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Prognostic Roles of APLNR Expression in Non-Small Cell Lung Cancer

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Keywords: APLNR; NSCLC; Prognostic roles; OS; Xenograft

Abstract

Apelin (APLN)/APLN receptor (APLNR) system has been proved to involve in tumor progression of various cancers. The prediction role of APLNR for non-small cell lung cancer (NSCLC) are not been studied. We first investigated the gene alteration frequency and expression in cancer tissues from NSCLC patients, and analyzed the prognostic roles and potential mechanism of APLNR in NSCLC patients. We observed that the APLNR gene expression was highly mutated and its protein level was lower in cancer tissues than normal tissues. Low expression of APLNR strongly associated with overall survival (OS) for NSCLC patients, predicated to worse survival in LUAD and LUSC. In NSCLC cells (A549 cells), knockdown of APLNR promoted the in vitro cell proliferation, migration, colony formation in vitro. And the in vivo xenograft mouse model proved that knockdown the APLNR expression augmented the tumor growth. These data suggested that APLNR induced inhibition of tumor progression in NSCLC may through suppressing the cell proliferation, migration and colony formation. This information will help us to pathological development NLCLC.

Abbreviations: ATCC: American Type Culture Collection; caBIG: Cancer Biomedical Informatics Grid; NSCLC: The European Genome-phenome Non-small cell lung cancer; LC: Lung cancer; LUAD: lung adenocarcinoma; TFF1: Trefoil factor family 1; LUSC: lung squamous cell carcinoma; KM plotter: The Kaplan-Meier plotter; HR: hazard ratio; OS: overall survival; TCGA: The Cancer Genome Atlas; EGA: Gene Expression Omnibus Archive; GEO: Gene Expression Omnibus; ECM: extracellular matrix; APLN: Apelin; APLNR: Apelin receptor; FABP4: Fatty acid binding protein 4; IGF1: insulin-like growth factor 1; PCOS: polycystic ovary syndrome; hGCs: human luteinized granulosa cells; FOXO1: Forkhead box protein O1; hGCs: human granulosa cells; LECs: lymphatic endothelial cells

Introduction

Apelin (APLN), peptide hormone, has been firstly identified in bovine stomach extracts [1]. The preproapelin gene of human originally includes the 77 total amino acids which is cleaved and subsequently form the active Apelin [2]. And among these active forms, the APLN-36, is the most abundant form in human circulation with 36 total amino acids [3]. The APLN receptor (APLNR), a class A GPCR, is the only APLN receptor has been identified [4]. In rat and human tissues, including lung, the mRNA of both APLN and its receptor (APLNR) is highly expressed [4]. Once binding with APLN, APLN/APLNR system modulates various MAPK3/1, AKT, and PRKA signaling pathways in various types of cell [1], regulates the progression of cancers and other different pathologies via regulation of angiogenesis, cell proliferation and energy metabolism [4,5]. *In vivo* investigation in humans indicated that the protein levels of APLN are higher in serum from people with diabetes (Type

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2) and obesity than that in normal people. In bovine granulosa cells, progesterone stimulates APLNR expression, suggesting that APLN/APLNR system may be essential to dominance [1,6].

Recent studies in patients with polycystic ovary syndrome (PCOS) have proved that APLN regulated the metabolic and hormonal systems in patients [7], and suggested the essential role of APLN and APLNR to human-granulosa-cells (hGCs) [8]. In lymphatic endothelial cells (LECs), APLN is highly expressed, and stimulates apelinergic signaling cascade [9]. However, pretreatment of APLN in LECs failed to regulate cell proliferation, but strongly enhanced cell migration, attenuated cell apoptosis under UV irradiation, and promoted the spheroid formation [10]. APLN and APLNR are strongly expressed in blood vasculature from adult and embryo to promote endothelial cell proliferation in many angiogenesis models [11]. And in tumors, APLN/APLNR signaling can also promote the vascular generation and tumor formation [5]. APLN/APLNR signaling pathways is associated with accelerated *in vivo* tumor formation and metastasis to lymph node [5]. But, the expression and roles of APLNR in lung carcinoma have not been well investigated. APLN/APLNR signaling pathways have been proved to involve in the inflammation-related diseases, atherosclerosis and adiposity [5,11,12].

