RESEARCH ARTICLE

ATF3 Activates Stat3 Phosphorylation through Inhibition of p53 Expression in Skin Cancer Cells

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Abstract

Aim: ATF3, a member of the ATF/CREB family of transcription factors, has been found to be selectively induced by calcineurin/NFAT inhibition and to enhance keratinocyte tumor formation, although the precise role of ATF3 in human skin cancer and possible mechanisms remain unknown. Methods: In this study, clinical analysis of 30 skin cancer patients and 30 normal donors revealed that ATF3 was accumulated in skin cancer tissues. Functional assays demonstrated that ATF3 significantly promoted skin cancer cell proliferation. Results: Mechanically, ATF3 activated Stat3 phosphorylation in skin cancer cell through regulation of p53 expression. Moreover, the promotion effect of ATF3 on skin cancer cell proliferation was dependent on the p53-Stat3 signaling cascade. Conclusion: Together, the results indicate that ATF3 might promote skin cancer cell proliferation and enhance skin keratinocyte tumor development through inhibiting p53 expression and then activating Stat3 phosphorylation.

Keywords: ATF3 - Stat3 phosphorylation - p53 expression - skin cancer cell

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Introduction

Skin cancer, categorized as melanoma and non-melanoma (basal and squamous cell carcinoma) skin carcinoma, is the common and life-risky type of cancer in the world and the incidence of skin cancer has reached epidemic proportions (Boni et al., 2002; Diepgen and Mahler, 2002; Gordon, 2013). The incidence of skin cancer is increasing yearly, and identification of risk factors is needed to stop this increasing trend. Multiple risk factors exist, including endogenous factors (genetic factors and gene mutations) and exogenous factors (sun exposure, chemical carcinogens and other environmental stress) (Gordon, 2013). Early diagnosis and treatment are needed to decrease the number of deaths due to skin cancer. Whereas, the precise molecular mechanism of skin cancer remains unclear, which make it difficult for treatment of the disease.

Activating transcription factor 3 (ATF3) is an ATF/CREB family member, which contains the basic region and leucine zipper (bZIP) motif characteristic of the bZIP (Liang et al., 1996; Allan et al., 2001). ATF3 is expressed at low levels in normal cells but can be rapidly induced by multiple and diverse extracellular signals including growth factors, cytokines and some genotoxic stress agents (Chen et al., 1996; Hai et al., 1999; Hai and Hartman, 2001; Fan et al., 2002; Taketani et al., 2012, Wang et al., 2012; Lee et al., 2013). The physiological function of ATF3 has been addressed in several cell lines that ATF3 might be involved in homeostasis, wound healing, cell adhesion, cancer cell invasion, apoptosis and signaling pathways (Chen et al., 1996; Wolfgang et al., 1997; Ishiguro and Nagawa, 2000; Ishiguro et al., 2000; Wolfgang et al., 2000; Allen-Jennings et al., 2001; Gold et al., 2012; Jang et al., 2012; Rose et al., 2012). Over-expression of ATF3 protein moderately suppresses cell growth through slowing down progression from G1/S transition in Hela cells (Fan et al., 2002). Conversely, ATF3 promotes growth factor-independent proliferation in chick embryo (Perez et al., 2001) and enhance serum-induced cell proliferation in rat fibroblasts (Tamura et al., 2005). Although ATF3 has been reported to promote skin tumor formation through suppression of p53-dependent senescence (Wu et al., 2010), the precise role of ATF3 and the underlying molecular mechanism in skin cancer remain unclear.

In this study, we found that ATF3 was upregulated in skin cancer tissues from patients and that ATF3 promoted skin cancer cell proliferation through activating Stat3 phosphorylation, which is the hallmark of various cancers development (Hodge et al., 2005; Yu et al., 2009; Kamran et al., 2013). Furthermore, activation of Stat3 by ATF3 was dependent on repression of p53 expression and the induction of p53-Stat3 signaling cascade accounted for the promotion effect of ATF3 on skin cancer cell proliferation. Together, ATF3 might increase the risk of skin cancer and serve as an important prognostic indicator for the disease.

