The circadian clock gene ARNTL overexpression suppresses oral cancer progression by inducing apoptosis via activating autophagy

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Research Article

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The circadian clock gene ARNTL overexpression suppresses oral cancer progression by inducing apoptosis via activating autophagy

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Abstract

Objective

The study aimed to explore tumor suppressor mechanism of ARNTL from the perspective of autophagy in oral cancer

Methods

Human oral squamous carcinoma HN6 cells stably overexpressing ARNTL were established, cell viability and apoptosis were detected by CCK-8 and TUNEL assays, and intracellular autophagosomes were observed under electron microscopy. Western Blot detected expressions of Beclin1, LC3 II/I, ATG-12, P62, BAX and BCL-2. Bafilomycin A1 was used to detect autophagic flux, and Western Blot was used to detect changes of LC3II and P62 proteins. Autophinib was added to cells with ARNTL overexpression for recovery experiments, and cell proliferation and apoptosis were detected by flow cytometry. In vivo tumorigenesis experiment was used to evaluate the in vivo anti-tumor efficacy of ARNTL, and Western blot simultaneously detected ARNTL, LC3 II/I, Beclin1, P62 and ATG-12 expressions.

Results
ARNTL overexpression promoted apoptosis and autophagy and inhibited cell viability. In ARNTL-overexpressing cells, expressions of Beclin1, LC3 II/I, and BAX were significantly up-regulated, while P62 and BCL-2 expressions were decreased, and ATG-12 expression wasn't significantly changed. When the autophagy inhibitor Autophinib was used, expressions of elevated BAX and decreased BCL-2 were reversed effectively, as were decreased cell proliferation index and increased apoptosis index. An in vivo tumorigenesis assay also showed ARNTL overexpression inhibited tumor growth, and autophagy-related protein expressions were consistent with the in vitro data.

**Conclusions**

The research demonstrated for the first time that ARNTL induced apoptosis and inhibit cell proliferation dependent on autophagy in oral cancer, which provides theoretical basis for potential therapeutic targets.

**Keywords:** ARNTL, autophagy, clock genes, oral cancer, apoptosis.

**Acknowledgments**

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Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world[1]. The incidence is increasing year by year, and HNSCC expected to increase by 30% by 2030 (ie 1.08 million new cases per year) [2]. Oral squamous cell carcinoma (OSCC) is the most common type of HNSCC, which is usually treated by surgical resection, followed by adjuvant radiotherapy or chemotherapy plus radiotherapy according to the disease stage[1]. Despite the gradual improvement of treatment technology, the 5-year survival rate of OSCC patients still has not increased significantly[2]. Moreover, due to the complexity of anatomical structure of head and neck, patients often face many serious disabilities after treatment, such as swallowing and speech disorders1, and the quality of their life has not been significantly improved[3]. Therefore, it is of great significance to explore effective treatment methods and prevention mechanisms for OSCC.

Circadian clock genes exist in almost all cells of life[4]. ARNTL (also known as BMAL1, MOP3) protein interacts with CLOCK or NPAS2 in the form of heterodimer to active the transcriptions of downstream clock genes[4]. When the translation products of downstream clock genes reach a certain concentration, they will negatively act on ARNTL: CLOCK or ARNTL: NPAS2 dimers. Finally, it will form a positive and negative transcription-translation feedback loop[4,5,6]. The ARNTL gene, expressed in almost all suprachiasmatic nuclear cells and most peripheral cells, is one of the core components of circadian clock system[4]. It is also the only clock gene that leads to the loss of all rhythmic behaviors in mice after knockout[5], which shows the important role of ARNTL. In addition, abnormal ARNTL expression will disrupt the physiological function of normal cells, and leads to a variety of diseases including tumors[6].

