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microRNA-365-targeted nuclear factor I/B transcriptionally represses cyclin-dependent kinase 6 and 4 to inhibit the progression of cutaneous squamous cell carcinoma

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ABSTRACT

Cyclin-dependent kinases are either post-transcriptionally regulated by interacting with cyclins and cyclin-dependent kinase inhibitors or are transcriptionally regulated by transcription factors, but the latter mechanism has not been extensively investigated. Dysregulated transcription factors resulting from aberrantly expressed microRNAs play critical roles in tumor development and progression. Our previous work identified miR-365 as an oncogenic microRNA that promotes the development of cutaneous squamous cell carcinoma *via* repression of cyclin-dependent kinase 6, while miR-365 also targets nuclear factor I/B. However, the underlying mechanism(s) of the interaction between nuclear factor I/B and cyclin-dependent kinase 6 are unclear. In this work, we demonstrate that miR-365-regulated nuclear factor I/B transcriptionally inhibits cyclin-dependent kinases 6 and 4 by binding to their promoter regions. *In vivo* and *in vitro* experiments demonstrate that the loss of nuclear factor I/B after miR-365 expression or treatment with small interfering RNAs results in the upregulation of cyclin-dependent kinases 6 and 4. This upregulation, in turn, enhances the phosphorylation of retinoblastoma protein and tumor progression. Characterizing this transcriptional repression of cyclin-dependent kinases 6 and 4 by nuclear factor I/B contributes to the understanding of the transcriptional regulation of cyclin-dependent kinases by transcription factors and also facilitates the development of new therapeutic regimens to improve the clinical treatment of cutaneous squamous cell carcinoma.

1. Introduction

Cutaneous squamous cell carcinoma (CSCC) is the second most commonly diagnosed cancer, with an annual incidence that exceeds one million patients worldwide (Lomas et al., 2012). CSCC mainly affects Caucasian populations and most frequently develops in skin that receives chronic sun exposure. CSCC can progress to poorly differentiated cancer and, eventually, metastases can develop. Based on the observed metastatic capacity of CSCC to reach regional lymph nodes, CSCC is considered more malignant than basal cell cancer (Lomas et al., 2012). Exposure to high doses of ultraviolet (UV) light (generally from the sun) can damage the DNA of normal keratinocytes in the epidermis, leading to the development of skin cancer (Lee et al., 2014; Lomas et al., 2012). However, the underlying molecular mechanism(s) responsible for this transition remain obscure, despite ongoing investigations over the past decade.

Our current understanding of the molecular mechanism(s) of CSCC has led to particular interest in the role of transcription factors (TFs), such as p53 and nuclear factor-kappa B (NF-κB), in this process (Cooper et al., 2007). The dysregulated expression and mutation of relevant TFs play key roles in the development of cancer hallmarks (Hanahan and Weinberg, 2000), which, in turn, can promote the tumorigenesis of CSCC. Correspondingly, mutations in the tumor suppressor p53 protein have been identified as common causes of CSCC (Giglia-Mari and Sarasin, 2003; Ziegler et al., 1994). Once activated by UV irradiation, NF-κB can translocate into the nucleus and upregulate IL-6 expression and secretion, thereby

Abbreviations: CSCC, cutaneous squamous cell carcinoma; NFIB, nuclear factor I/B; CDK, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; UV, ultraviolet; p-Rb, phosphorylated retinoblastoma protein; IHC, immunohistochemistry.

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encouraging the development of chronic inflammation. This same dysregulated signaling pathway may contribute to the tumorigenesis and progression of CSCC (Sidransky, 2002; Xia et al., 2005).

Loss of cell cycle control in normal cells is considered to be a major factor in cancer development (Malumbres and Barbacid, 2009). The accumulation of mutations in cancer cells can result in constitutive mitogenic signaling and dysregulated responses to tumor suppressive signals, leading to uncontrolled proliferation (Malumbres and Barbacid, 2009). Cyclin-dependent kinases (CDKs) have important roles in controlling cell division and modulating transcription in response to extracellular and intracellular cues (Malumbres, 2014). The activities of CDKs are regulated by the physical binding of activators (cyclins) and inhibitors (Ink4, Cip, and Kip) (Malumbres and Barbacid, 2001). In response to mitogenic stimuli, CDKs promote cell cycle progression *via* phosphorylation and tumor suppressor protein retinoblastoma (Rb) inactivation. Phosphorylated Rb protein (p-Rb) is then able to dissociate from E2F proteins, resulting in the formation of activated E2Fs (Komori, 2013). The CDK-Rb pathway helps regulate the initiation of DNA replication, and control of this pathway is disrupted in almost all of the human cancers that have been characterized to date (Nevins, 2001; Sherr and McCormick, 2002). Thus, the aberrant expression of CDKs is considered to be a common prerequisite for carcinogenesis (Baker and Reddy, 2012).

We previously identified microRNA-365 (miR-365) as an oncomiR that is highly expressed in CSCC cells and clinical tumors (Zhou et al., 2013). Further study of this microRNA led to the identification of a downstream target of miR-365, nuclear factor I/B (NFIB) (Zhou et al., 2014). NFIB is a member of the nuclear factor I gene family in vertebrates and has been associated with a diverse set of transcriptional regulatory activities. Two-hundred residues at the N-terminus of NFIB constitute a DNA-binding domain. Loss of this domain eliminates the DNA-binding activity of this protein and stimulates adenovirus DNA replication (Gronostajski, 2000; Pjanic et al., 2011). Approximately 300 residues at the C-terminus of NFIB comprise the transcriptional modulation region that is responsible for the transcriptional regulatory function of NFIB. This TF may regulate hundreds of genes in various organs (Persson et al., 2009), especially genes related to cell proliferation and differentiation in lung maturation (Hsu et al., 2011).

