited detectable levels of nuclear Axl protein (Fig. 1A and B). Furthermore, we employed confocal immunofluorescent microscopy to visualize the nuclear expression of Axl. Nuclear Axl was observed in YD-38 and SAS cells (Fig. 1C).

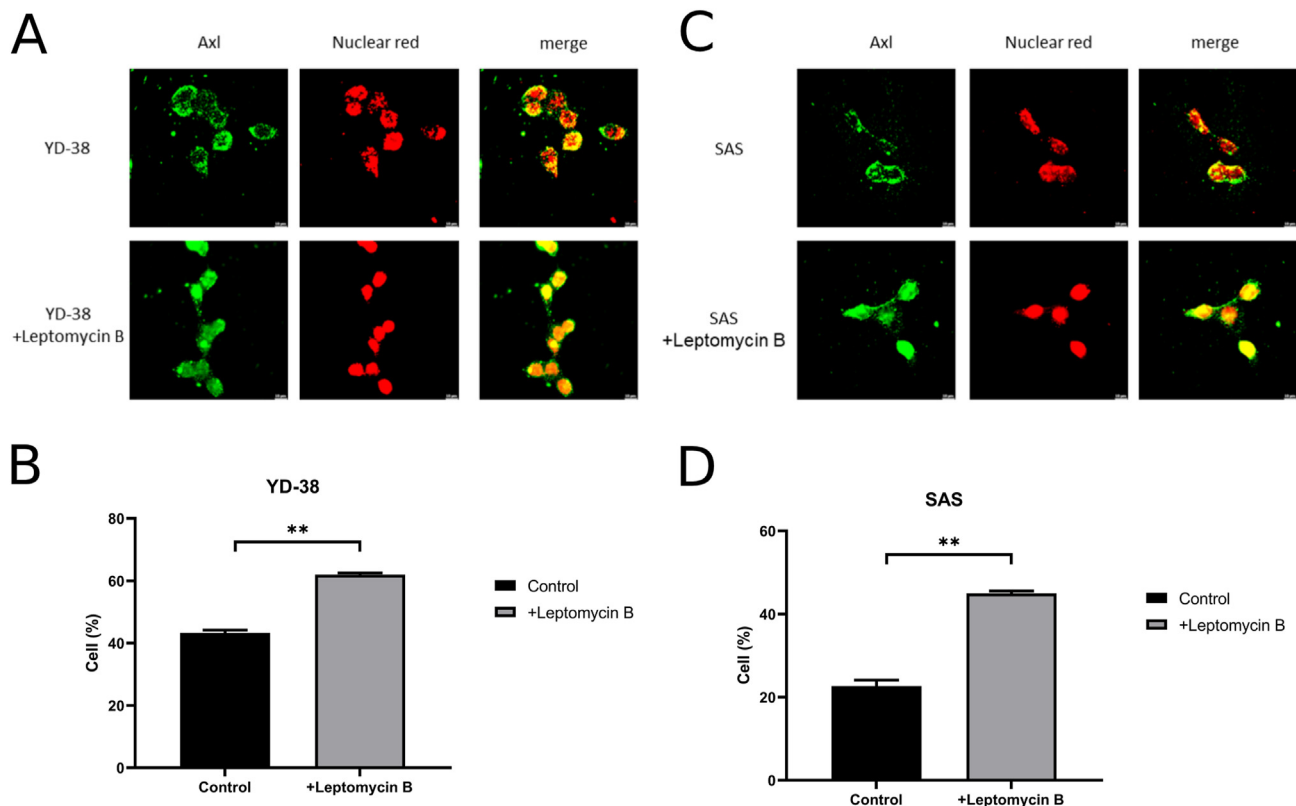


Figure 2 The nuclear fraction of Axl increased upon treatment with a nuclear export inhibitor. (A, C) Two oral cancer cell lines were cultured in growth media and treated with the nuclear export inhibitor, leptomycin B. Subsequently, cells were fixed, labeled, and analyzed using confocal microscopy. Scale bars: 10 μ m. (B) and (D) represent quantification of nuclear Axl staining from (A) and (C), respectively. The vertical axis indicates the number of positively stained nuclei per 100 cells. Data are shown as mean $\pm$ SEM. ***P* < 0.01.