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Materials and Methods

Samples and cell lines

This study was approved by the ethics review board of General Hospital of Beijing Region of PLA. Written informed consent was obtained from all study participants. A total of 30 surgically resected patients with skin cancer were enrolled. Patients were histologically examined by the pathology department of General Hospital of Beijing Region of PLA. As normal control, 30 specimens from healthy donors were collected. All samples were individually fresh-frozen in TRIzol Reagent (Invitrogen, Carlsbad, CA). Each sample was subjected for RNA extraction and quantitative real-time PCR (qRT-PCR). After ATF3 expression was examined, the 3 pieces of cancer tissues with highest ATF3 (C1, C2, C3) and the 3 pieces of normal tissues with lowest ATF3 (N1, N2, N3) were primarily cultured and established as cell lines in fibroblast standard conditions (DMEM medium with 10% FBS). The human squamous carcinoma SCC-13 cells and p53-null RTS3b cells were cultured in RPMI-1640 medium with 10% FBS and 100 units/mL PS (penicillin/streptomycin) at 37°C in 5% CO_2.

RNA isolation and real-time RT-PCR

Total RNA was extracted using TRI Reagent Kit (Ambion, USA) according to the manufacturer's instructions. Reverse transcription was performed using random primers with SuperScript III (Invitrogen, USA) to derive cDNA samples. Gene expression was analyzed using Taqman Universal PCR Master Mix. The relative expression levels of genes were normalized to GAPDH. The $2^{-\Delta\Delta Ct}$ method was applied to quantify the relative expression of genes. Primer sequences were following: p53 forward: 5'-ACTTGTCGCTCTTGAAGCTAC-3', p53 reverse: 5'-GATGCGGAGAATCTTTGGAACA-3'; ATF3 forward: 5'-CGAAGACTGGAGCAAAATGATG-3', ATF3 reverse: 5'-CATCCAGGCCAGGTCTCTGCCTCAG-3'; GAPDH forward: 5'- GATTCCACCCATGGCAAATTC-3', GAPDH reverse: 5'- AGCATCGCCCCACTTGATT-3';

Western blot analysis

Whole cells were directly lysed in 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton containing aprotinin (0.15 U/ml), 20 mM leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, IN, USA). The membrane was then blocked with 5% non-fat milk for 1 h, followed by incubation with primary and secondary antibodies, and finally developed with an enhanced chemiluminescence (ECL) kit (Pierce, USA). The following antibodies were used: anti-ATF3 (1:400, Santa Cruz Biotechnology, Santa Cruz, CA), p53 (1: 1:500, Santa Cruz Biotechnology, Santa Cruz, CA), anti-p-Stat3 (Tyr705) (1:1,000, Cell Signaling), anti-Stat3 (1:2000, Millipore), and anti-GAPDH (1:8000, Abcam). The secondary antibodies were diluted at 1: 5000.

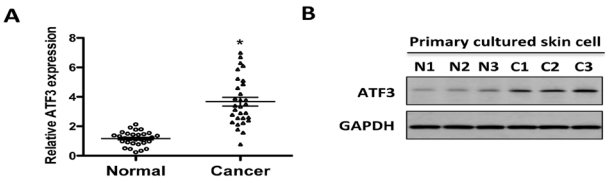

Figure 1. ATF3 Was Accumulated in Skin Cancer Cells. (A) qRT-PCR analysis of ATF3 expression in 30 skin cancer tissues and 30 normal skin tissues. (B) 3 primary cultured normal fibroblasts (N1-N3) and 3 cancer-skin derived primary cells (C1-C3) were subjected for ATF3 and GAPDH protein analysis by Western blot

Luciferase reporter assay

The SIE luciferase reporter activity represents the transcriptional activity of Stat3. The luciferase reporter construct (100 ng) was co-transfected with pRL-TK (20 ng) and ovexexpressing plasmids or vectors (200 ng) into SCC-13 or RTS3b cells. After transfection for 48 h, cells were harvested for the luciferase reporter assay (Promega, WI).

