An oncogenic function of retinoic acid receptor-α in the development of laryngeal squamous cell carcinoma

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Abstract. The aberrant expression of retinoic acid receptor-α (RARα) has been reported in various types of cancer. However, its association with the prognosis and development of laryngeal squamous cell carcinoma (LSCC) has not yet been determined. Therefore, the present study aimed to examine the expression and function of RARα in patients with LSCC. The expression of RARα in LSCC tissues was investigated using immunostaining. An MTT assay and flow cytometry analysis were also performed to investigate the function of RARα in the proliferation and cell cycle of LSCC cells. The expression of RARα was significantly elevated in LSCC tissues compared with adjacent noncancerous tissues (78.1 vs. 6.3%, P<0.05). The overexpression of RARα was associated with poorly differentiated features of LSCC (P<0.05). Furthermore, the downregulation of RARα inhibited the proliferation of LSCC cells, and arrested the cell cycle at the G1 phase via upregulation of cyclin dependent kinase inhibitor 1A, which may be associated with inhibition of the protein kinase B signaling pathway. Therefore, the overexpression of RARα may contribute to the development of LSCC through the regulation of the cell cycle. The results of the present study provide evidence that RARα serves an important function in LSCC development and may be a potential therapeutic target or prognostic predictor for LSCC.

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Introduction

Laryngeal carcinoma, of which >95% of cases are laryngeal squamous cell carcinoma (LSCC), is a prevalent malignancy of the head and neck, with an incidence of 3.5-5.5/100,000 people and a mortality rate of 2.1-2.4 per 100,000 people worldwide in 2008 (1,2). Although there have been significant improvements in terms of diagnosis and treatment, the clinical outcome of therapy for patients with LSCC, particularly those with advanced stages of the disease, remains poor (3). Therefore, it is necessary to explore the molecular mechanisms of LSCC, which will contribute to increasing survival rates and improving prognosis.

Previous studies have revealed that retinoic acid receptors (RARs) serve important functions in various types of cancer (4). The RAR family contains three different subtypes (namely, RARα, RARβ and RARγ). As RARs are important members of the nuclear receptor superfamily, the three subtypes function as transcription factors that are essential in embryonic development, the maintenance of differentiated cellular phenotypes, metabolism and cell death (5). The three RAR subtypes are encoded by different genes and they differ in terms of structure and distribution as well as possessing unique functions. Increasingly, the evidence has indicated that the aberrant expression of RARα is present in various types of cancer. RARα was revealed to regulate the cell differentiation of neurocytoma through the activation of the transcription of associated target genes (6). The activation of RARα competed with the fusion protein promyelocytic leukemia gene-RARα, induced by a mutation to combine with the Fas cell surface death receptor domain structure, promoting the apoptosis of myeloid leukemia (7). The activation of RARα also promoted cell cycle arrest and the apoptotic process in lymphoma cells through the upregulation of reactive oxygen species (8). The protein stability of RARα, enhanced by ubiquitin, inhibited the proliferation of gastric cancer cells (9). The contribution of RARα to the development of hepatocellular carcinoma was associated with its regulation of cytochrome P450 family 1 subfamily A member 1 expression (10,11). RARα is also involved in the development of breast cancer and prostate carcinoma (12-14). However, little is known about the expression and function of RARα in human LSCC.

In the present study, the function and mechanisms of RARα in primary LSCC tissues and adjacent normal tissues were investigated. It was revealed that the overexpression of RARα in LSCC tissues promoted the proliferation of leukocyte common antigen (LCA) cells through the regulation of the cell cycle, with definitive clinical significance. The results of the present study may provide a reference to gain additional insight into the potential function of RARα in the development of LCA, suggesting a potential target for LSCC therapy.

Materials and methods

Patients and tissue samples. Fresh human laryngeal carcinoma and adjacent noncancerous tissues were obtained from 32 patients who underwent surgery at the First Affiliated Hospital of Xiamen University (Xiamen, China). Tumor tissues and adjacent non-tumor tissues were sampled and immediately soaked in formalin overnight, and then paraffin embedded. The pathological characteristics were confirmed by pathological examination. The present study was approved by the ethics committee of the First Affiliated Hospital of Xiamen University, and all patients provided written informed consent prior to participation. None of the patients with LSCC were treated with radiotherapy, chemotherapy or other types of therapy prior to surgery. Metastatic tumors from other tissues were excluded from the study. The clinicopathological characteristics are presented in Table I.