There is a close connection between the circadian clock system and autophagy activity, and the direct regulation of autophagy-related genes transcription is considered to be the main way that the circadian clock regulates autophagy activity[7,8]. Recent study has shown that ARNTL can enhance the protection of diabetic mice from cerebral ischemia-reperfusion injury through autophagy mediated by SIRT1 pathway[9]. Besides, ARNTL can also improve glucose homeostasis through autophagy pathway in Clock(Δ19/+ ) heterozygous mice[10]. However, it is unclear whether ARNTL can regulate autophagy activity of tumor cells to affect tumor development.

In this study, by establishing ARNTL-overexpressing OSCC cells, we confirmed ARNTL as a tumor suppressor gene in OSCC, and explored its tumor suppressor mechanism from the perspective of autophagy. For the first time, it was demonstrated that the clock gene ARNTL could induce OSCC cell
apoptosis and inhibit cell proliferation through directly regulating autophagy.

Materials and Methods

Cell culture
OSCC cells HN6, SCC15, and CAL27 were obtained from the Stomatological Hospital of Chongqing Medical University. The DMEM (Sigma, USA) medium containing 10% inactivated fetal bovine serum (MP Biomedicals, New Zealand) and 1% penicillin-streptomycin mixture was used for cell culture in an incubator at 37°C with a volume fraction of 5% CO2. Cells were passaged every 2d-3d, and cells in logarithmic growth phase were used for subsequent experiments.

Construction of ARNTL Overexpression Lentiviral Vector
Synthetic primers were designed according to the ARNTL gene sequence in NCBA. The primer sequences are: upstream primer: 5’-GAGGATCCCCGGTACCACCACGTGCGCAGACCAG AGAATGGAC-3; downstream primer: 5’-TCTTTGTAGTCCATACCCAGCGCCATGGCAAGTCA CTAAGG-3’. ARNTL amplification products were obtained by PCR. The amplified product was recombined with the linearized vector digested by Age I, then the product was added to competent E. coli cells, and heat shock promoted the transformation of the product. After culture, it was sent to a sequencing company for sequencing identification. The 293T cells were inoculated in petri dishes, and the lentivirus infection was carried out when they grew to 70% to 80%. GV365 vector plasmid, pHelper1. 0 vector plasmid and pHelper2.0 vector plasmid were co-transfected with Genechem transfection reagent for 48h. After the medium was changed, the cell supernatant was aspirated and centrifuged, filtered and centrifuged to collect the virus concentrate and stored in a -80°C refrigerator.

Lentivirus infection of target cells
OSCC cells were made into a suspension and inoculated in a six-well plate. The frozen virus stock solution was thawed in an ice bath, diluted according to the appropriate MOI value. The original medium of the experimental group and the control group was aspirated, and the lentivirus dilution solution and lentivirus negative control dilution were added dropwise to the cells of the experimental group and the control group, respectively. Fluorescence expression was observed by an automatic fluorescence imaging system (Life, USA) after 48 h.

Real-time quantitative polymerase chain reaction (RT-qPCR)
Total RNA was extracted from each group of cells according to the instructions of TaKaRa RNAiso
Plus. The optical density values at wavelengths of 260 nm and 280 nm were measured with a nucleic acid protein analyzer (Thermo Scientific, USA), and the RNA concentration and purity were calculated. The cDNA was reverse transcribed using PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Japan). The reaction system was 10μl, and the reaction conditions were 37 °C for 15 min, 85 °C for 5 s, and 4 °C for 4 min. Primers were designed using Oligo7.0 software. Amplification was performed on a Real-time PCR Amplifier (Bio-Rad, USA). The reaction conditions were: pre-denaturation at 95°C for 30s, denaturation at 95°C for 5 seconds, annealing and extension at 60°C for 30 seconds, and 40 cycles. The 2\(^{-\Delta\Delta Ct}\) method was used to calculate the mRNA expression of each gene, and the experiment was repeated three times.