Cell transfection and treatment

SCC-13 or RTS3b cells were transfected with control shRNA plasmids, ATF3 shRNA plasmids, pcDNA3 vector, pcDNA3-p53, pcDNA3-Stat3C and/or pcDNA3-ATF3 plasmids using Lipofectamine 2000 (Invitrogen, USA) following the manufacturer's instructions. The cells were analyzed at indicated time points after transfection. shRNAs against human ATF3 in GV118 vector were purchased from Genechem (Shanghai, China) and the sequences were: control, GCCTAAACAATCAGCCTTCGGAGT; KD sequence, GAATAAACACCTCTGCCATCGGATG. ATF3 was cloned from human cDNA library and constructed into pcDNA3 vector. Plasmid Miniprep kit (QIAGEN, Canada) was used to extract plasmids used in this study. The Stat3 inhibitor Stattic was purchased from Calbiochem (San Diego, CA, USA) and the final concentration was 20 μM.

Cell proliferation assay

Cell proliferation of primary cultured fibroblasts, SCC-13 cells and RTS3b cells was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described (Yamaue et al., 1992). The cell growth curve was constructed by cell OD570 values after cells were seeded in 24-well plates for 24 hours. For transfection assay, OD570 value was determined at 48 hour post-transfection. The mean value of 3 wells presents the relative proliferation of cells.

Statistical analysis

The data were presented as the mean±SD. Data were analyzed using the SPSS software (Version 10.0). All results shown in this study were repeated at least for three times. Student's t-test was used for statistical analysis and $*p<0.05$ were considered statistically significant.

Results

ATF3 was upregulated in skin cancer cells

Previously, ATF3 was revealed to enhance keratinocyte

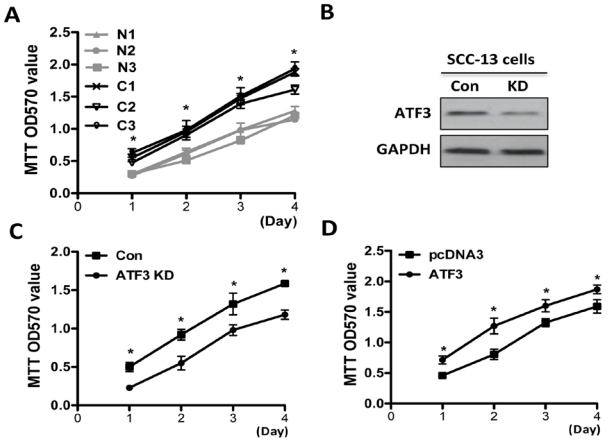

Figure 2. ATF3 is Required for Cell Proliferation of Skin Cancer Cells. (A) MTT assay was performed to analyze the proliferation of 6 primary cultured fibroblasts (N1-N3, C1-C3). (B) The knockdown efficiency of ATF3 shRNA (KD) was determined in SCC-13 cells. GAPDH served as loading control. (C) Cell proliferation rate was determined in control shRNA (Con) or ATF3 shRNA (KD) transfected cells. (D) ATF3 was overexpressed in SCC-13 cells and cell proliferation was determined by MTT assay