Blocking CXCR4 signaling via treatment of pharmacological inhibitor or suppressing the expression of CXCR4 through augmentation the expression of miR-139-5p, which directly block CXCR4 mRNA expression, were proved to attenuate APLN/APLNR induced vascular phenotype [13]. In endothelium, APLN binding with APLNR to inhibit the fat transport across the endothelial layer through the suppression of Forkhead box protein O1 (FOXO1) signals and subsequently attenuated the protein level of fatty acid binding protein 4 (FABP4) in endothelial cells [11]. Interestingly, recent investigations have proved that FABP4 is critical to promote the oncogenesis in NSCLC [14], which may through increasing the cancer progression. Lung carcinoma (LC), a primary reason of cancer associated death in the whole world, results in over 8.2 million death per year [15]. Among these LC patients, around 80% of patients are diagnosed as non-small cell lung cancer (NSCLC), mainly including adenocarcinoma (LUAD) and squamous cell carcinoma (LUSC) [16]. In last two cascades, numbers' improvements in LC treatment approaches, including radiotherapy, chemotherapy, surgical resection and targeted therapy [17]. However, the overall 5-year survival rate of LC patients is ~ 15%, which mainly due to the severe resistance to drug treatments and metastasis to other organs [18]. Thus, we firstly analyzed the mRNA and protein expression of APLNR in cancer tissues from patients with NSCLC and assessed the correlation with the OS rate and clinicopathological features in NSCLC. Next, we analyzed the cell proliferation, migration in LC cells with knockdown of APLNR *in vitro*, and investigated its role in tumor growth via *in vivo* xenograft mouse model.

Methods and Materials

Human NSCLC Tissue Samples

9 NSCLC tissue and related non-tumor control lung tissues from same patients were collected from No.4 West China Teaching Hospital and froze in −80 °C in 2017. This study was recorded and approved by No.4 West China Teaching Hospital's ethical Committees. This experiment follows the Declaration of Helsinki.

Cell Culture

A549 human LC cell line (CCL-185 from ATCC) was maintained in DMEM medium containing 10% FBS and 5% CO2 at 37°C [19].

sh-RNA Transfection

Vehicle control sh-RNA (Veh sh-RNA, SHC016) and targeting human APLNR (sh-APLNR, TRCN0000356634) from Sigma aldrich, Inc. A549 cells with ~60% confluence were transiently transfected with sh-RNAs using transfection kit from Qiagen, Inc. (Gaithersburg, MD, USA). Then, transfected cells were incubated culture medium by adding 1 μg/ml of puromycin. After selection in puromycin medium for two weeks, the mRNA and protein expression of APLNR in cells was analyzed by using real-time quantitative PCR (RT-qPCR for mRNA) and immunoblotting (for protein).

RT-qPCR

Trizol reagent (Invitrogen, Inc; Carlsbad, Ca, USA) was used to isolate total RNA from cells or tissues. mRNA was converted to cDNA using the kit for reverse transcription from Bio-Rad, Inc (Hercules, CA, USA). Primer sequences were listed bellow. APLNR, Forward primer (F): CTCTGGACCGTGTTTCGGAG; APLNR, Reverse primer (R): GGTACGTGTAGGTAGCCCACA. FABP4, F: ACTGGGCCAGGAATTTGACG; R CTCGTGGAAGTGACGCCTT. GAPDH, F: ACAACTTTGGTATCGTGGAAGG; R: GCCATCACGCCACAGTTTC. SYBR Premix Ex Taq (Takara) was used for RT-qPCR in an Mx3000P QPCR system (Agilent, Santa Clara, CA, USA) by using the thermal condition as: 95°C for 10 seconds, 40 cycles of 5 seconds at 95°C plus 30 seconds at 60°C. GAPDH was used to normalize the expression of genes.

Materials

Cocktail for protease inhibitor, mouse anti-APLNR (sc-517300), anti-GAPDH (sc-47724) antibodies, Horseradish peroxidase (HRP)-linked second antibodies were purchased from Santa Cruz, Inc. (Dallas, TX, USA).

Immunoblotting

Cells were lysate in lysate buffer with 1X protease inhibitors, centrifugated at 4°C (10,000 g,10 min), and incubated with 1X sample buffer at 95°C for 5 min. Proteins were transferred to membranes, blocked in 5% BSA TBST solution for 1 h at room temperature, incubated in first antibodies (1:2000 dilution, 4°C) overnight, and HRP-linked secondary antibodies (1:2000 dilution at room temperature, 120 mins). Protein expression was analyzed

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and quantified by using ImageJ from Molecular Dynamics, Inc. (Sunnyvale, CA, USA).

Cell Proliferation Assay

Serum starved cells seeded in 96 well plates were challenged with FBS (10%, 0-24 h). The proliferated cells were analyzed using kit from Promega Corporation (Fitchburg, WI, USA) [20].

Wound Closing Assay

Cells with 100% confluence were scraped with a pipette tip to form 2 mm wound at 0 h. After removing the floating cells by changing medium, cells with wound were incubated in medium containing 1 µg/ml of mitomycin C [21]. Wound was recorded at h and 24 h, quantified by using ImageJ from Molecular Dynamics (Sunnyvale, CA, USA). % of wound closing= (Wound 0 h – wound 24 h)/ wound 0 h X 100% [22].

