Nicotine exposure increases PIK3CA, AKT1, HIF-1α, GLUT1, CA9 and VEGF expression in oral potentially malignant lesions

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Abstract

Background:

Oral squamous cell carcinoma (OSCC) is a highly aggressive malignancy often arising from oral potentially malignant lesions (OPMD). Nicotine triggers pathways associated with tumor progression, angiogenesis, and metastasis, notably PI3K/AKT and HIF-1. This study investigated the impact of nicotine on cell viability, migration, and gene expression (*PIK3CA, AKT1, HIF-1α, GLUT1, CA9* and *VEGF*) in DOK and SCC9 cell lines.

Methods and Results:

DOK and SCC9 cell lines, were cultured in standard media and conditions, exposed to different nicotine concentrations (control, 1 µM, and 10 µM) for 8 and 24 hours. Cell viability assay, wound healing scratch assay cell, RNA extraction and RT-qPCR analysis of target genes were performed. Nicotine increased DOK cell migration and mRNA expression of *PIK3CA, AKT1, HIF-1α, GLUT1, CA9*, and *VEGF* after 8-hour exposure to 10 µM nicotine.

Conclusions

Our results suggest a relationship between nicotine exposure and the increased expression of genes that have a strict association with metabolites, survival, proliferation and inhibition of apoptosis in DOK, where the expression patterns were well-defined within 8 hours and in a dose-dependent manner. Further studies are warranted to comprehend nicotine's intricate mechanisms impacting oral cancer progression.

Introduction

Oral squamous cell carcinoma (OSCC) is known for its aggressive and rapidly advancing malignancy, often associated with a high mortality rate [1]. The presence of oral potentially malignant lesions (OPMD) is associated with an increased risk of developing this type of malignancy [2, 3]. The estimated worldwide prevalence of OPMD is approximately 4.47%, with higher prevalence rates observed in Asian (10.54%) and South American/Caribbean (3.93%) populations, compared to other regions, with a rate of malignant transformation estimated at around 12%; however, the absence of a consistent pattern and molecular hallmarks makes it challenging to predict which lesions are most likely to evolve into OSCC [4–6].

Continuous consumption of cigarettes is linked to an increased likelihood of developing OPMD and OSCC [7]. This association may be attributed to substances in cigarette smoke, many of which are carcinogenic, co-carcinogenic, and contribute to the tumorigenic process [8]. Nicotine is the main natural alkaloid found in large quantities in cigarette smoke and electronic nicotine delivery systems (ENDS) [9]. Although not considered a carcinogenic compound, nicotine inherently contributes to the activation of pathways

involved in tumor growth, cell progression, migration, angiogenesis, apoptotic evasion, and the induction of metastasis, through the activation of the PI3K/AKT and HIF-1 pathways [10–15].

The PI3K/AKT signaling pathway is dysregulated in a wide range of human cancers. The acquisition of these characteristics leads to heightened oncogenic signaling, which results in increased signal transduction associated with diverse cellular functions, such as cell growth, differentiation, survival, and intracellular trafficking. Normally, this gain in function indicates a worse prognosis for patients diagnosed with head and neck, colon, prostate, and breast cancers [15–19].

HIF-1 transcriptional complex is comprised of an alpha subunit (HIF-1α) and a nuclear beta subunit (HIF-1β). This transcription factor is responsible for activating the expression of genes involved in various physiological aspects, such as angiogenesis (VEGF), cellular pH regulation (CA9), glucose transport (GLUT-1) and metabolism, among others [20]. Activation of this pathway not only helps normal cellular adaptation to hypoxic environments, but also, when activated in tumor cells, induces metabolic reprogramming from oxidative to glycolytic states (Warburg effect), even under normoxic conditions, which leads to a more aggressive phenotype in OSCC [21–24].

In recent years, there has been a rise in the utilization of electronic nicotine delivery systems (e-cigarettes), especially among younger people in Brazil, as an alternative to traditional cigarettes [25]. These devices pose a higher risk of inducing addiction in users compared to conventional cigarettes, potentially serving as a gateway to the use of traditional tobacco products [26, 27].

The aim of this study was to assess the impact of nicotine exposure on cell viability, migration, and the mRNA expression of *PIK3CA, AKT1, HIF-1α, GLUT1, CA9* and *VEGF* in the DOK and SCC9 cell lines.