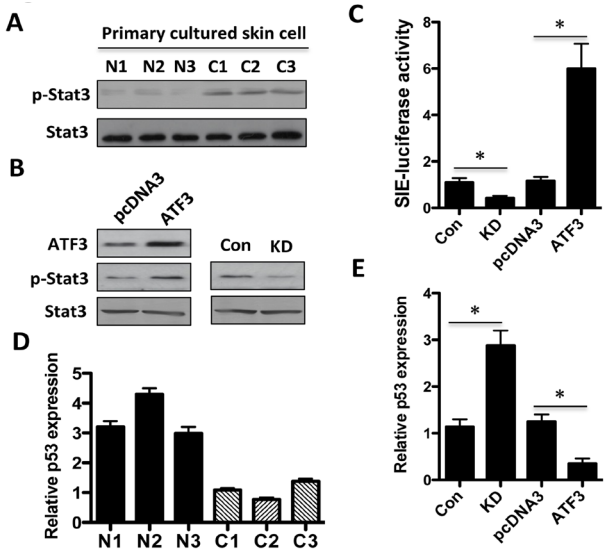

Figure 3. ATF3 Overexpression Activated Stat3 and Suppressed p53 Expression in Skin Cancer Cells. (A) Phosphorylated Stat3 (p-Stat3) and total Stat3 were examined in 6 primary cultured fibroblasts. (B) The vector (pcDNA3) and pcDNA3-ATF3 were transfected in SCC-13 cells. The protein levels of ATF3, p-Stat3 and Stat3 were determined by western blot. p-Stat3 level was also determined in ATF3 knockdown (KD) cells. (C) The luciferase activity of SIE-reporter, which represents the transcriptional activity of Stat3, was examined in ATF3 overexpressed or knocked-down cells. (D) The expression of p53 in 6 primary cultured fibroblasts was examined by qRT-PCR. (E) p53 expression was analyzed in SCC-13 cells when ATF3 was overexpressed or knocked down

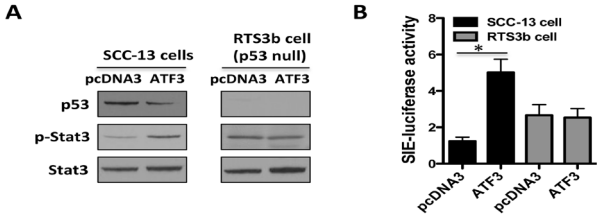

Figure 4. ATF3 Activates Stat3 Through Suppressing p53 Expression. (A) ATF3 was overexpressed in SCC-13 cells or p53 null RTS3b cells. p53, p-Stat3 and total Stat3 protein levels were determined. (B) The SIE-luciferase reporter activity was determined in ATF3 overexpressed SCC-13 cells and RTS3b cells

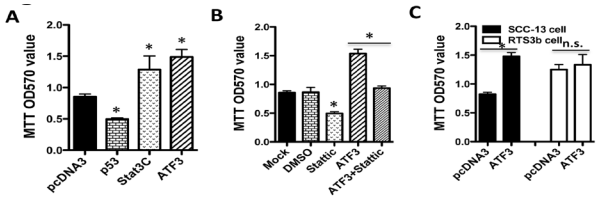

Figure 5. ATF3 Promoted Skin Cancer Cell Proliferation Through p53-Stat3 Signal Cascade. (A) p53, Stat3C and ATF3 were overexpressed in SCC-13 cells and the empty vector pcDNA3 served as control. The proliferation ability of these transfected cells was determined after transfection for 48 hours by MTT assay. or pcDNA3-ATF3 was transfected into SCC-13 cells and p53 null RTS3b cells. Western blot were performed to p53, p-Stat3 and total Stat3. (B) Control or ATF3 overexpressed cells were treated with Stat3 inhibitor Stattic or DMSO for 48 hours. Then the proliferation rate of cells under indicated conditions was determined. The cells without treatment (mock) served as control. (C) Determination of cell proliferation in ATF3-overexpressing SCC-13 cells and RTS3b cells

tumor formation (Wu, Nguyen, Dziunycz, Chang, Brooks, Lefort, Hofbauer and Dotto), while the expression levels of ATF3 in skin cancer tissues were not analyzed. In this study, 30 skin cancer tissues from patients and 30 normal tissues from healthy donors were collected for ATF3 expression analysis. qRT-PCR results showed that ATF3 was significantly upregulated in skin cancer while it was maintained at low level in normal epitheliums (Figure 1A). To verify the protein level of ATF3 in skin cancer, primary cultured skin fibroblasts from cancer and normal tissues with highest or lowest ATF3 mRNA level were established. The protein level of ATF3 was determined by western blot, showing that primary cultured skin cancer fibroblasts possessed higher ATF3 protein level, which is consistent with transcriptional difference in cancer and normal tissues.