Nucleocytoplasmic transport and NLS sequence analysis in Axl nuclear translocation

Nucleocytoplasmic transport processes are often mediated by karyopherins.¹⁰ These karyopherins include exportins and importins, which facilitate nuclear export and import, respectively.⁵ Leptomycin B has been shown to serve as an inhibitor for the nuclear export signal.⁶ In this study, we employed leptomycin B to block nuclear protein export. Cells were treated with leptomycin B for 4 h and examined the nuclear level of Axl. As shown in Fig. 2A–D, a significant accumulation of nuclear Axl was observed using confocal immunofluorescent microscopy. To identify potential NLS sequences, we employed bioinformatics tools including cNLS mapper,¹⁸ NucPred,¹⁹ and NLStradamus,²⁰ and the 465–468th amino acids were identified as a putative NLS sequence. We further generated a point mutation in the 465–468th amino acids (Fig. 3A and B), which was

subsequently validated by sequencing. To assess the functional significance of the NLS, we established stable clones of the YD-10B cell line with either the wild-type Axl or the NLS mutant. In the Axl-transfected cell lines, a more pronounced nuclear translocation was observed compared to the NLS mutant-expressing cell lines, as shown in Fig. 3C. Additionally, we employed cellular fractionation followed by Western blot analysis, which revealed a significant increase in nuclear Axl in the continuously expressing Axl cell lines compared to the mutant cell lines (Fig. 3D).

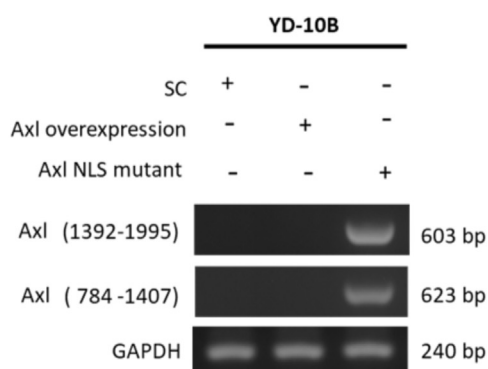
Transfection of the Axl NLS mutant in oral cancer cells led to reduced growth, invasion, and migration

To investigate the impact of Axl nuclear translocation, the YD-10B cells transfected with the Axl NLS mutant demonstrated reduced growth compared to those transfected

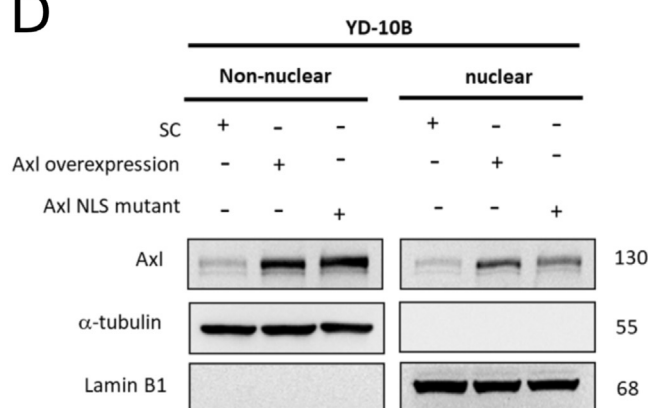
A

Axl NLS 465-8 mutant	RRKK->AAAA
Forward mutant:	GCGGCAGCGGCGAGACCCGTTATGGAGAAGTGT
AXL Reference Sequence :	TTGGCTCTCTCCTGTCCACGCGCGAAAGAAGGAGACCCGTTATGGAGAAGTGT
Reverse mutant:	TTGGCTCTCTCCTGTCCACGCGGCAGCGGCG