Colony Formation Assay

A549 single-cell suspensions with or without stable knockdown of APLNR were seeding in 6 well plates with approximately 300 cells/well. All plates were cultured to form visible colonies at 37 °C. Wash the plates with PBS (2X, 3 min) at 14 days post seeding. Then the plates were fixed via cold methanol (100 %, -20°C, 5 min), stained by crystal violet (Sigma–Aldrich, 5 min at room temperature). After 3 times washing by water, the plates were dried under air at room temperature, the colonies images were analyzed via ImageJ software (Molecular Dynamics, Sunnyvale, CA, USA).

Gene Expression Alteration

APLNR gene alteration frequency in NSCLC patients from TCGA data base was analyzed via cBioportal [23].

APLNR Expression

We queried APLNR expression in lung cancer from GEO database (Gene Expression Omnibus). The original data from GSE10072 were analyzed.

Analysis the Prognostic Role of APLNR and FABP4 in NSCLC

The data about NSCLC patients used for KM plotter analysis were pooled from TCGA (The Cancer Genome Atlas), GEO (Gene Expression Omnibus), EGA (European genome-phenome archive), and NCBI [24]. The KM plotter database was used to obtain Kaplan-Meier plots in NSCLC patients [25].

In vivo Tumor Growth

We ordered the 6 weeks female nude via the Animal center in Shanghai's belong to Chinese Academy of Science (China). Mice were maintained in nude mice care center of the No.4 West China Teaching Hospital. 50 µl of cells (1 × 107 cell/ml) were injected (subcutaneously) into the right flank of animal. Measured the size of tumor (0-8 week post injection) once a week, and quantified the tumor volume (mm^3) = $\pi/6 \times a \times b^2$; a, longest diameter; b, shortest diameter.

Statistical Analyses

Results were displayed as mean±sd of at least three independent experiments. ANOVA (one-way) and Student t test (two-tailed) were used for statistical analysis, and below 0.05 was identified as significant different [25].

Results

Gene Expression and Alternation Frequency of APLNR in NSCLC

First, we analyzed the APLNR mRNA expression alternation in LC tissues. In Figure 1, we observed that the APLNR expression was highly mutated in NSCLC tissues. We further investigated the mRNA expression level of APLNR in NSCLC tissues. As displayed in GSE#10072, the APLNR expression was lower in NSCLC tissues by comparing that in adjacent non tumor tissues ($P=1.77e^{-9}$; Figure 1). Next to conform the expression of APLNR in human LC tissue via real-time qPCR and western blot. As shown in Figure 1, the APLNR expression (mRNA, Figure 1; protein, Figure 1) was lower in NSCLC tissues.

Association Between APLNR Expression and Clinico-pathological Characteristics of Patients with NSCLC

High expression of APLNR (213592_at) was correlated to better overall survival (OS) for all NSCLC patients with hazard ratio (HR) 0.73 (0.64-0.83), $P=1.6e^{-6}$ (Figure 2, n=1,926). In detail, we observed that APLNR high expression was strongly associated with better OS in LUAD patients, HR 0.72 (0.57- 0.91), P =0.0058 (Figure 2, n =720), and in LUSC patients, HR 0.69 (0.53-0.89), P=0.0046 (Figure 2, n=524). And APLNR mRNA high expression was also strongly correlated to better OS in male NSCLC patients, HR 0.76 (0.64- 0.91), P =0.0027 (Figure 3), and in female NSCLC patients, HR 0.62 (0.49-0.79), $P=7.8e^{-5}$ (Figure 3). Next, we compared the expression of APLNR in NSCLC patients in different smoking status. As shown in Figure 4, the APLNR expression was similar between the smoked patients and patients never smoked. And the high APLNR expression predicted better OS in NSCLC patients with smoking (Figure 4, HR 0.77 (0.61- 0.96), P =0.021) or without smoking (Figure 4), (HR 0.48 (0.28- 0.84), P =0.0082). Next, we studied the correlation of APLNR expression with different clinical stages (Table 1), and treatments (Table 1). As from Table 1, APLNR expression was not shown strong association with difference of prognosis in patients with various chemotherapy and radiotherapy treatments. Interestingly, high APLNR expression indicated better OS of patients in grade I and II but not for III, with HR 0.6 (0.6–0.78, P = 0.00014) to grade I patients;, HR 0.63 (0.44–0.91, P = 0.014) to grade II patients (Table 1).