ATF3 promotes skin cancer cell proliferation

During the culture of the 6 lines of primary cultured skin cells, we observed that skin cancer fibroblasts (C1-C3) proliferated much faster than the normal skin fibroblasts, which was determined by MTT assay (Figure 2A). We considered the possibility that ATF3 enhanced skin carcinogenesis through promoting skin cancer cell proliferation, and we therefore knocked down ATF3 expression in human epidermal squamous carcinoma cell line SCC-13 by ATF3 specific shRNA transfection (Figure 2B). As expected, the proliferation rate of SCC-13 cells was suppressed by ATF3 knockdown (KD) (Figure 2C). Conversely, forced expression of ATF3 in SCC-13 cells significantly promoted cell growth (Figure 2D). These results demonstrated that ATF3 is a positive regulator of skin cancer cell proliferation.

ATF3 upregulation activates Stat3 and suppresses p53 expression in human skin cancer cells

To explore the underlying mechanism of ATF3 in

skin cancer, the ATF3 downstream signaling cascades were mainly considered in this study. Stat3 was frequently activated and functionally connected with cancer cell proliferation (Corvinus et al., 2005; Yu et al., 2009). Moreover, EGF mediated Stat3 activation was required for skin cancer development. Whereas, the relationship between ATF3 and Stat3 has not been reported previously. To determine this point, we fist checked the phosphorylation state of Stat3 in our established fibroblast cell lines. It was found that phosphorylated Stat3 level was much higher in skin cancer cells with higher ATF3 expression (Figure 1B, 3A), suggesting that ATF3 might correlate with Stat3 phosphorylation. To test the hypothesis, the Stat3 phosphorylation state was determined when ATF3 was overexpressed or knocked-down in SCC-13 cells. The results in Figure 3B showed that ATF3 was required for Stat3 phosphorylation and that ATF3 overexpression upregulated Stat3 phosphorylation level. Furthermore, SIE-luciferase reporter assay revealed that ATF3 positively regulated Stat3 transcriptional activity (Figure 3C). Besides, ATF3 was reported to regulate p53 expression or trans-activity (Yan et al., 2002; Yan et al., 2005; Wu et al., 2010). The regulatory relationship between ATF3 and p53 was also determined in skin cancer. p53 showed lower mRNA level in established skin cancer cell lines with high ATF3 expression (Figure 3D). Correspondingly, ATF3 knockdown upregulated p53 expression and ATF3 overexpression strongly inhibited p53 transcriptional level, which is consistent with previous findings in primary human keratinocytes (Wu et al., 2010).

ATF3 promotes Stat3 activation through suppressing p53 expression

Give that ATF3 simultaneously activated Stat3 phosphorylation and suppressed p53 expression in skin cancer, we supposed to study the correlation between Stat3 and p53 regulation. Previous studies reported that p53 regulated Stat3 phosphorylation and DNA binding activity in human prostate cancer cells (Lin et al., 2002). We hypothesized that the activation of Stat3 by ATF3 might depend on p53 inhibition. Therefore, the effect of ATF3 on Stat3 activation was performed in p53 null RTS3b cells and SCC-13 cells served as positive control. As expected, Stat3 phosphorylation and transcriptional activity was not affected by ATF3 overexpression (Figure 4). We also observed that Stat3 activity were higher in RTS3b cells than that in SCC-13 cells (Figure 4). It indicates that ATF3 might downregulate p53 expression to release the inhibition effect of p53 on Stat3 activation.

ATF3 enhances skin cancer cell proliferation through p53-Stat3 signal cascade

Based on these data, ATF3 promoted skin cancer cell growth and regulated p53-Stat3 signaling cascade, we were wondering whether the functional effect of ATF3 is associated with that regulation. It was found that the constitutive active Stat3 form, Stat3C overexpression significantly promoted skin cancer cell growth, which was consistent with ATF3 functions (Figure 5A). Conversely, the Stat3 inhibitor Stattic inhibited cell proliferation of SCC-13 cells and the promotion effect of ATF3 was prevented by Stattic (Figure 5B). It suggests that ATF3 enhances skin cancer cell proliferation mainly through regulation of Stat3 activity. Moreover, the proliferation rate of skin cancer cells was suppressed by p53 overexpression in SCC-13 cells (Figure 5A). Whereas, ATF3 had no significant effects on the cell proliferation of p53-null RTS3b cells. Collectively, these data demonstrate that ATF3 might act trough modulating p53-Stat3 signaling cascade to enhance skin cancer cell proliferation.