Materials and Methods

Cell Culture

The malignant cell line SCC9 (ATCC catalog number CRL-1629), derived from tongue squamous cell carcinoma, and the dysplastic oral keratinocyte cell line (DOK) (European Collection of Authenticated Cell Cultures – ECACC), derived from dysplastic oral keratinocytes of the tongue, were utilized in the assay. SCC9 cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham (DMEM/F12, Gibco, Germany) supplemented with 10% fetal bovine serum, antimycotic/antibiotic, and hydrocortisone (50 ng/mL). DOK cells were cultured in DMEM (Gibco, Germany) supplemented with 10% fetal bovine serum, antimycotic/antibiotic, and hydrocortisone (5 ng/mL). All cell cultures were maintained in a 5% CO_2 environment at 37°C.

Nicotine exposure

Nicotine (N3876 - ≥99%, GC, liquid, Sigma–Aldrich St. Louis, MO, USA) concentrations used during the study were: Control (0 μM), 1 and 10 μM. The stock solution of nicotine was passed through a 0.22 μm

pore filter and then diluted in culture media. The cells were seeded in a p6 plate in triplicate and exposed to nicotine after reaching 80% confluent, during a period of 8 and 24 hours.

Cell Viability Assay

The effect of different nicotine concentrations on the proliferation of SCC9 and DOK cells was estimated by the Sulforhodamine B (SRB) method describe by VICHAI &¨KIRTIKARA, 2006. SCC9 and DOK were seeded in 96-well plates in quintuplicates for 24 hours before being treated with different concentrations of nicotine (0, 1 and 10µM) for 8 and 24 hours. SRB absorbance was measured at 515 nm.

Wound healing Scratch Assay Cell

SCC9 and DOK cells were seeded on a 6-well cell culture plate (10^5 cells per well) and incubated at 37°C in 5% CO_2. After reaching confluency, a scratch was made using a p200 pipette tip, and cell debris were washed out with phosphate saline (PBS 1x), before cells were exposed to different concentrations of nicotine. Cells without nicotine were used as a control. Images of the scratch were taken at 0h, 8h, and 24h after exposure using a phase-contrast inverted microscope. Three images were taken for each well under 10x magnifications after incubation to estimate the migration of cells. The ImageJ software was used to calculate the scratch area. The wound closure percentage was calculated compared to the initial scratch area control.

RNA extraction and RT-qPCR

Total RNA from cell culture was extracted using TRIZOL® (Invitrogen, California, USA) according to the manufacturer's instructions. Quantity and quality of total RNA were accessed by NanoDropTM 2000/2000c spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) and 0.8% agarose gel.

To remove genomic DNA, the samples of total RNA were treated with DNase I (Invitrogen, California, USA). After treatment, the synthesis of cDNA was performed from 1µg of RNA using High-Capacity cDNA Reverse Transcription kit (Invitrogen, California, USA), according to the manufacturer's instructions.

RT-qPCR was performed using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The reactions were performed in triplicate in the 7500 Real-Time PCR System thermal cycler (Applied Biosystems, Foster City, CA, USA). Primers (Table 1) used were designed using the tools PrimerQuest Tool and OligoAnalyzer Tool, made available by the company IDT™ on its website (https://www.idtdna.com/pages). The transcript levels were normalized in relation to the housekeeping B2M (Beta-2-Microglobulin) and then corrected in relation to the control group according to method $2^{-\Delta\Delta Ct}$ described by LIVAK & SCHMITTGEN, 2001.

Table 1
qPCR primers.