Cell culture. The LSCC cell line AMC-HN-8 purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Science (Shanghai, China). Cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) at 37°C in a humidified atmosphere of 5% CO_2.

Cell transfection. RARα-specific small interfering (si)RNA (siRARα), 5'-AUCUCUUCAGAACUGCUGCUCUGGG-3' and the Stealth RNAi™ siRNA negative control (siCtrl), 5'-UAU CCUUACGAGUACCUUGCGGCUG-3' were designed and obtained from Invitrogen; Thermo Fisher Scientific, Inc. LSCC cells were seeded in 6 well plates at a density of $0.5x10^6$ cells per well. siRARα and siCtrl were transfected into cells using the LipoRNAiMAX transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Following transfection at 37°C in a humidified atmosphere with 5% CO_2 for 24 h, cells were collected for subsequent experiments.

Cell proliferation assay. Cell proliferation was analyzed using an MTT assay (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The cells were seeded at a density of $5x10^3$ cells per well into 96-well plates overnight. Following the transfection of cells with siRARα and siCtrl, 20 µl MTT (5 mg/ml) was added to each well, and the cells were cultured for a further 4 h at 37°C. Then the formazan crystals formed were dissolved in DMSO. The absorbance was measured at 490 nm using an ELISA microplate reader. All experiments were performed in triplicate.

Table I. Associations between RARα and clinical features of laryngeal squamous cell carcinoma.

Features	N	RARα Low	RARα High	χ^2	P-value
Age, years				0.0185	0.8918
<59	13	3	10		
>60	19	4	15		
Sex				0.0261	0.8716
Male	28	6	22		
Female	4	1	3		
Pathologic differentiation				6.8183	0.0331[a]
High	14	6	8		
Middle	8	1	7		
Low	10	0	10		
T stage				0.4780	0.9237
T1	7	1	6		
T2	13	3	10		
T3	9	2	7		
T4	3	1	2		
Lymph node metastasis				1.0135	0.3141
N0	19	3	16		
N+	13	4	9		
Distant metastasis				0.9874	0.3204
M0	30	6	24		
M+	2	1	1		
Clinical stage				0.5644	0.9045
1	6	1	5		
2	14	3	11		
3	11	3	8		
4	1	0	1		

[a]$P<0.05$ was considered to indicate a statistically significant difference. RAR, retinoic acid receptors.

Colony formation. A total of 600 AMC-HN-8 cells were cultured in 6-well plates for 14 days and then fixed and stained with 0.005% crystal violet for 30 min at room temperature. Colonies >100 µm in diameter were counted under a light microscope (Olympus BH-2, Olympus Corporation, Tokyo, Japan).

Reverse transcription quantitative RT-(q)PCR. Total RNA was extracted from cells using an RNA Extraction kit (cat. no. DP405-02; Tiangen, Beijing, China) according to manufacturer's protocol. Reverse transcription was carried out using the SuperScript III First-Strand Synthesis system (Invitrogen; Thermo Fisher Scientific, Inc.) for qPCR. The qPCR was carried out by ABI 7500 Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with SYBR-Green (Promega Corporation, Madison, WI, USA) as a fluorophore. GAPDH was used as a control. The primer sequences are listed

Table II. Primer sequences for reverse transcription-quantitative polymerase chain reaction analysis.

Gene	Sense (5'-3')	Antisense (5'-3')
RARα	AATACACTACGAACAACAGC	CGAACTCCACAGTCTTAATG
P21	CTAGTTCTACCTCAGGCAGCT	GTCGCTGGACGATTTGAGG
P27	GTTAGCGGAGCAATGCGC	CAGGCTTCTTGGGCGTCTG
Cyclin A	AAGCACTCCCTGACTGTGG	ACTGATGTTTCTTGGTGAC
Cyclin B	CCTCAACCATCCTGGCTGCG	CTGTTCTTGGCCTCAGTCC
Cyclin D	CCGAGTCACCAGGAACTCGA	AGGACAGACTCCGCTGTGC
Cyclin E	AATGTCAAGACGAAGTAGCC	ATTTCCTCAAGTTTGGCTGCA

RARα, retinoic acid receptor-α; P21, cyclin dependent kinase inhibitor 1A; P27, cycin dependent kinase inhibitor 1B.

in Table II. All experiments were performed in triplicate, and the data were relatively calculated using the $2^{-\Delta\Delta Cq}$ method (15).