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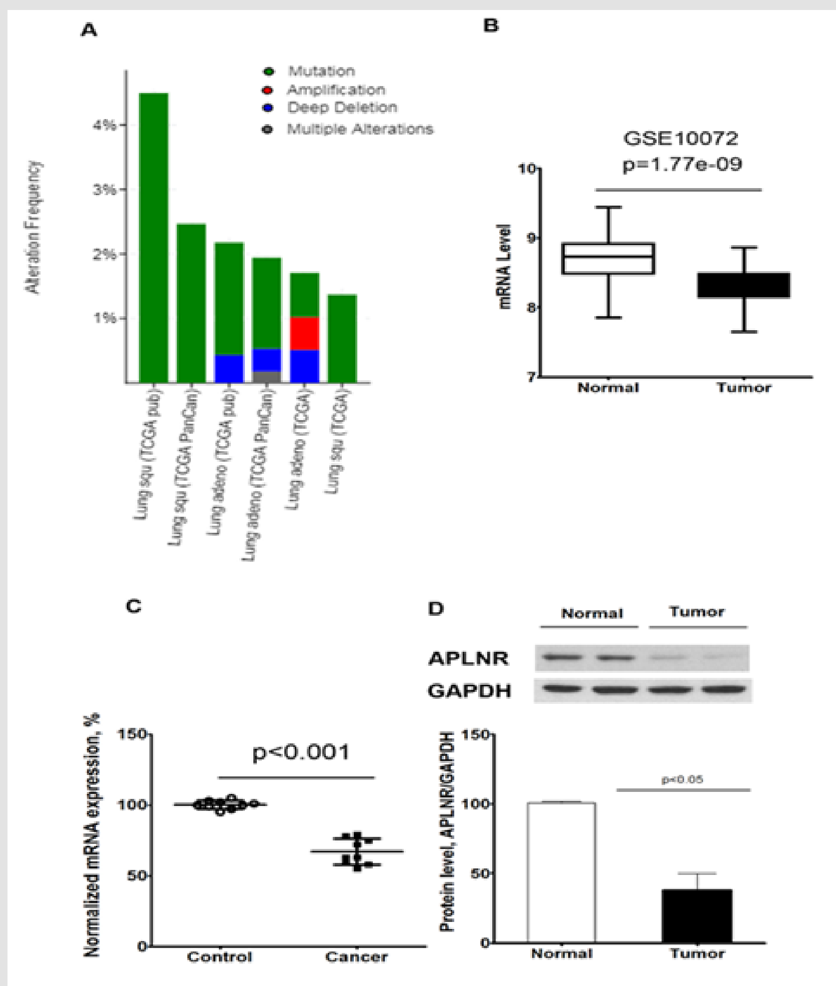

Figure 1: APLNR expression in human LC tissues.

(A) The alteration of APLNR gene expression.

(B) APLNR mRNA expression level in lung tissue in GSE10072. APLNR expression

(C) mRNA, (D) protein) expression in tumor tissue and adjacent normal tissues from NSCLC patients.

(D) Upper panel, representative images protein expression from cancer tissue (tumor) and adjacent normal tissues (normal); lower panel, quantification of normalized protein expression (n=4).

Table 1: Correlation of APLNR expression with NSCLC patients.

Variables		Number	HR	CI (95%)	P
Chemotherapy	Yes	176	1.27	0.84-1.91	0.26
	No	310	0.8	0.56-1.13	0.21
Clinical stage	I	577	0.6	0.6-0.78	0.00014
	II	244	0.63	0.44-0.91	0.014
	III	70	1.32	0.74-2.37	0.35
Radiotherapy	Yes	70	0.7	0.4-1.21	0.2
	No	271	0.68	0.43-1.07	0.095