Gene	Forward Primer (5' – 3')	Reverse Primer (5' – 3')	Amplicon size (pb)
AKT1	AGATCCTCAAGAAGGAAGTC	TCTGGAAAGAGTACTTCAGG	120
B2M	ACTTTGTCACAGCCCAAGAT	CCAAATGCGGCATCTTCAAA	80
CA9	GACATCCTAGCCCTGGTTTT	CCTTTGGTTCCCCTTCTGTG	95
GLUT1	CCAAGAGTGTGCTAAAGAAG	CGACTCTCTTCCTTCATCT	76
HIF-1α	TTCAAGCAGTAGGAATTGGAAC	CGTTTCCAAGAAAGTGATGTAGTAG	115
PIK3CA	GCTTTCTGTCTCCTCTAAAC	CAGAGGACATAATTCGACAC	75
VEGF	AGGGCAGAATCATCACGAAG	GTCTCGATTGGATGGCAGTAG	72

Statistical Analysis

SRB test was carried out through one-way ANOVA and Dunnett's post-hoc test. Wound healing Scratch Assay Cell was carried out through one-way ANOVA with Bonferroni post-hoc test. The difference in gene expression between the control and treated groups was performed using one-way ANOVA and the means were compared by Tukey's HSD post-hoc test. Significance level of 5% ($p \leq 0.05$) was considered in all analyses. All analyses were conducted using RStudio (version 2023.06.0 + 421 and R version 4.3.1).

Results

Effect of nicotine in cell viability and migration of SCC9 and DOK

The SRB assay revealed an increase in cell viability, specifically in SCC9 cells (Fig. 1B), associated with different nicotine concentrations and exposure durations. We observed an increase in SCC9 cell viability at concentrations of 1 μM (17% in 8 hours, $p = 0.005$; 8% in 24 hours, $p = 0.003$) and 10 μM (14% in 8 hours, $p = 0.01$; 7% in 24 hours, $p = 0.004$) compared to the control group (Fig. 1B). However, this effect was not observed in DOK cells (Fig. 1A).

The wound assay demonstrated heightened cell migration in DOK cells, particularly notable at 8 hours with 10 μM of nicotine (Fig. 2B). At this concentration, there was a 44% ($p > 0.01$) increase in cell migration and wound closure compared to the control.

Nicotine concentration modulation of PIK3CA and AKT1 expression

The DOK cell line showed a significant increase in the expression of *PIK3CA* and *AKT1* at 10μM (*PIK3CA*: 4.17-fold, p = 0.0001; *AKT1*: 2.84-fold, p = 0.001) after 8 hours and significant decrease at 1 μM (2.94-fold, p < 0.0001; 8.38-fold, p < 0.0001) and 10 μM (1.99-fold, p < 0.0001; 1.88-fold, p = 0.001) after 24 hours compared to control. Furthermore, the expression was significantly higher under 10 μM compared to 1 μM after both timepoints (Fig. 3).

The expression of *PIK3CA* and *AKT1* in SCC9 cells was not associated with nicotine concentration after 8 hours of exposure compared to control. Gene expression was decreased at 1 μM (*PIK3CA*: 1.44-fold, p = 0.01) and at 10 μM (*PIK3CA*: 1.85-fold, p = 0.002; *AKT1*: 2.85-fold, p = 0.0002), compared to control after 24 hours (Fig. 3). Furthermore, *AKT1* expression was significantly different between 1 μM and 10 μM for both timepoints (Fig. 3B).

Nicotine Impact on HIF-1α and its Target Genes in DOK and SCC9 Cells

The RT-qPCR analyses in DOK cells demonstrated an increased expression of *HIF-1α* at a concentration of 10μM (3.81-fold, p = 0.0001) after 8 hours compared to control (Fig. 4A). Subsequently, after 24 hours, the mRNA expression decreased notably at both 1 and 10 μM concentrations in *HIF-1α* (5.26-fold, p < 0.0001; 1.53-fold, p < 0.0001). We observed that SCC9 cells showed a slight increased mRNA expression only for *HIF-1α* at both concentrations (1 μM: 1.34-fold, p = 0.02; 10 μM: 1.52-fold, p = 0.003) after 24 hours (Fig. 4A).

Analyses of the effect of nicotine on the expression of *CA9, GLUT1* and *VEGF*, the target genes of the transcription factor HIF-1, showed that treatment of DOK cells at a dose of 10 μM promoted an increase in the expression of these genes after 8 hours (*CA9*: 23.27-fold, p = 0.0003; *GLUT1*: 14.41-fold, p < 0.00001; *VEGF*: 2.80-fold, p = 0.0009). However, a significant decrease in the mRNA expression of *CA9* (1μM: 11.1-fold, p < 0.0001), *GLUT-1* (1 μM: 2.73-fold, p = 0.002) and *VEGF* (1 μM: 8.19-fold, p = 0.00001; 10 μM: 1.72-fold, p = 0.001) after 24 hours (Fig. 4).