Western blotting. Whole-cell lysates were prepared in lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 50 mM NaF, 1 mM Na3VO4, 30 mM sodium pyrophosphate, 0.5% NP-40 and 0.5 mM PMSF (Sigma-Aldrich; Merck KGaA) supplemented with EDTA-free protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland), and insoluble debris was pelleted by centrifugation for 20 min at 4°C and 13,000 x g and removed. Protein concentration was determined by Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and lysate proteins denatured by boiling for 5 min in reducing SDS-sample buffer. Lysate proteins (15 μg total protein/lane) were separated by 12% SDS-PAGE and transferred to polyvinylidene fluoride membranes (PVDF, Millipore, Billerica, MA, USA). Membranes were blocked in 7% bovine serum albumin in TBS and Tween-20 (TBST) for 1 h at room temperature, and probed overnight at 4°C in primary antibody solutions (RARα, RARβ and RARγ; Abcam, Cambridge, UK; dilution, 1:1,000) [cyclin-dependent kinase inhibitor 1A (p21), proliferating cell nuclear antigen (PCNA), protein kinase B (AKT), phosphorylated (p)-AKT and GAPDH (dilution, 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) in TBST. They were then washed in TBST 3 times for 5 min, and incubated in TBST solution containing 25% (v/v) non-fat dry milk powder and goat anti-rabbit (cat. no., 31430) and goat anti-mouse (cat. no. 31460) secondary antibodies (1:300) conjugated to horseradish peroxidase (Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. Finally, the membranes were washed in TBST 3 time for 5 min, and bands imaged using Western Lightning Plus-ECL (PerkinElmer, Inc., Waltham, MA, USA) and HyBlot ES autoradiography film (Denville Scientific Inc.). The band intensities for western blot analysis were quantified using ImageJ 1.48 software (National Institutes of Health, Bethesda, MD, USA) and normalized to GAPDH. All experiments were performed in triplicate.

Hematoxylin and eosin staining (H&E) and immunohistochemistry (IHC). LSCC and the adjacent noncancerous specimens were fixed in 10% formalin for 24 h at room temperature, paraffin embedded and cut into 3-μm thick sections for H&E or IHC. The H&E staining were performed according to manufacturer's protocol of H&E Staining kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). IHC staining using anti-RARα antibody (1:300; cat. no. ab117728; Abcam) was detected using the EliVisionTM Plus kit (Fuzhou Maixin Biotech Co., Ltd., Fuzhou, China) according to the manufacturer's protocol. PBS without primary antibody was used as the negative control. The IHC staining result was evaluated based on the proportion of stained tumor cells. RARα-positive tumor cells were counted in 10 randomly selected high power fields under a light microscope (Olympus BH-2; Olympus Corporation) at a magnification of x400 and was evaluated according to the following criteria: <5% was defined as -; 5-25% as +; 25-50% as ++; >50% as +++. Expression of RARα protein was categorized as low (- to +) or high (++ to +++).

Cell cycle analysis. To detect the cell cycle distribution, cells were collected following transfection with siRARα or siCtrl at 37°C for 48 h and washed twice with ice cold PBS, and then fixed in ice-cold 70% ethanol at 4°C overnight. Following centrifugation for 5 min at 4°C and 2,000 x g, the cells were resuspended in 100 μg/ml RNase A at 37°C for 30 min and subsequently stained with 50 μg/ml propidium iodide (Sangon Biotech Co., Ltd., Shanghai, China) at 4°C for 30 min in the dark. The cells were analyzed using a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) at 488 nm and the data was analyzed using ModFit 3.3 (Verity Software House, Topsham, ME, USA) software.

Statistical analysis. Data analysis was conducted using SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA). Continuous data was expressed as the mean ± the standard error of the mean and analyzed using one-way analysis of variance with a Tukey's post hoc test, whilst χ^2 or Fisher's exact tests were used to examine the association between RARα and the clinicopathological parameters of LSCC. $P<0.05$ was considered to indicate a statistically significant difference.