**Western blot**

Total protein was isolated using RIPA lysis buffer (Beyotime, China) containing 2% phosphatase inhibitor and 1% PMSF. The protein concentrations were quantified using a BCA protein quantification kit (Beyotime, China). Proteins (30 μg-50 μg) were separated on an 8%-15% SDS-PAGE gel (Epizyme, China) and transferred onto PVDF membranes (Immobilon-P, Ireland). After blocking for 1h with 5% fat-free milk diluted in TBST at room temperature, the membranes were incubated with primary antibodies against ARNTL, LC3, P62, Beclin1, ATG-12, BAX, BCL-2 and GAPDH, respectively, at 4°C overnight. The antibodies information were listed in Supplementary Table 1. Subsequently, the membranes were washed with TBST 3 times, and incubated with HRP-conjugated secondary antibody for 1h at room temperature. After washed with TBST, the protein bands were detected using an ECL-Advance Western blot Imaging System (Bio-Rad, USA) with a hypersensitive chemiluminescent substrate (Beyotime, China). ImageJ 5.0 software (Windows, 64-bit Java 1.8.0_112) was used to analyze the gray values of the bands.

**CCK-8**

Three groups of OSCC cells in good growth condition were taken and counted after digestion with trypsin. The cells were seeded in a 96-well plate at 5\(\times\)10\(^3\) cells/well, and each group consisted of 3 replicate wells. After culturing for 1, 2, 3, 4, and 5 days, the original medium was aspirated, 100 μL of fresh medium and 10 μL of CCK8 solution (Bioground, China) were added to each well of cells, and incubated at 37°C for 2 h in the dark. A multifunctional microplate reader (Molecular Devices, USA) was used to detect the OD values by each group of cells at a wavelength of 450 nm. The growth curves were drawn with the time and the corresponding OD values.
Tunel apoptosis detection

The experimental operation was carried out according to the instructions of the apoptosis detection kit (TUNEL, Beyotime, China). The cells were plated in a 12-well plate. When the cells grew to about 50%, the culture medium was discarded. The cells were washed twice with PBS, fixed with 4% paraformaldehyde solution for 15 min, and then incubated with PBS containing 0.3% Triton X-100 (Solarbio, China) for 5 min at room temperature. Next, each well of the cells were added 50 μl of TUNEL detection solution and incubated at 37°C for 60 minutes in the dark, then added an appropriate amount of anti-fluorescence quenching mounting solution (Solarbio, China) containing DIPA dropwise, and observed under the automatic fluorescence imaging system (Life, USA). The experiment was repeated 3 times and 5 different fields were randomly selected. Cell numbers were counted using ImageJ-5.0 software. The TUNEL positive rate was equal to the number of TUNEL positive cells divided by the total number of cells × 100%.

Transmission Electron Microscopy (TEM)

The cells in the logarithmic growth phase were digested with 0.25% trypsin, centrifuged at 1200 r/min for 10 min at room temperature, and slowly added 4% glutaraldehyde for fixation in a 4°C refrigerator overnight. Then the cells were added 2.5% osmium tetroxide and dehydrated with increasing concentrations of ethanol. Cells were embedded in paraffin and sectioned at 80 nm. The morphology and number of autophagosomes in each group of samples were observed and photographed by transmission electron microscope (JEM-1400Plus, Japan). Ten cells were randomly selected from each group of samples and calculated by the following formula: autophagosome density = autophagosome number/cell number.

Flow cytometry

Cell proliferation assay

The cells in the logarithmic growth phase were digested with 0.25% trypsin, removed the supernatant by centrifugation, and resuspended in 100 μl of PBS and 500 μl of cold 75% ethanol for 12h at 4°C. After washed twice with pre-cooled PBS and centrifugated, 400 μl propidium iodide staining solution (1 mg/ml) and 100 μl RNase A (100 μg/ml) were added and incubated at 4°C for 30 min in the dark. Cell cycle distribution was detected by flow cytometry. Cell proliferation index (PI) = (S+G2/M)/(G0/G1+S+G2/M) ×100%.