B



D



C

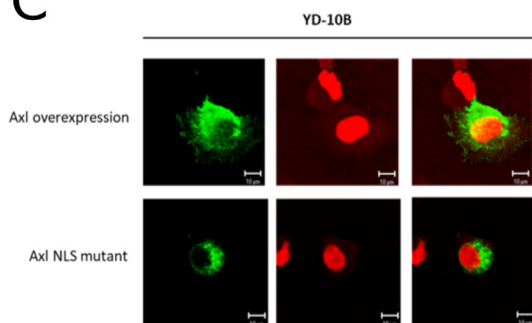


Figure 3 Establishment of Axl NLS and reconstituted mutant. (A) Schematic representation of Axl mutants. Potential NLS candidates were identified using the cNLS Mapper, NucPred and NLStradamus databases. The NLS mutant consists of alanine substitutions for four clustered basic amino acids. (B) Validation of the NLS mutant using primer pairs. (C) Confocal microscopy analysis of cells expressing wild-type Axl and the NLS mutant. Cells were fixed, sequentially labeled, and analyzed. (D) Generation and validation of stable clones overexpressing wild-type Axl and NLS mutant Axl cell lines using Western Blotting. GAPDH was used as housekeeping protein. NLS, nuclear localization signals; R, Arginine; K, Lysine; A, Alanine; SC, scramble control.

with the wild-type Axl (Fig. 4A–C). Furthermore, stable transfection of the Axl NLS mutant in YD-10B cells led to decreased invasion and migration capabilities relative to the cell line transfected with the wild-type Axl (Fig. 4D–G). Western blot analysis of YD-10B cells showed that the Axl

NLS mutant group exhibited significantly increased expression of the epithelial marker E-cadherin, while displaying decreased expression of mesenchymal proteins, including slug and MMP9, compared to the wild-type Axl (Fig. 4H).