Results

RARα was elevated in laryngeal carcinoma tissues. IHC was performed to detect the expression levels of RARα in LSCC tissues and the adjacent noncancerous tissues. As presented in Fig. 1, the expression of RARα in the paired adjacent paratumor tissues was weak or negative, but was strong in the LSCC tissues. In addition, RARα exhibited high expression levels in
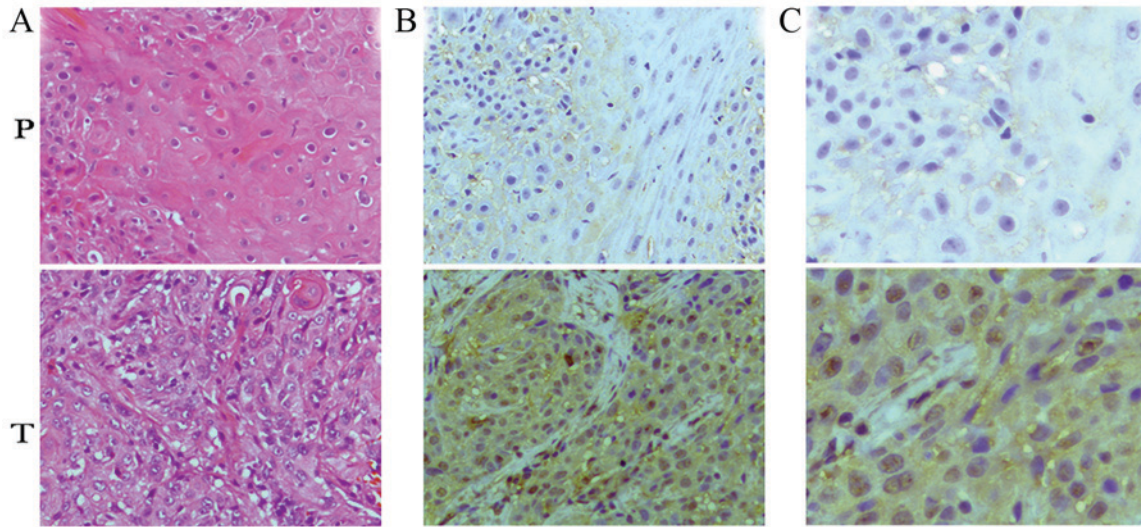
Figure 1. Expression of RARα in laryngeal squamous cell carcinoma tissues and paired paratumor tissues. (A) Representative images of H&E staining for T and P tissues at magnification, x100. (B) Representative images of IHC staining for RARα protein in T and P tissues at magnification, x100. (C) Representative images of IHC staining for RARα protein in T and P tissues at magnification, x400. RARα, retinoic acid receptor-α; H&E, hematoxylin and eosin staining; IHC, immunohistochemical staining; T, tumor; P, paratumor.
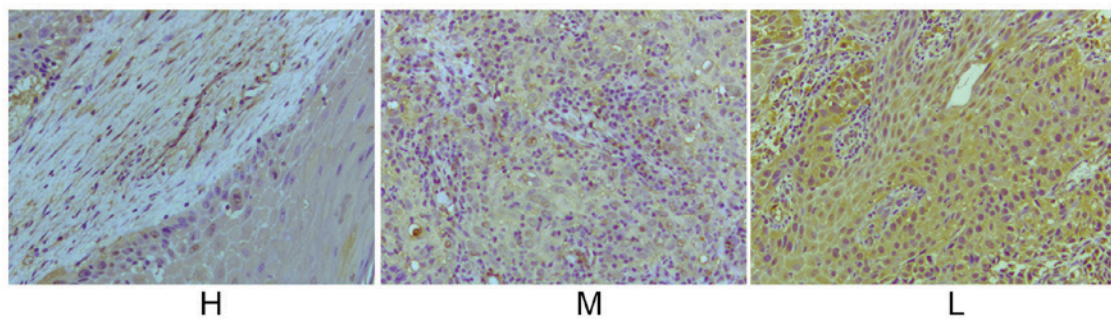

Figure 2. Expression of RARα protein in tumor tissues of different pathologic differentiation. Representative images of immunohistochemical staining for RARα protein in L, M and H laryngeal squamous cell carcinoma tissues at magnification, x100. RARα, retinoic acid receptor-α; L, low; M, moderate; H, high.

the cytoplasm of cancer cells, with comparatively low expression levels in the nucleus. Furthermore, in order to compare the expression level of RARα protein in LSCC tissues and the adjacent noncancerous tissues, the expression level was classified into two groups (low expression, - to +; high expression, ++ to +++), which were presented in Table III. The rate of high expression in LSCC tissues was 78.1%, and the rate of high expression in paired adjacent paratumor tissues was 6.3%. The χ^2 test statistics demonstrated that the expression of RARα was significantly higher in LSCC tissues compared with adjacent tissues (P<0.05).