Apoptosis detection
The cells were digested and resuspended to the concentration of approximately $10^6$ cells/ml. 1ml of cell suspension and 200µl of Annexin V-APC staining solution were mixed and incubated at room temperature for 15 min in the dark, and then added 1 ml of propidium iodide staining solution. The apoptosis of cells in each group was detected by flow cytometry. Apoptotic index=number of apoptotic cells/the total number of detected cellsx100%.

**Subcutaneous xenograft models**

The use of 8 SPF-grade BALB/c nu/nu female nude mice aged 4-5 weeks in the study was approved by the Chongqing Medical University Laboratory Animal Research Institute. Nude mice were randomly divided into the ARNTL-OE group and the ARNTL-NC group. Then, 0.2 mL of ARNTL-OE and ARNTL-NC cell suspensions at a concentration of $5\times10^6$ cells/mL were injected into the right back of each mouse. Tumor size was measured every 3 days after injection. After 3 weeks, tumor formation was obvious. The nude mice were killed by cervical dislocation, and the tumor tissues were stripped. The tumors were weighed with an electronic balance, and the tumor volume ($V$) was calculated using the following formula: $V$ (cm$^3$) = ($a \times b^2$) / 2 ($a$ is the length, and $b$ is the width). Western blot was used to detect the protein expression levels of ARNTL, LC3 II/I, Beclin1, P62 and ATG-12 in the tumor tissues.

**Statistical analysis**

Graph Pad Prism 8.0 was used for statistical analysis, and data were expressed as mean ± SD. The student’s t-test was used for comparison between two groups, and the one-way analysis of variance (ANOVA) was used for comparison between multiple groups. Values of $P < 0.05$ were considered to be statistically significant.

**Results**

**Establishment of stably overexpressing ARNTL OSCC cells**

The expression levels of ARNTL mRNA and protein in OSCC cell lines HN6, SCC15 and CAL27 were detected by RT-qPCR and Western Blot. The results showed that the ARNTL expression levels of mRNA and protein in HN6 cells were significantly lower than those of the other two groups of OSCC cells ($P<0.05$). See Fig.1a, b. HN6 cells were selected for subsequent transfection of the overexpressing lentiviral vector. The cells after transfection were shown in Fig.1c below. The green fluorescent light
densities indicated efficient integration of the lentiviral plasmid into the genome of HN6 cells. RT-qPCR and Western Blot results showed that the expressions of ARNTL mRNA and protein in the ARNTL-OE group were significantly higher than that in the Blank and ARNTL-NC groups ($P<0.05$). See Fig.1d, e.

**ARNTL overexpression inhibits OSCC cells viability and promotes apoptosis and autophagy**

CCK-8 and TUNEL experiments were carried out to investigate the effects of ARNTL overexpression on OSCC cells viability and apoptosis. The CCK8 assay showed that the cell viability in the ARNTL-OE cells was significantly lower than that of the Blank and ARNTL-NC cells on 4 and 5 days ($P<0.05$), as shown in Fig.2a. There was no significant change in results of the first three days. TUNEL analysis showed that the proportion of TUNEL-positive cells in the ARNTL-OE cells was significantly elevated than the other groups ($P<0.05$). See Fig. 2b. These consequences suggest that ARNTL overexpression inhibits OSCC cells viability and promotes apoptosis. Transmission electron microscopy is the current gold standard for detecting the level of autophagy in cells. As shown in Fig.2c compared with the ARNTL-NC cells, more double-membrane autophagolysosomes that engulfed organelles were seen in the ARNTL-OE cells, and the density of autophagosomes elevated significantly ($P < 0.05$).

Western Blot detection of autophagy-related genes showed that the expressions of Beclin1 and LC3 II/I ratio were notably increased after ARNTL overexpression, while the expression of P62 was significantly decreased ($P<0.05$), and the ATG-12 expression had no significant change ($P>0.05$) (Fig.3a). To further determine the changes in dynamic autophagy flux, the autophagy inhibitor Bafilomycin A1 (Baf A1) was used, and the reduced P62 and the LC3II protein were significantly reverted due to the addition of Baf A1 ($P<0.05$), as shown in Fig.3b. These results clarifies that ARNTL overexpression promotes autophagy in OSCC cells.