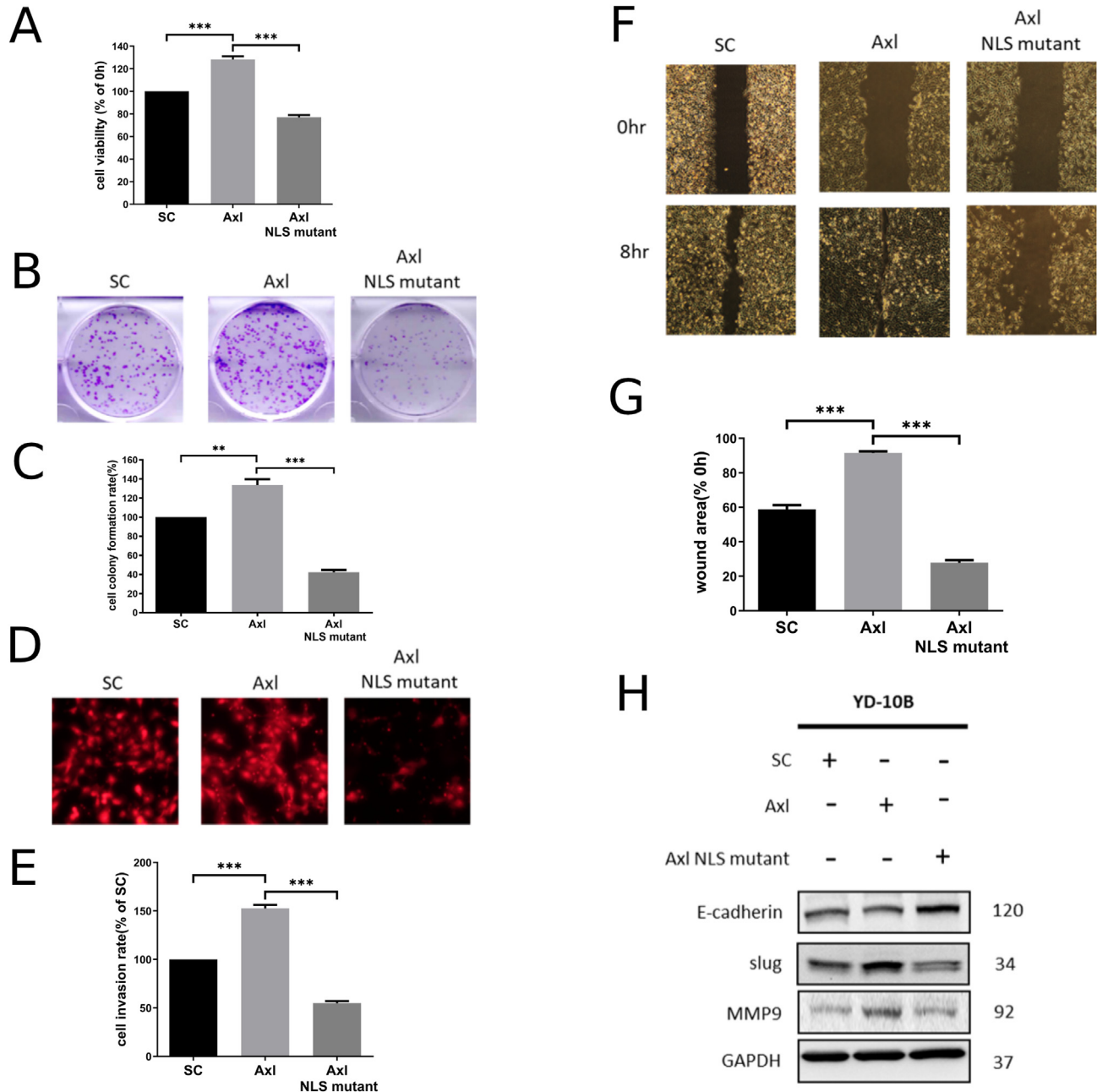


Figure 4 Nuclear translocation of Axl regulates multiple aspects of the transformed phenotype in oral cancer cells. (A) Constitutive expression of Axl significantly promotes cell growth, as shown by the MTT assay, and this effect is abolished by the NLS mutant (B) Axl expression enhances colony formation capability, but this enhancement is lost with the NLS mutant (C) Quantification of the colony formation assay is presented. (D) Constitutive expression of Axl NLS mutant significantly decreased cell invasion, as determined by the Boyden chamber assay. (E) Quantification of the invasion assay is shown. (F) Constitutive expression of Axl significantly promotes cell migration, as assessed by the wound-healing assay. (G) Quantification of the migration assay is provided. (H) Western blotting reveals that constitutive expression of Axl induces epithelial-mesenchymal markers. Data are shown as mean $\pm$ SEM. ** $P < 0.01$, *** $P < 0.001$. NLS, nuclear localization signals; SC, scramble control; MTT assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; MMP9, matrix metalloproteinase 9.