The clinical significance of RARα overexpression in laryngeal carcinoma. The association between RARα and the clinicopathological characteristics of LSCC was further analyzed. There was no significant association between RARα expression and age, sex, TNM stage or clinical stage (P>0.05). However, high RARα expression was significantly associated with advanced pathological differentiation (P<0.05). As presented in Fig. 2, the expression levels of RAR protein in poorly differentiated LSCC tissues was visibly higher than that in moderately differentiated and highly differentiated LSCC tissues. The results of the present study indicate that

Table III. Expression of RARα in LSCC and paired adjacent paratumor tissues.

Tissues	RARα expression		
	Low	High	P-value
Paratumor (N=32)	30	2	<0.0001
LSCC (N=32)	7	25	

RARα, retinoic acid receptor-α; LSCC, laryngeal squamous cell carcinoma.

RARα may be involved in the regulation of the differentiation of LSCC cells.

Downregulation of RARα inhibited laryngeal carcinoma cell proliferation in vitro. To assess the effect of downregulating RARα on the proliferation of LSCC cells, the expression of RARα was knocked down using siRARα, and the control group siCtrl was transfected with non-specific fragments. The specificity of siRNA was confirmed by the observation that the expression
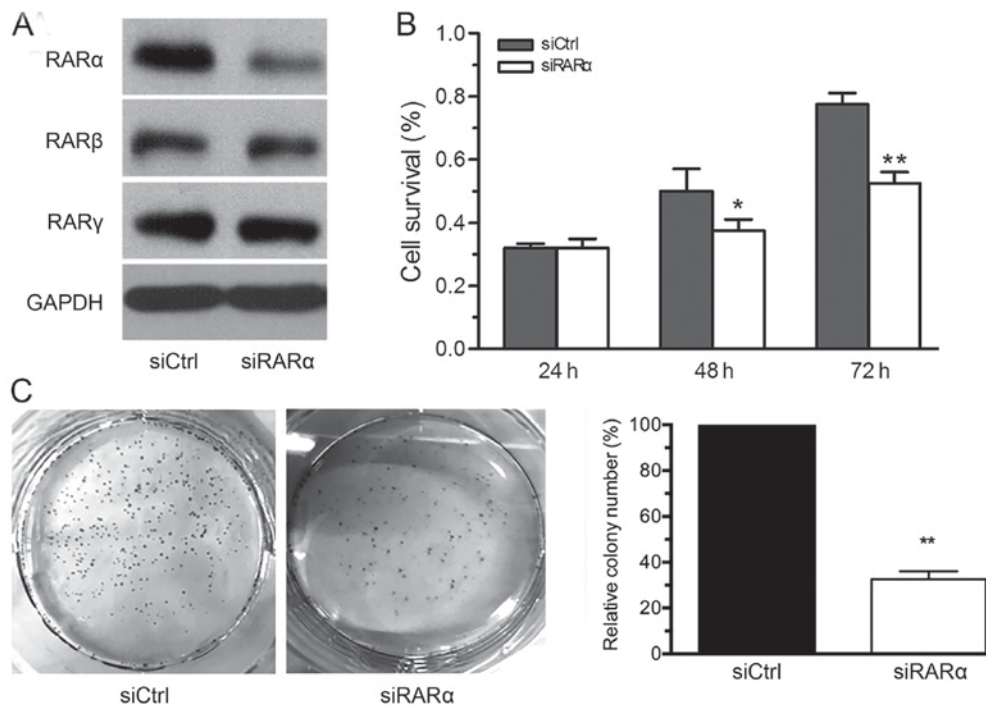
Figure 3. Effect of RARα knockdown on the proliferation of laryngeal squamous cell carcinoma cells. (A) RARα, RARβ, and RARγ protein expression was detected using western blotting with GAPDH as the loading control. (B) MTT assays to analyze cell proliferation were performed in AMC-HN-8 cells following transfection with siRARα or siCtrl for 24 h. (C) Colony formation assays were performed in siCtrl and siRARα cells. The mean number of foci formed by siCtrl-AMC-HN-8 cells was normalized to 100%, whilst all other colonies were compared with it. *P<0.05 and **P<0.01 vs. control group. RARα, retinoic acid receptor-α; siRARα, RARα-specific small interfering RNA; siCtrl, small interfering RNA negative control.
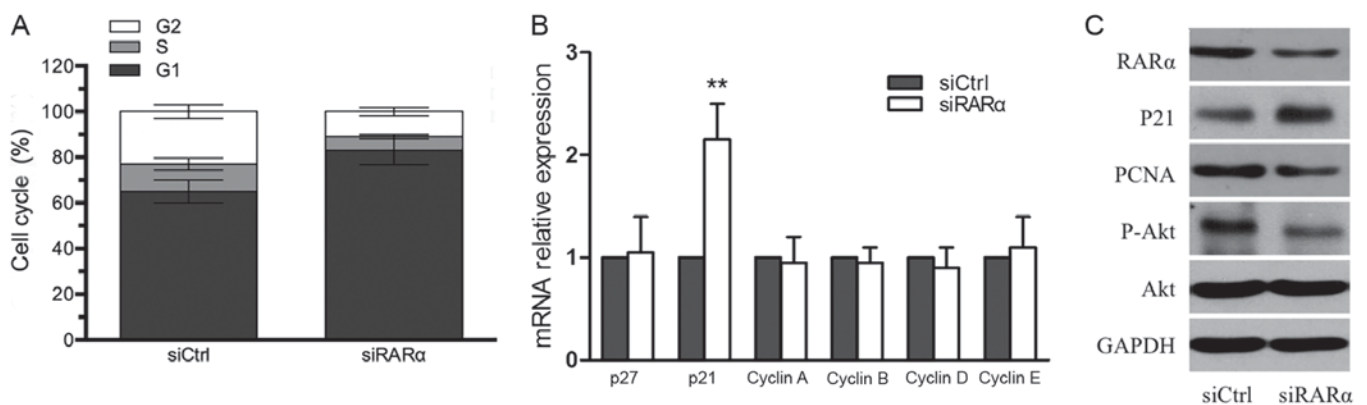