We also examined the effects of nicotine in SCC9 and found that *CA9* and *GLUT1* mRNA expression increased at 1 μM after 8 hours (1.45-fold, p = 0.04; 2.25-fold, p = 0.00002), and *VEGF* after 24 hours (1.56-fold, p = 0.01) (Fig. 4). The expression of *GLUT1* was lower compared to the control at 10 μM after 8 hours (1.47-fold, p = 0.02), but increased significantly after 24 hours (1.40-fold, p = 0.03) at the same concentration (Fig. 4C).

Discussion

We analysed the influence of nicotine on *PIK3CA, AKT1, HIF-1α, GLUT1, CA9* and *VEGF* mRNA gene expression in the dysplastic oral keratinocyte (DOK) and tongue cancer (SCC9) cell lines. Our results showed that nicotine induced an increase in SCC9 cell viability and DOK cell migration in a dose- and time-dependent manner. We found that an 8 hour exposure to 10 μM of nicotine increased mRNA

expression of all genes associated with proliferation and survival pathways. Nicotine exposure in SCC9, on the other hand, was shown to affect the expression of genes related to the HIF1 pathway at different concentrations and times. These results suggest that nicotine induces the activation of gene transcription in DOK and SCC9 in different ways.

There is a paucity of studies in the literature linking nicotine exposure and its effect on cell migration of oral dysplastic keratinocytes. We found only one study that showed that nicotine at a concentration of 10 µM promoted and increased cell migration in DOK after 24 hours of exposure, but not after 8 hours [28]. Our results showed different effect, where we observed that exposure to 10 µM nicotine for a period of 8 hours resulted in an increase in cell migration in DOK, but not after 24 hours. This could be due to differences in the wound closure migration assay, as Wisniewski et.al. (2018) starved cells of serum or growth factor overnight and followed it with to 2-hour pre-treatment with the inhibitors.

The PI3K-AKT signalling pathway is a widely recognised catalyst for cancer progression and acts on cell proliferation, growth, differentiation and motility, particularly in OSCC [15, 19]. The literature reports a marked increase in the expression of the *PI3K* gene and p-AKT protein in dysplastic and cancerous oral tissues compared to normal epithelium, suggesting an association between activation of the PI3K-AKT pathway and the progression of oral carcinogenesis [29–31]. Our results showed that nicotine also increases the expression of *PIK3CA* and *AKT1*, which may contribute to the increased migration of DOK cells we observed at the 10 µM nicotine concentration after 8 hours.

Nicotine's effects on cancer cells could mimic the effects of hypoxia even under normal oxygen conditions, leading to an increase in transcription, translation and cytoplasmic stabilisation of HIF1-α, which consequently increases gene expression of *GLUT, CA9* and *VEGF* [21, 32–34]. We observed the same pattern in our results, where 10uM nicotine increased HIF1 and its targets expression in DOK cells after 8 hours. To the best of our knowledge no studies analysed the effects of nicotine on the HIF pathway in dysplastic oral cells. However, studies in oral tissues suggest that the expression of HIF1-α, and its targets (CA9, GLUT1, VEGF) is an early event in oral carcinogenesis and can contribute to a great risk of malignant transformation [35–38]. Therefore, we hypothesize that changes in the DOK metabolic pathway caused by nicotine exposure may favour increased expression, translation and migration of CA9, GLUT1, VEGF in the cell, contributing to the malignancy process.

Although our study was limited to observing the gene expression of *PIK3CA, AKT1, HIF-1α, GLUT1, CA9* and *VEGF*, there are studies showing a positive correlation between in gene and protein expression [39–41]. This is corroborated in our results since each target in the PI3K/AKT and HIF-1 pathway were increased. In addition to protein expression analyses, future studies could also use pathway inhibitors, to support these results.

Our results showed that nicotine was able to increase dysplastic oral keratinocytes migration and gene expression of *PIK3CA, AKT1, HIF-1α, GLUT1, CA9*, and *VEGF* after 8 hours of exposure. These findings may help understand the impact of nicotine's action on the pre- and post-transcriptional regulation, since mRNA expression of these genes is poorly understood in dysplastic oral keratinocytes.