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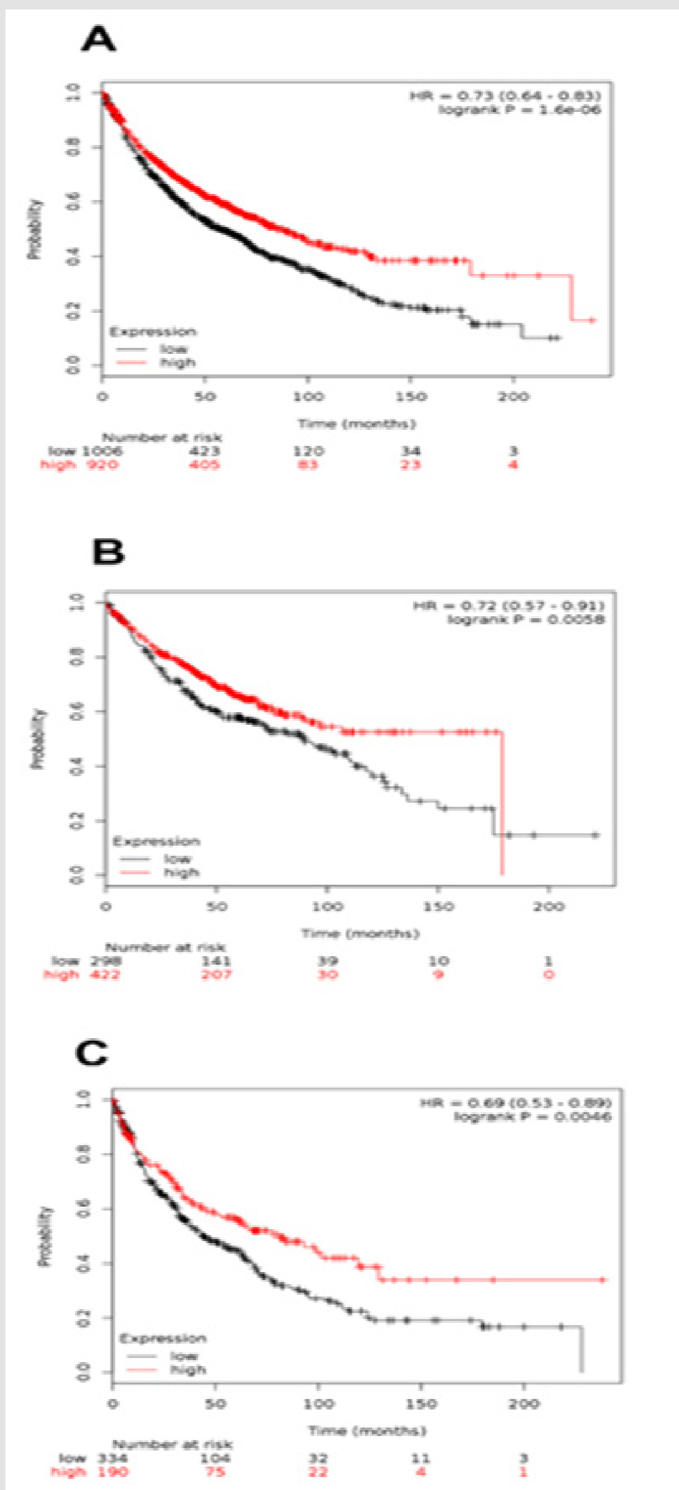

Figure 2: Prognostic role of APLNR in NSCLC. Survival curves is plotted the expression of APLNR (Affymetrix IDs: 213592_ at) in NSCLC patients (A, n =1,926); adenocarcinoma patients (B, n =720) and squamous cell carcinoma patients (C, n =524).

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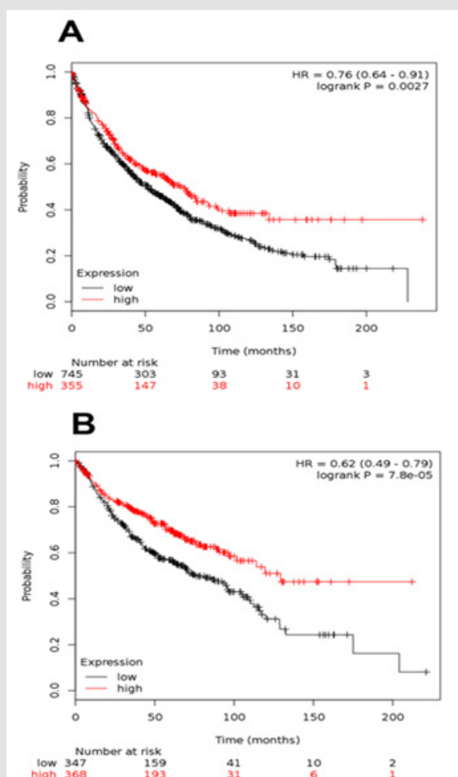

Figure 3: Prognostic role of APLNR expression in female and male patients with NSCLC. (A & B) Survival curves is plotted for all male patients (A, n =1100) and female patients (B, n =715).