Discussion

ATF3, a member of the enlarged AP-1 family transcription factors, is responding to physiological stresses and stress signaling pathways such as TGF-beta (Chen et al., 1996; Allen and Sivaprasad, 1999; Hai et al., 1999; Tsujino et al., 2000). ATF3 can be also induced by UV and ionizing irradiation, which was the main environmental causes of skin carcinogenesis (Fan et al., 2002; Kool et al., 2003; Koike et al., 2005). ATF3 has been revealed to enhance keratinocyte tumor formation in mice model (Wu et al., 2010), while how ATF3 promotes skin cancer development remains unclear. ATF3 is reported to be involved in various cancer cell invasion, apoptosis and cell growth with inconsistent effects (Chen et al., 1996; Lianget al., 1996; Wolfgang et al., 1997; Ishiguro and Nagawa, 2000; Ishiguro et al., 2000; Wolfgang et al., 2000; Allen-Jennings et al., 2001), suggesting the complexity of ATF3 functions in cancer progression.

In this study, we first identified the physiological role of ATF3 in skin cancer cell growth. Transcription factors rely on their expressed proteins to act in kinds of cell activity. ATF3 was found to accumulate in skin cancer tissues, while it maintained at the low level in healthy donor skin cells (Figure 1). It hints us that ATF3 might be a positive regulator of skin cancer in vivo. In our primary cultured fibroblasts from skin caner patients or normal people, we found that cancer-derived fibroblasts grow faster than normal skin cells. Functional assay revealed that ATF3 was sufficient and necessary for skin cancer cell proliferation (Figure 2). We postulate that ATF3 might enhance skin carcinogenesis through promoting skin cancer cell proliferation, which is consistent with reported function of suppressing cancer cell senescence (Wu et al., 2010). Interestingly, the down-regulated ATF3 expression was observed in several human cancers, and ATF3 played as a key mediator of KLF6-induced apoptosis in prostate cancer cells (Huang et al., 2008; Yin et al., 2008; Thompson et al., 2009). As an adaptive-response gene, ATF3 might play distinct roles in different biological systems through specific regulatory mechanisms.

ATF3 has been reported to activate c-Jun and JNK pathway in human vascular endothelial cells (Cai et al., 2000; Zhang et al., 2001) and ATF3 mutually regulates gadd153/Chop10 in liver cells (Wolfgang et al., 1997). Notably, ATF3 could activate p53 by blocking its ubiquitination in malignant transformation and conversely antagonize p53 trans-activity in Human fibrosarcoma cells. To explore the mechanism of ATF3 in skin cancer, we found that ATF3 suppressed p53 expression in skin cancer cells (Figure 3), consistent with previous

reports (Wu et al., 2010). We firstly found that Stat3 was activated by ATF3 in skin cancer cells (Figure 3) and ATF3 promoted Stat3 phosphorylation and transcriptional activity through suppressing p53 expression (Figure 4). More importantly, ATF3 enhanced skin cancer cell growth through p53-Stat3 signaling cascade (Figure 5). Consistently, DNA binding activity of Stat3 is regulated by p53 in human prostate cancer cells (Lin et al., 2002), and ATF3 and Stat3 synergistically modulate neuronal injury-inducible gene expression. Reversely, Stat3 could regulate p53 expression and function (Niu et al., 2005). These reports are coordinated with our findings that ATF3-p53-Stat3 signaling cascade indeed regulates skin cancer cell proliferation (Kiryu-Seo et al., 2008).

In summary, ATF3 was upregulated in skin cancer tissues and promoted cell proliferation of skin cancer cells. The molecular mechanism of ATF3 in skin carcinogenesis was investigated and we found that ATF3 enhanced skin cancer cell growth through modulating p53-Stat3 signaling activity. This study discovered the functions of ATF3-p53-Stat3 signal cascade in skin cancer development and will assist to understand the pathology of skin carcinogenesis.

Acknowledgements

The author(s) declare that they have no competing interests.

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