Declarations

Data Availability

Data are available from the corresponding author on reasonable request.

Funding

Author Contributions

JGS, MMO, FDN and AMAS conceived and designed the study. JGS, MMO, GTP, LLM, AFCSP and ABA performed the molecular analysis. JGS, MMO, SOM, ARB, IAAM and LJO assisted with data analysis and interpretation of results. JGS, MMO, LJO, FDN and AMAS drafted and performed a critical review of the manuscript. All authors critically reviewed content and approved final version for publication.

Ethics declarations

Competing interest

 The authors declare no competing interests.

Consent to participate

Not applicable.

Consent for publication

The authors hereby consent to publication of this study.

Research involving human participants and/or animals

This article does not contain any studies with human or animal subjects.

Ethical approval

Not applicable.

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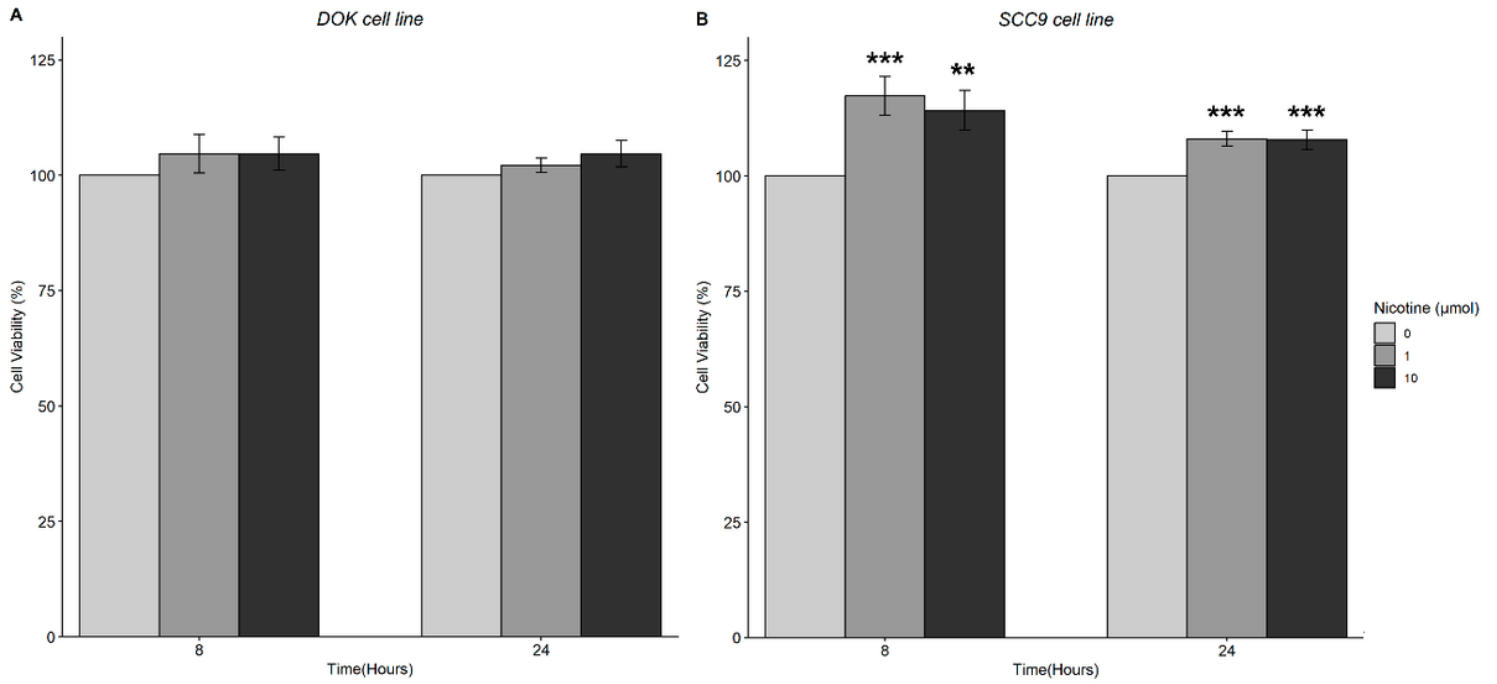
Figures