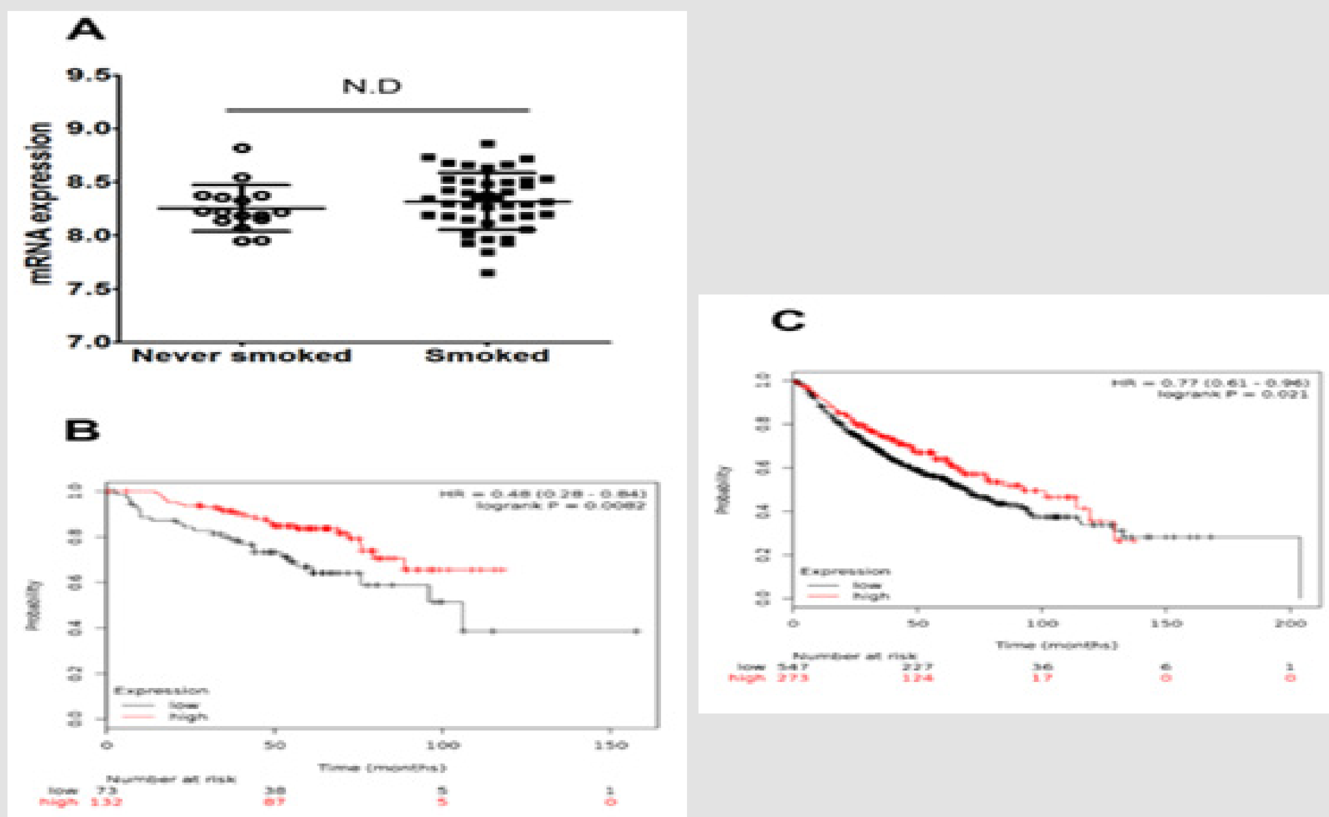

Figure 4: Expression and prognostic role of APLNR expression in NSCLC patients with or without smoking. (A) mRNA expression of APLNR in cancer tissues from NSCLC patients with or without smoking. N.D, non-significant different. (B & C) Survival curves are plotted for all non-smoked patients (B, n =205) and smoked patients (C, n =820).

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Knockdown of APLNR Promotes Cell Proliferation, Migration, Colony Formation and Tumor Growth

To further analyze role of APLNR in tumor progression for NSCLC, we studied the cell proliferation in different APLNR expression levels. The expression of APLNR in A549 cells was knockdown via transfection of sh-APLNR. As shown in Figure 5, stable knockdown of APLNR decreased the APLNR the mRNA level (Figure 5) or protein level (Figure 5). Next, we also observed that lower APLNR expression promotes cancer cell (A549) proliferation (Figure 5), colony formation (Figure 5) and migration (Figure 5).