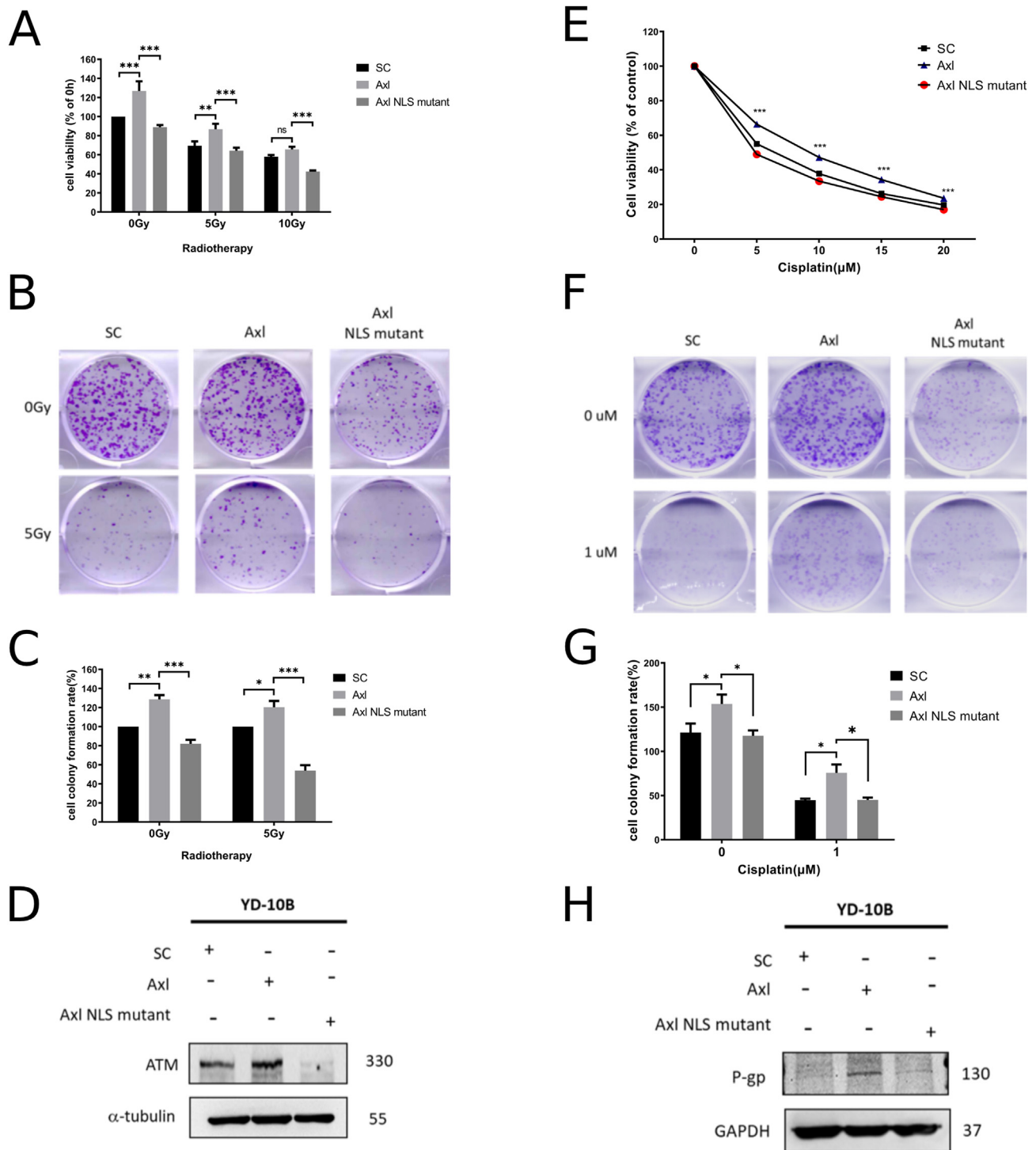


Figure 5 The expression of Axl in the nucleus contributes to the chemoradioresistance of cancer cells. (A) Constitutive expression of Axl significantly enhances cell growth after radiotherapy, as assessed by the MTT assay. (B) The colony formation assay further confirms the promotive effect of Axl expression on cell growth after radiotherapy. This effect is abolished in cells expressing a NLS mutant of Axl. (C) Quantification of the colony formation assay results is presented. (D) The NLS mutant significantly diminishes the ATM expression induced by Axl expression. (E) The NLS mutant significantly reduces the cell growth induced by Axl expression after chemotherapy, as assessed by the MTT assay. (F) The colony formation assay further confirms the promotive effect of Axl expression on cell growth after chemotherapy. This effect is abolished in cells expressing an NLS mutant of Axl. (G) Quantification of the colony formation assay results is shown. (H) Constitutive expression of Axl induces the expression of P-gp, a known chemoresistant protein. Data are shown as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. NLS, nuclear localization signals; SC, scramble control; MTT assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; P-gp, P-glycoprotein; ATM, ataxia telangiectasia mutated; ns, not significant.