Figure 1

Nicotine concentration and nicotine exposure time influence cell viability of SCC-9 and DOK. (A) SCC-9 cell viability after 8 and 24 hours of nicotine exposure. (B) DOK cell viability after 8 and 24 hours of nicotine exposure. Asterisks indicate significant difference by *Dunnett's post-hoc* test. * p ≤ 0.05.
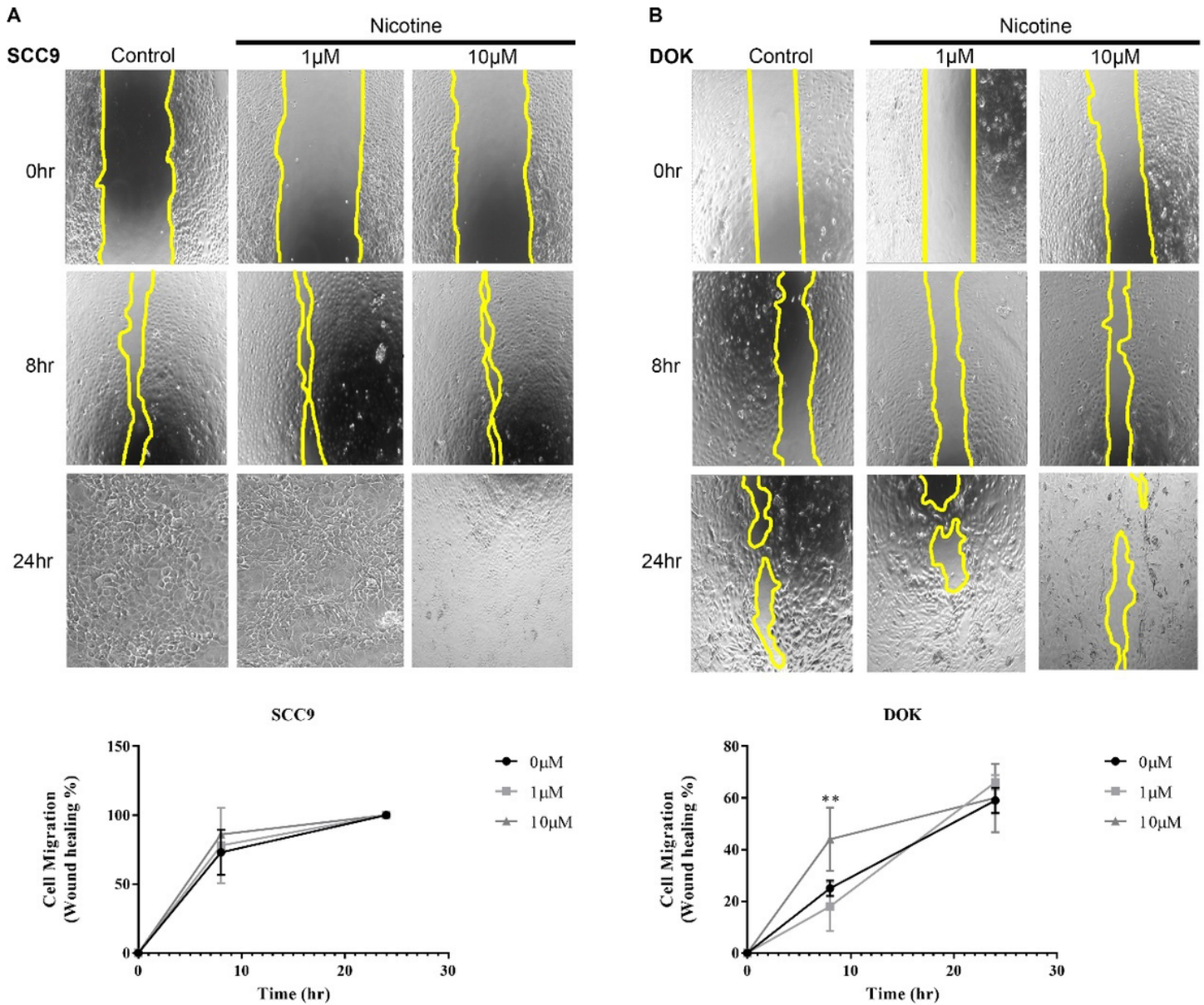
Figure 2