**ARNTL induced apoptosis and inhibited cell proliferation by promoting autophagy in OSCC cells**

To investigate the relationship between autophagy, apoptosis and proliferation, the autophagy inhibitor Autophinib was added to the ARNTL-OE cells. As shown in Fig.4a, the expression of LC3 II/I was significantly decreased, and the P62 expression was significantly up-regulated ($P<0.05$), which proved that Autophinib could effectively inhibit the autophagy activity of cells. Furthermore, ARNTL overexpression promoted the BAX expression and inhibited the expression of the anti-apoptotic gene
BCL-2 ($P<0.05$). The proliferation index of the ARNTL-OE cells was significantly decreased, while the apoptosis index was significantly increased ($P<0.05$) (Fig.4b). These results prove once again that ARNTL overexpression inhibits OSCC cells proliferation and promotes apoptosis. After adding the autophagy inhibitor Autophinib, the elevated BAX and decreased BCL-2 expressions were both significantly recovered ($P<0.05$). The results of flow cytometry experiments also showed that the decreased cell proliferation index and the increased apoptosis index were reversed effectively due to the addition of Autophinib ($P<0.05$) (Fig.4b). Taken together, these consequences suggest that ARNTL can induce apoptosis and inhibit cell proliferation dependent on autophagy in OSCC cells.

**ARNTL overexpression inhibits tumor growth in vivo**

The above studies confirmed the anti-tumor activity of ARNTL in vitro. Next, we evaluated the in vivo anti-tumor efficacy of ARNTL using subcutaneous xenograft models of the ARNTL-OE cells. The results revealed that the weights and volumes of tumors in the ARNTL-OE group were significantly lower than those in the ARNTL-NC group ($P<0.05$) (Fig. 5a). Western blot showed no significant difference in the ATG-12 protein expression between the ARNTL-OE group and the ARNTL-NC group ($P>0.05$), whereas the P62 protein expression was significantly reduced, and the ARNTL, LC3 II/I ratio and Beclin1 protein expressions were significantly increased in the ARNTL-OE group compared to those in the ARNTL-NC group ($P<0.05$) (Fig.5b). Consistent with the in vitro data, the above results indicate that ARNTL overexpression can significantly inhibit the growth of tumors and promote autophagy in vivo.

**Discussion**

Tumor formation is an extremely complex process, which is affected by various established or uncertain factors. The circadian clock controls various biological processes in the life system, of course including the core feature of tumor: cell division[4]. As a core circadian clock gene, abnormal ARNTL expression is closely related to the occurrence and development of tumors. Our results showed that ARNTL could promote OSCC cell apoptosis, inhibit cell proliferation and play the role of tumor suppressor gene in vitro and in vivo. This conclusion is consistent with previous studies on ARNTL in nasopharyngeal carcinoma[11], tongue cancer[12], and pancreatic cancer[13]. Although researchers have made a lot of efforts, the exact anti-cancer mechanism of ARNTL is still unclear, so it is necessary to further explore its underlying mechanism.
Autophagy is a universal life activity in eukaryotic cells. It provides necessary raw materials for cell reconstruction and regeneration by degrading damaged or aging organelles in cells, and timely removes misfolded proteins, so as to maintain the stability of the internal environment[14]. Abnormally reduced or elevated autophagy can lead to cell canceration or death[14]. LC3 is a characteristic marker on the membrane of autophagosomes. LC3 I is hydrolyzed and cleaved into LC3 II, which wraps around the damaged organelles to form autophagosomes, and the formation of autophagosome is the central link of autophagy activity[15]. LC3 II is the only protein specifically localized to autophagic structures throughout the phagosome-to-lysosomal degradation process, therefore, the ratio of LC3 II/I can evaluate the level of autophagy to a certain extent[16]. In the process of monitoring autophagy, the increase of LC3 II may be caused by the increase of LC3 II bound to autophagosomes, or the decrease of lysosomal degradation of LC3 II. Therefore, it is necessary to use Baf A1, a late autophagy inhibitor, to inhibit the degradation of intracellular autophagolysosomes. The changes in LC3 II level observed at this time only represent changes in the number of autophagosomes, reflecting dynamic autophagosomes[15]. The results of this study showed that the number of autophagosomes in OSCC was significantly increased after ARNTL overexpression, the expressions of Beclin1 and LC3 II/I were significantly increased, and the P62 expression was significantly decreased. After use of Baf A1, the expressions of LC3II and P62 proteins increased significantly. Thus, it can be proved that ARNTL promotes autophagy in OSCC cells.