Transfection of the Axl NLS mutant in oral cancer cells reduced chemoradioresistance

To investigate the potential involvement of nuclear Axl in cellular physiology, YD-10B cells were subjected to radiation doses of 5 Gy or 10 Gy. The results demonstrated that there was a slower cell growth rate in the Axl NLS mutant compared to the wild-type Axl (Fig. 5A–C). Additionally, Western blot analysis, as shown in Fig. 5D, revealed reduced ATM expression in the Axl NLS mutant compared to wild-type Axl. This implies the NLS mutant may modulate radiotherapy resistance via ATM, a known factor in radioresistance. Furthermore, we investigated their response to cisplatin treatment. Similarly, the group transfected with the Axl NLS mutant demonstrated decreased growth compared to the wild-type Axl group (Fig. 5E–G). Immunoblotting analysis showed a downregulation of the

multidrug resistance gene P-gp in the Axl NLS mutant group compared to the wild-type Axl group.

Discussion

In this study, we demonstrated the entry of Axl into the cell nucleus using different experiments. To further investigate the functional implications of Axl's nuclear localization, we conducted transfection experiments in which cells were transfected with mutated NLS. Our findings revealed that reducing the nuclear entry of Axl led to a decrease in the malignancy of cancer cells. Significantly, we observed enhanced sensitivity to radiation and the chemotherapeutic drug cisplatin in cells with reduced levels of nuclear Axl. These results strongly suggest a potential role of Axl's nuclear translocation in promoting cancer malignancy. The experimental framework is depicted in Fig. 6.