Figure 4. Effect of RARα knockdown on the cell cycle of AMC-HN-8 cells. (A) Flow cytometry analysis revealed that RARα knockdown increased the proportion of cells in G0/G1 phase and decreased the number in the G2 phase, compared with the control group. (B) The mRNA expression of several cell cycle associated genes were assessed using reverse transcription-quantitative polymerase chain reaction, following transfection with siRARα or siCtrl for 24 h. (C) The protein expression of RARα, p21, PCNA, p-AKT and AKT in siCtrl and siRARα-AMC-HN-8 cells were detected using western blotting analysis. **P<0.01 vs. control group. RARα, retinoic acid receptor-α; siRARα, RARα-specific small interfering RNA; siCtrl, small interfering RNA negative control; p21, cyclin dependent kinase inhibitor 1A; PCNA, proliferating cell nuclear antigen; AKT, protein kinase B; p-, phosphorylated.

of RARα, but not RARβ or RARγ, was downregulated using siRARα (Fig. 3A). The results from the MTT assay revealed that the proliferation of siRARα-AMC-HN-8 cells were significantly inhibited from 48 h following transfection, compared with the siCtrl-AMC-HN-8 cells (Fig. 3B). Furthermore, the down-regulation of RARα significantly decreased the forming foci of AMC-HN-8 cells (Fig. 3C). This data indicated that the down-regulation of RARα inhibited the growth of LSCC cells.