The regulatory mechanism of autophagy on tumor cells is quite complex, and there is an interaction between autophagy, cell proliferation and apoptosis. In some studies, autophagy is considered to inhibit tumor cell proliferation and promote apoptosis. When apoptosis is activated, it can specifically induce the cleavage of autophagy-related proteins, so as to inhibit cellular protective autophagy[17,18]. However, in other studies, the role of autophagy is diametrically opposite. In glioblastoma and prostate cancer, autophagy is thought to promote tumor growth by providing energy to tumor cells and assisting the tumor in responding to changes in the microenvironment [19,20]. Autophagy, as a "double-edged sword", often plays different roles in the occurrence and development of tumors, and the function is often affected by many factors, such as tumor type and classification[21]. In this study, after using early autophagy inhibitor Autophinib to effectively inhibit autophagy, the apoptosis was inhibited and cell proliferation was promoted in OSCC cells. Elevated Bax and decreased BCL-2 expressions were also significantly restored. It can be proved that the regulation of ARNTL on the proliferation and apoptosis
of OSCC cells depends on autophagy, and the specific regulation mechanism and related signal pathways, for example PI3K/AKT/mTOR or JNK/Bcl-2 pathway, need to be further explored.

In summary, our study demonstrated for the first time that the circadian clock gene ARNTL could induce OSCC cell apoptosis and inhibit cell proliferation by directly regulating autophagy, so as to play the role of tumor suppressor gene. This discovery improves the anti-cancer mechanism of ARNTL from the perspective of autophagy, and also provides a theoretical basis for finding valuable potential therapeutic targets.

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**Statements & Declarations**

No potential conflicts of interest were disclosed.
**Fig. 1** ARNTL was successfully overexpressed in OSCC cells

a RT-qPCR detection of ARNTL mRNA expression in OSCC cells. b Western Blot detection of ARNTL protein expression in OSCC cells. **P < 0.01, ***P < 0.001, compared to HN6 cells. c OSCC cells transfected with ARNTL overexpression lentivirus under a fluorescence microscope. d RT-qPCR detection of ARNTL mRNA expression in OSCC cells before and after ARNTL overexpression. e Western Blot detection of ARNTL protein expression in OSCC cells before and after ARNTL overexpression. The data are presented as the means ± SD from at least three independent experiments. **P < 0.01, ***P < 0.001, compared to Blank or ARNTL-NC group.

**Fig. 2** ARNTL overexpression inhibits OSCC cells viability and promotes apoptosis and autophagy activities

a CCK-8 assay to detect OSCC cells viability. b TUNEL assay to detect OSCC cells apoptosis. Scale bar = 200um. c Observation of intracellular autophagosomes under TEM. The lower picture is the image of the upper picture with the red box area under high magnification. The autophagosome can be distinguished by its contents (partially cytoplasm, including mitochondrion, ribosomes, and rough endoplasmic reticulum) indicated by red arrows. Scale bars are 5um and 1um respectively. The data are presented as the means ± SD from at least three independent experiments.*P < 0.05, **P < 0.01, ***P < 0.001, compared to Blank or ARNTL-NC group.