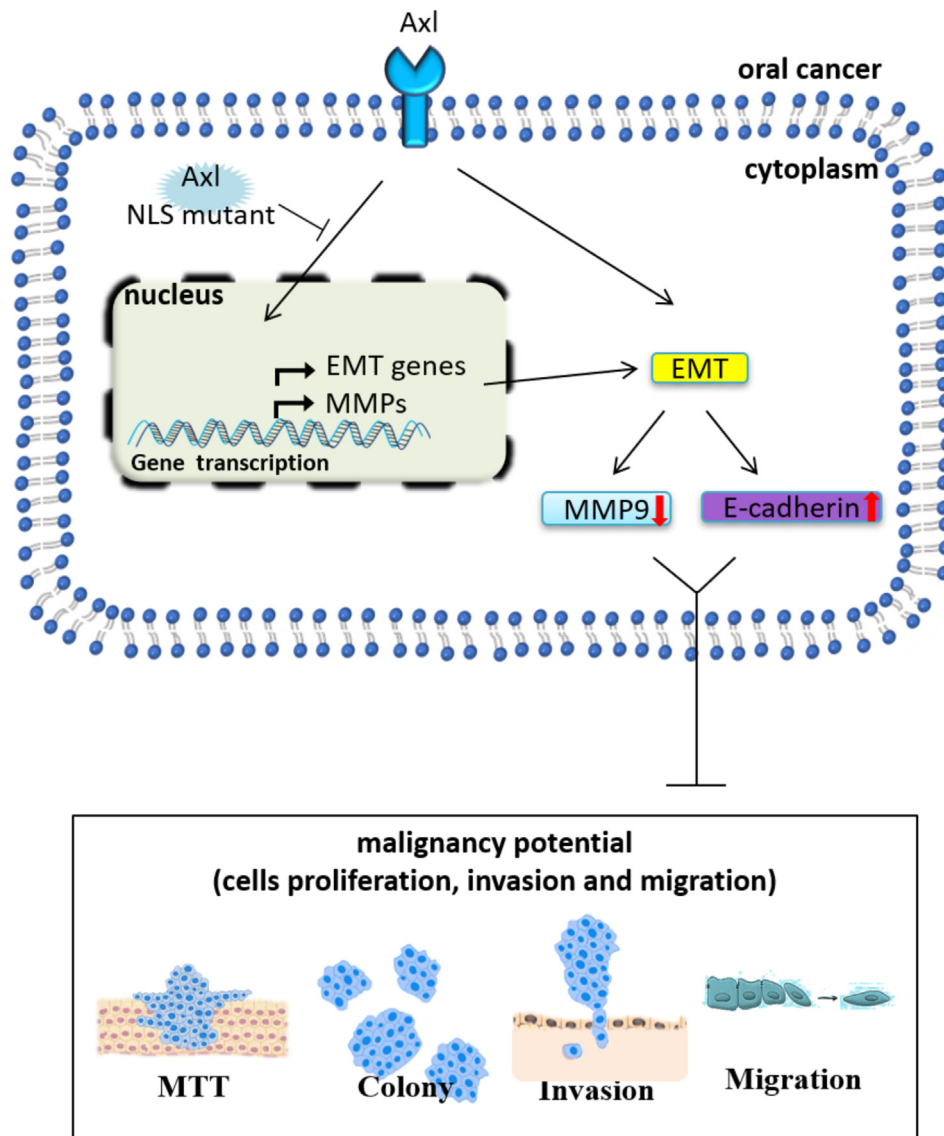


Figure 6 Schematic representation of constitutive Axl expression and NLS mutants. The NLS mutant attenuates the effect of Axl in inducing oral cancer progression.

The specific factors driving the nuclear translocation of the membrane protein Axl in our study remain unclear. Previous cases of membrane protein nuclear translocation, such as the well-known example of the epidermal growth factor receptor (EGFR), have shed light on various factors, including ligand stimulation,²¹ chemical agents like cisplatin,²² and radiation therapy.²³ The nuclear localization of EGFR has been implicated in tumor malignancy in various cancers, including breast cancer,²⁴ lung cancer,²⁵ and ovarian cancer.²⁶ In our investigation, we explored the potential induction of Axl nuclear translocation using radiation therapy, hypoxia, and the chemotherapeutic agent cisplatin. However, our data did not reveal significant translocation of Axl into the nucleus under these conditions (data not shown). Further research is needed to elucidate the mechanisms that trigger Axl nuclear translocation.

In previous research, Lu et al. also reported the presence of nuclear Axl in various cancer cells, where intracellular proteolytic processing of Axl leads to its translocation to the nucleus.²⁷ This finding may be relevant to lung cancer patients with certain EGFR mutations, who develop resistance to erlotinib treatment. Our study further confirmed Axl nuclear translocation in oral cancer cells and provided comprehensive evidence of its association with malignant behavior in this context. The correlation between nuclear Axl and the treatment of cisplatin and radiotherapy, commonly used for advanced oral cancer patients, highlights the potential clinical relevance of nuclear Axl in therapeutic responses.

The nuclear translocation of membrane proteins serves various purposes,^{9,28} with common roles as transcription factors or transcriptional co-factors. As transcription factors, they typically possess a DNA binding domain essential for binding to specific DNA sequences. However, Axl lacks a DNA binding domain in its structure, suggesting an alternative function upon nuclear entry. It is postulated that Axl enters the cell nucleus as a transcriptional co-factor, forming complexes with other proteins that possess nuclear localization properties. Through interactions with these proteins, the transcriptional co-factor is transported into the nucleus. Several receptor tyrosine kinases have been demonstrated to interact with transcription factors. For instance, EGFR interacts with signal transducers and activators of transcription 3 (STAT3),²⁹ while the fibroblast growth factor receptor 2 (FGFR-2) interacts with STAT5.³⁰ These interactions facilitate the translocation of activated STAT proteins into the nucleus, where they bind to specific DNA sequences and regulate the transcription of specific genes. Therefore, future investigations on Axl could explore its potential association with transcription factors during nuclear translocation.

In this study, we have reported a potential association between nuclear Axl and tumor malignancy. Our cellular experiments have provided valuable insights into the correlation of nuclear Axl with the aggressiveness of oral cancer and its involvement in conferring resistance to radiotherapy and the chemotherapy agent cisplatin. These findings suggest that targeting nuclear Axl could represent a promising avenue for a novel therapeutic approach in oral cancer treatment.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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