In our previous work, high levels of miR-365 were associated with the repression of NFIB expression. This repression affected downstream cancer-related effectors, including CDK6 (Zhou et al., 2014). Although the role of CDK6 in the initiation and development of tumors has been well-characterized (Kollmann et al., 2013), the underlying mechanism(s) responsible for the regulation of CDK6 by NFIB remain unclear. Interactions between CDK6 and CDK4 have been described as well (Kollmann et al., 2013). Thus, an important question is whether CDK4 is also regulated by NFIB.

Here, we confirm that NFIB transcriptionally represses both CDK6 and CDK4 by binding to the promoter regions of these two proteins. The results of *in vivo* and *in vitro* experiments showed that the loss of NFIB after the expression of miR-365 or treatment with a small interfering RNA (siRNA) targeting NFIB leads to the upregulation of CDK6 and CDK4, which, in turn, enhances the levels of p-Rb and contributes to CSCC progression.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Institutional Review Board of Nanfang Hospital, which is affiliated with the Southern Medical University of China. All participating patients provided written informed consent for the use of surgical samples. All animals were treated in accordance with standard guidelines for the care and use of laboratory animals.

2.2. Cell culture and tumor samples

The CSCC lines A431, Tca8113 (China Center for Type Culture Collection and Cell Bank of the Chinese Academy of Sciences, Shanghai, China), and HSC-1 (Dongguang Biojet Biotech. Co., Ltd, Guangzhou, China) as well as the human benign epidermal keratinocyte cell line HaCaT (China Center for Type Culture Collection, Wuhan, China) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and streptomycin (Invitrogen). Cells were maintained at 37 °C with 5% CO_2 in a humidified atmosphere. CSCC tissue samples were obtained from patients diagnosed with CSCC between January 2009 and August 2011 in the Departments of Dermatology, Pathology, and Oncology at Nanfang Hospital and Zhujiang Hospital, which are affiliated with Southern Medical University and The Third Affiliated Hospital of Sun Yat-sen University, respectively.

2.3. Isolation of RNA and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the CSCC cell lines (2×10^6 cells) and tissues (100 mg) with TRIzol reagent (Invitrogen), in accordance with the manufacturer's protocol. The optical density of the RNA samples at 260 nM was quantified by a Nanodrop 2000 (Thermo Scientific). Reverse transcription (RT) and qRT-PCR of the mRNA samples were performed with a M-MLV 1st Strand Kit (Invitrogen), Oligo(dT)20 primers (Invitrogen), and SYBR Select Master Mix (Invitrogen). RT and qRT-PCR of microRNAs were performed with the TaqMan MicroRNA Reverse Transcription Kit for miR-365 and U6 (Ambion), TaqMan MicroRNA Assay for miR-365 and U6 (Ambion), and TaqMan Universal Master Mix II without UNG (Ambion). The following primer sequences were used to amplify the indicated genes: *NFIB* (forward [F]: 5′-TCTCAGCAATGTCAACGAC-3′; reverse [R]: 5′-TTTATGCCTACAGCCTCCT-3′), *CDK6* (F: 5′-GAACCAAAATGCCACATACA CT-3′; R: 5′-TTCGGCCTTTCGCATAGG-3′), *CDK4* (F: 5′-GCCTAGATTTCCTTCATGCC AAT-3′; R: 5′-CGACGAAACATCTCTGCAAAGAT-3′), and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (F: 5′-TTGCCATCAATGACCCCTTC A-3′; R: 5′-CGCCCCACTTGATTTTGGA-3′).

The qRT-PCR reactions were performed with the TransStart TipTop Green qPCR SuperMix (TransGen Biotech) and a MX3005P instrument (Stratagene). The cycling parameters were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and an annealing/extension step at 60 °C for 40 s. Gene expression $\Delta\Delta Ct$ values for the mRNAs and miR-365 from each sample were calculated by normalizing the values with the internal controls (*GADPH* and *U6* snRNA, respectively). The fold change was calculated by the equation $2^{-\Delta\Delta Ct}$. All of the experiments were performed in triplicate.

2.4. DNA constructs

To construct the NFIB expression plasmid, full-length *NFIB* was cloned into the pcDNA3.1(+) vector (Life Technologies) between the *BamH*I and *Xho*I sites using the following primer pair: NFIB-FP (5′-agtggatcca tgatgtattc tcccatctgt ctc-3′) and NFIB-RP (5′-agtctcgagc tagcccaggt accaggactg ttgc-3′). For dual-luciferase assays to determine the promoter activities of *CDK6* and *CDK4*, the predicted promoter regions of *CDK6* and *CDK4* were amplified and cloned into the pGL3 vector between the *Nhe*I and *Hind*III sites using the following primer pairs: *CDK6* promoter_FP (5′-AGTGCTAGCGACCTGTGTTTCCTTGGAATCG-3′), *CDK6* promoter_RP (5′-AGTAAGCTTCCTCCGAGAAAAGCGAAGTTACTT-3′) and *CDK4* promoter_FP (5′-AGTGCTAGCTTCCCTCTGAGGCCAAGTTCTAC-3′),