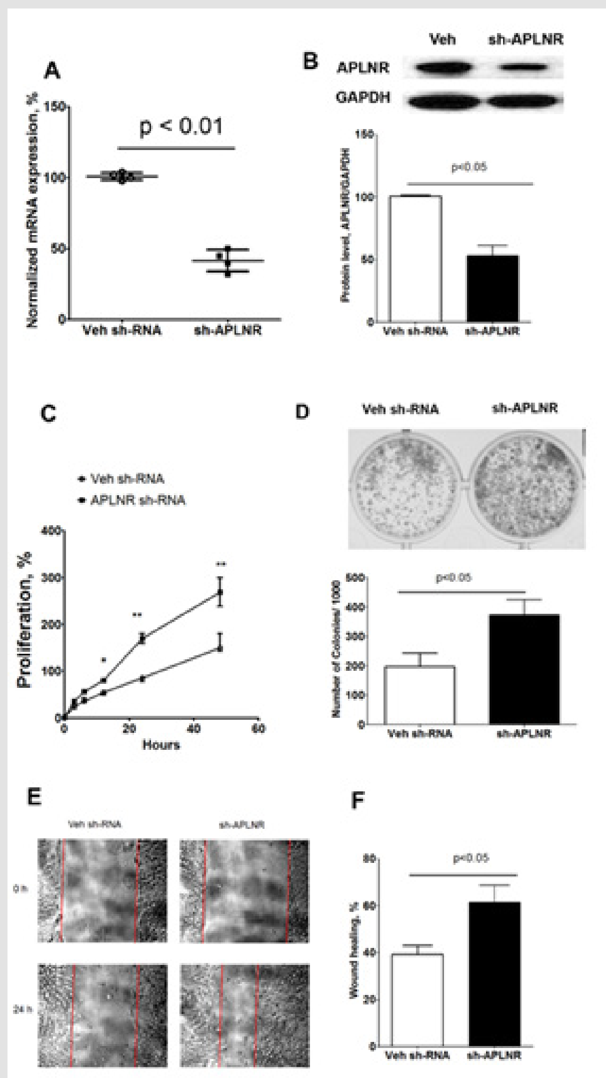


Figure 5: Knockdown APLNR expression in A549 cells stimulates cell proliferation, colony formation, cell migration and tumor formation.

(A) mRNA expression and protein (B) of APLNR in A549 cell with or without stable knockdown of APLNR. (B) Upper panel, representative images protein expression in cells; lower panel, quantification of protein expression. Data is displayed as mean ± sd. in different cells.

(B) Cell proliferation in A549 cells with or without stable knockdown of APLNR.

(C) Colony formation in A549 cell with or without stable knockdown or APLNR. Upper, representative images of colon; lower, quantification. (E & F) Wound healing assay to analyze the cell migration.

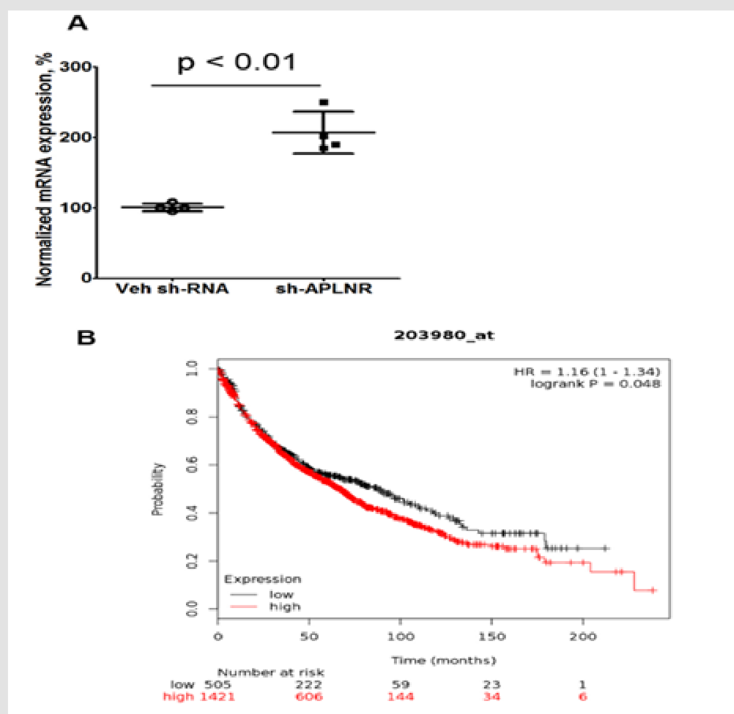
(D) Representative images and

(E) quantification of percentage of wound healing for cells post 24 h of FBS culture. Data is displayed as mean ± sd. in different cells. *, $p < 0.05$; **, $p < 0.01$, cells with knockdown of APLNR vs cell with transfection of Veh shRNA.

(F) Tumor volume,

(G) tumor weight form nude mice inoculated with A549 cells. Upper panel shown the xenografts from mice with subcutaneously inoculation of A549 cells with or without knockdown of APLNR. sh-APLNR (n=3) and veh sh-RNA (n=3).

(H) qPCR quantification of mRNA expression of APLNR from the xenografts. *, $p < 0.05$; **, $p < 0.01$, cells with stable knockdown of APLNR vs cells with stable transfection of veh shRNA.



Supplimentary Figure 1: Knockdown the expression of APLNR stimulates FABP4 expression.
(A) FABP4 mRNA expression in APLNR knockdown A549 cell. Data is displayed as mean ± sd in different cells.
(B) Prognostic role of FABP4 (Affymetrix IDs: 203980_at) in NSCLC (n =1,926).