Effect of nicotine concentrations on cell migration SCC9 (A) and DOK (B) at 0, 8 and 24 hours. The graph illustrates the percentage density of wound closure when comparing different nicotine concentrations with the control. The asterisk (*) indicates significant differences tested by one-way ANOVA with Bonferroni post-test ($p < 0.05$). The experiments were carried out in triplicate.
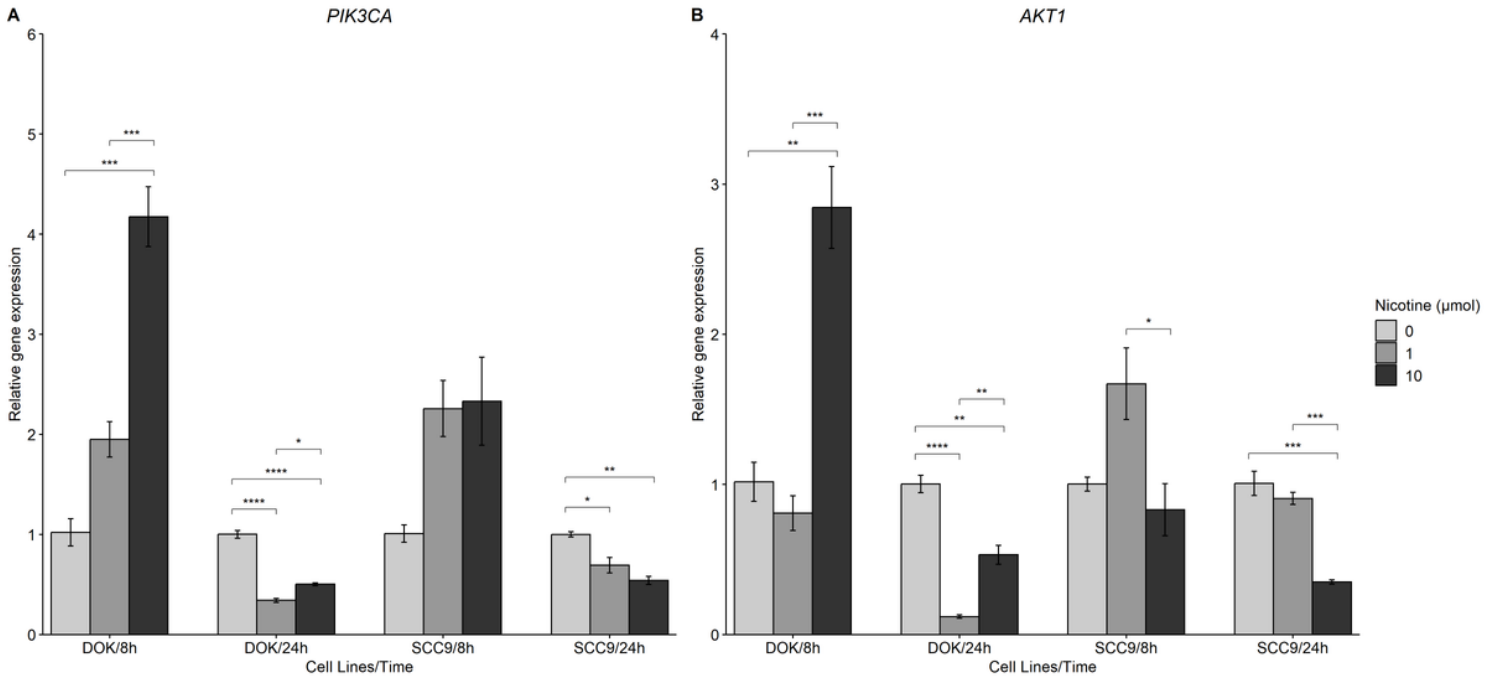
Figure 3