Downregulation of RARα induced laryngeal carcinoma cell cycle arrest via inhibition of the protein kinase B (AKT)

signaling pathway. The development of LSCC is associated with disorder of the cell cycle (16). Therefore, flow cytometry was performed to evaluate the effect of RARα downregulation on the cell cycle distribution of LSCC cells. The results demonstrated that the down-regulation of RARα in AMC-HN-8 cells reduced the proportion of cells in the S and G2/M phases, and a higher number of cells were arrested in the G0/G1 phase compared with cells in the control group (Fig. 4A). Furthermore, RT-qPCR was performed to detect the mRNA expression of cell cycle associated genes. As presented in Fig. 4B, the expression levels of P21 mRNA in the siRARα-AMC-HN-8

cells were >2x higher than those of the siCtrl cells, but the mRNA expression of p27, cyclin A, cyclin B, cyclin D, cyclin E was not significantly altered. Furthermore, the results from the western blot analysis confirmed the upregulation of the P21 protein in siRARα-AMC-HN-8 cells (Fig. 4C), which may be associated with the inhibition of the AKT signaling pathway. Thus, this data indicated that the downregulation of RARα resulted in P21 upregulation and induced G1 phase arrest via inhibition of the AKT signaling pathway.

Discussion

Laryngeal carcinoma is one of the most aggressive malignant tumors out of all head and neck squamous cell carcinoma, and generally has a poor prognosis (2). The survival time of laryngeal cancer has not increased, despite substantial progress in the developments of surgery and radiotherapy (3,17). Revealing the molecular mechanism underlying the development of LSCC is important for early diagnosis and targeted therapy. The development of LSCC is a complex process which is associated with changeable expression of multiple genes (18). Here, it was revealed that RARα was elevated in the LSCC tissues and was associated with poor pathological differentiation. Knockdown of RARα significantly inhibited the proliferation of LSCC cells through the induction of cell cycle arrest.

RARα, regarded as an important member of the retinoic acid receptor family, is involved in the regulation of cell differentiation, proliferation, apoptosis and metabolism. Accumulating evidence has indicated that RARα serves an important function in the development of malignant tumors. Notably, the functions of RARα in the development of tumors is dependent on the origin and the microenvironment of tumor cells. RARα served as a tumor suppressor gene in the development of gastric cancer, non-small cell lung cancer, melanoma tumor and prostate cancer. However, RARα may also serve as an oncogene in the development of hepatocellular carcinoma, leukemia and breast cancer. In the present study, it was identified that the expression of RARα in LSCC tissues was significantly higher than that in adjacent noncancerous tissues, suggesting that RARα may serve as an oncogene, participating in the process of the development of LSCC. Further analysis revealed that overexpression of RARα was associated with poor pathologic differentiation. Despite the small sample size of the present study, it was indicated that RARα may be involved in the regulation of LSCC cell differentiation.

Rapid proliferation, resistance to apoptosis, indefinite reproduction, angiogenesis, invasion and metastasis are the main features in the development of a tumor (19). These are also the biological functions of various types of tumor oncogenes and suppressor genes involved in the development of malignant tumors. In the present study, the downregulation of RARα significantly inhibited the proliferation of AMC-HN-8 cells through inducing cell cycle arrest. It was indicated that the overexpression of RARα served an important function in the development of LSCC. Consistent with the present study, previous studies have reported that RARα was also involved in the regulation of the cell cycle in breast cancer (13). Retinoic acid, a ligand of RARα, inhibited the growth of breast cancer cells through the induction of cell cycle arrest at the G1 phase and cell apoptosis (13). The mechanism of RARα in the regulation

of the development of LSCC is the focus of a number of studies; it has been reported that there are genomic and non-genomic influences (20). During genomic regulation, the conformation of RARα changes and recruits a coactivator protein once it is combined with its ligand. The complex forms the retinoic acid response element in the promoter region of the target gene, and then activates the transcription of target genes, resulting in the promotion of cell differentiation, apoptosis and other biological processes (20). In previous years, there was a greater focus on the non-genomic regulation of RARα. Previous studies have identified that RARα may regulate the phosphoinositide 3-kinase, AKT, c-Jun N-terminal kinase, mitogen-activated protein kinase 14 and protein kinase C signaling pathways following abnormal translocation into the cytoplasm (10,21-23). The present study identified that the overexpression of RARα was mainly located in the cytoplasm of LSCC cells and its downregulation significantly inhibited the activation of the AKT signaling pathway. Therefore, it was speculated that RARα promoted the development of LSCC through non-genomic transcriptional regulation.

Altogether, the present study has demonstrated that RARα expression was significantly elevated in patients with LSCC, and the downregulation of RARα induced cell cycle arrest and inhibited the proliferation of LSCC cells via the inhibition of the AKT signaling pathway. The present study may offer a potential molecular basis for the further development of RARα as a novel therapeutic target for patients with LSCC.

Acknowledgements

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