Next we also checked the role of APLNR in tumor growth by *in vivo* xenograft mouse model. We injected A549 cells into nude mice, and recorded the tumor size for 8 weeks. As shown in Figure 5, we observed that lower APLNR expression via knockdown APLNR speeded the tumor growth *in vivo* by comparing with those mice receive he control cells (Figure 5). And the qPCR data also demonstrated that these xenografts shown less expression of human APLNR (Figure 5) by comparing with the mice inoculated with control cells with Veh sh-RNA transfection. These data may suggest that APLNR induced inhibition of tumor progression through suppressing cell proliferation, migration and colony formation in NSCLC cells (Supplementary Figure 1).

Discussion

Apelin (APLN), a adipokine, regulates metabolic functions through binding its receptor (APLNR) [1,5]. APLNR was mainly expressed in reproductive tissues [26]. Accumulated experimental evidences have suggested that APLNR expressed in respiratory bronchi epithelial cells [27] and lung endothelial cells [28]. In our study, we firstly analyzed the APLNR expression in human NSCLC tissue. We observed that APLNR expression was decreased in tumor tissue from NSCLC patients by comparing with that in control tissue, that may relate to the high mutation ration of APLNR in NSCLC patients. Recent investigations indicated that APLN/APLNR pathways contributes to the development and pathogenesis of various human diseases [26]. In polycystic ovary syndrome (PCOS) both APLN and APLNR were detected in nuclei and cytoplasm from human luteinized granulosa cells (hGCs) and tissues via RT-qPCR and immunohistochemical staining [1,6,8]. Additionally, insulin-like growth factor 1 (IGF1) was proved to stimulate APLNR expression through the activation of AKT and MAPKs pathways in hGCs [1]. And APLNR also regulates the apoptosis in human ovarian cells, which may through its critical roles in energy metabolism and insulin sensitivity [1].

The APLN/APLNR signaling pathways were implicated in a lot of key physiological processes, including cell proliferation [29]. Tumor cells secreted APLN was represented as an angiogenic factor which promotes the formation of new blood vessels system to stimulate tumor progression [5]. Hypoxia has been well established as the vital factor correlated to the pathogenesis of tumor [30]. Hypoxia was well known to stimulate APLN expression in tumor cells [31], and constitutive expression of APLN rapidly promotes the angiogenic of tumor [32]. Inside of the solid tumor, due to lower level of oxygen, hypoxia induces the upregulation of APLN and further stimulates the tumor progression via promoting endothelial cell proliferation and blood vessel generation [31]. Our studies suggested that APLNR expression associated to the OS in LUAD and LUSC patients. Stable knockdown of APLNR augmented the proliferation and colony formation in cancer cells. And our *in vivo* data in xenograft mouse model also proved that knockdown the expression of APLNR promotes the tumor growth, that may result in the worsen survival for the patients with lower expression of APLNR of NSCLC.

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APLN/APLNR systems are also involved in energy metabolism under physiological and pathological conditions [1]. APLN enhances glucose utilization and improves insulin sensitivity in cancer cells. Mice with deficient expression of APLNR in endothelial cells resulted in less utilization of glucose [12]. Additionally, Forkhead box protein O1 (FOXO1) inactivation or fatty acid binding protein 4 (FABP4) inhibition was also related to the APLNR signaling [11]. FABP4 inhibitor administration in mice with specific deletion of Aplnr expression in endothelial cells restored the glucose metabolism [11]. Since APLNR regulates the FABP4 expression in endothelial cells, and FABP4 is known an oncogene in NSCLC [14]. As shown in Supplemental Figure 1A, lower APLNR expression significantly increased the expression of FABP4. Additionally, the higher expression of FABP4 predicted less survival time to NSCLC patients (Supplemental Figure 1B, HR 1.16 (1–1.34, P = 0.048)). These data may suggest that the role of APLNR in NSCLC at least partly relates to the regulation of FABP4 expression and related energy metabolism, and inhibitor of FABP4 may be used to treat the patients with less expression of APLNR.

High APLNR expression predicted better survival time for in LUAD and LUSD patients. In detail analysis of patients in different stages of NSCLC, the data indicated that higher expression of APLNR correlated to better OS in earlier stages (1 and 2) of NSCLC patient. *In vitro* studies in NSCLC cells, knockdown of APLNR expression in NSCLC cells promotes cell migration, proliferation and colony formation. *In vivo*, knockdown the expression of APLNR augments the tumor growth in xenograft mouse model. In summary, our data suggested that the lower expression of APLNR might predict worsen survival in NSCLC patients, and this may due to the lower expression of APLNR promotes cancer cell proliferation, migration and colony formation.

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Authors have no conflicts of interest to declare for this study.

Disclosure

The authors have no conflicts of interest to declare.

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