**Fig. 3** ARNTL overexpression promotes autophagy in OSCC cells

a The autophagy-related protein expressions of LC3 II/I, Beclin1, P62 and ATG-12 after ARNTL overexpression in OSCC cells. b The protein expressions of LC3 II and P62 in OSCC cells after adding the autophagy inhibitor Baf A1. All data represent three independent experiments. The results are shown as the mean ± SD.*P < 0.05, **P < 0.01.

**Fig. 4** ARNTL induces apoptosis dependent on autophagy pathway

a The autophagy-related and apoptosis-related protein expressions of LC3 II/I, P62, BAX and BCL-2 after adding the autophagy inhibitor Autophinib. b Flow cytometry results showed that the decreased cell proliferation index and the increased apoptosis index were reversed effectively after adding Autophinib. All data represent three independent experiments. The results are shown as the mean ± SD.*P < 0.05, **P < 0.01, ***P < 0.001.

**Fig. 5** ARNTL overexpression inhibits OSCC cells tumorigenesis in vivo
a The weights and volumes of tumors in the ARNTL-OE group were significantly lower than those in the ARNTL-NC group. b Western blot was used to detect the autophagy-related protein expressions in tumor-forming tissues in vivo. All data represent three independent experiments. The results are shown as the means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.
Figures

**Figure 1**

ARNTL was successfully overexpressed in OSCC cells a RT-qPCR detection of ARNTL mRNA expression in OSCC cells. b Western Blot detection of ARNTL protein expression in OSCC cells. **P < 0.01, ***P < 0.001, compared to HN6 cells. c OSCC cells transfected with ARNTL overexpression lentivirus under a fluorescence microscope. d RT-qPCR detection of ARNTL mRNA expression in OSCC cells before and after ARNTL overexpression. e Western Blot detection of ARNTL protein expression in OSCC cells before and after ARNTL overexpression. The data are presented as the means ± SD from at least three independent experiments. **P < 0.01, ***P < 0.001, compared to Blank or ARNTL-NC group.
ARNTL overexpression inhibits OSCC cells viability and promotes apoptosis and autophagy activities. 

a) CCK-8 assay to detect OSCC cells viability. 

b) TUNEL assay to detect OSCC cells apoptosis. Scale bar = 200um. 

c) Observation of intracellular autophagosomes under TEM. The lower picture is the image of the upper picture with the red box area under high magnification. The autophagosome can be distinguished by its contents (partially cytoplasm, including mitochondrion, ribosomes, and rough endoplasmic reticulum) indicated by red arrows. Scale bars are 5um and 1um respectively. 

The data are presented as the means ± SD from at least three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, compared to Blank or ARNTL-NC group.
Figure 3

ARNTL overexpression promotes autophagy in OSCC cells. 

a The autophagy-related protein expressions of LC3 II/I, Beclin1, P62 and ATG-12 after ARNTL overexpression in OSCC cells. 

b The protein expressions of LC3 II and P62 in OSCC cells after adding the autophagy inhibitor Baf A1. All data represent three independent experiments. The results are shown as the mean ± SD.*P < 0.05, **P < 0.01.
ARNTL induces apoptosis dependent on autophagy pathway. a The autophagy-related and apoptosis-related protein expressions of LC3 II/I, P62, BAX and BCL-2 after adding the autophagy inhibitor Autophinib. b Flow cytometry results showed that the decreased cell proliferation index and the increased apoptosis index were reversed effectively after adding Autophinib. All data represent three independent experiments. The results are shown as the mean ± SD.*P < 0.05, **P < 0.01, ***P < 0.001.

ARNTL overexpression inhibits OSCC cells tumorigenesis in vivo. a The weights and volumes of tumors in the ARNTL-OE group were significantly lower than those in the ARNTL-NC group. b Western blot was used
to detect the autophagy-related protein expressions in tumor-forming tissues in vivo. All data represent three independent experiments. The results are shown as the means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1InformationaboutAntibodies.docx