Relative expression of *PIK3CA* and *AKT1* after 8- and 24-hours exposure to different concentrations of nicotine. (A) Relative expression of *PIK3CA* in SCC9 and DOK cells. (B) Relative expression of *AKT1* in SCC9 and DOK cells. Statistical analyses were performed by one-way ANOVA and Tukey's post hoc test (p <0.05). The expression levels were calibrated according to $2^{-\Delta\Delta Ct}$ method (LIVAK; SCHMITTGEN, 2001). RT-qPCR reactions were prepared in triplicate.
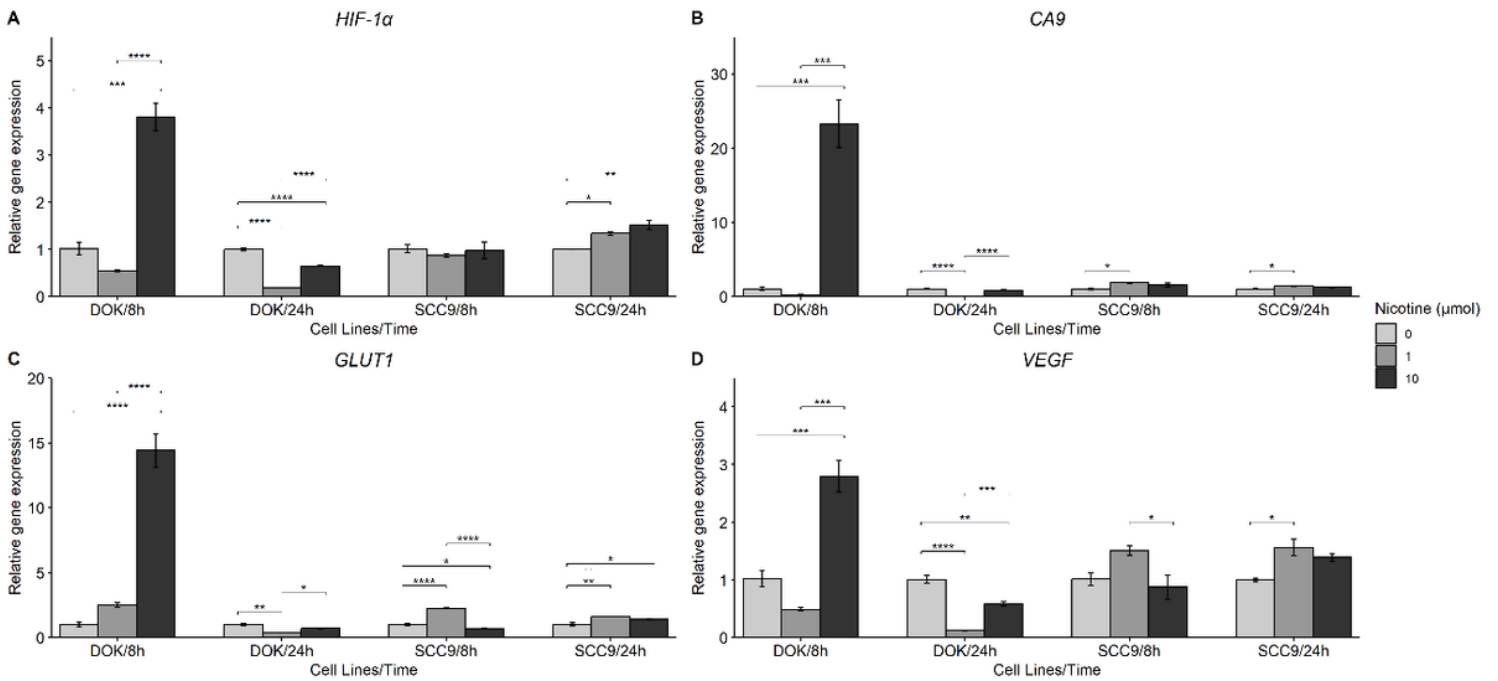

Figure 4

Relative expression of *HIF-1α, GLUT1, CA9* and *VEGF* after 8- and 24-hours exposure to different concentrations of nicotine. (A) Relative expression of *HIF-1α* in SCC9 and DOK cells. (B) Relative expression of *CA9* in SCC9 and DOK cells. (C) Relative expression of *GLUT1* in SCC9 and DOK cells. (D) Relative expression of *VEGF* in SCC9 and DOK cells. Statistical analyses were performed by one-way ANOVA and Tukey's post hoc test (p <0.05). The expression levels were calibrated according to $2^{-\Delta\Delta Ct}$ method (LIVAK; SCHMITTGEN, 2001). RT-qPCR reactions were prepared in triplicate.