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Expression levels of NF-Y target genes changed by CDKN1B correlate with clinical prognosis in multiple cancers

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ABSTRACT

CDK inhibitors CDKN1B (p27) and CDKN2A (p16) inhibit cell cycle progression. A lower expression level of only p27 has been correlated with poorer prognosis in various types of clinical cancers. The difference may be the result of distinct genes downstream of these CDK inhibitors. Here, we report that NF-Y transcription factor-targeted genes specifically down-regulated by p27 correlate with poor prognosis in multiple tumor types. We performed mRNA expression profiling in HCT116 cells over-expressing either p16 or p27 and identified their regulatory genes. *In silico* transcription factor prediction indicated that most of the genes specifically down-regulated by p27 are controlled by NF-Y. Under the hypothesis that NF-Y-targeted genes are responsible for poor prognosis, we predicted prognosis in four types of cancer based on genes with the NF-Y motif, and found a significant association between the expression of NF-Y-targeted genes and poor prognosis.

Introduction

Cell cycle control is a highly coordinated process mediated by a diversity of genes and proteins. Cyclin-dependent kinases (CDKs) play a pivotal role in regulating the initiation and transition of cell cycle phases by inactivating phosphorylation of the retinoblastoma (RB) family of proteins, which leads to activation of the E2F transcription factors [1]. The E2F transcription factors cooperating with DP1/2 to sequentially transactivate the genes required for each phase of cell cycle progression [2]. While CDKs are positively regulated by several types of cyclins, their negative regulation is mediated by the CDK inhibitors (CDKIs), which are divided into two classes; the INK4 and CIP/KIP families based on their CDK inhibition spectrum [3]. Given the fundamental functions of the CDK/RB/E2F pathway in cell cycle control, deregulation of the components in this pathway, including genetic/epigenetic alterations, over-expression and post-translational aberrations, is a critical step in multistage carcinogenesis.

An INK4 family CDKI, CDKN2A (p16), binds to and inactivates CDK4 and CDK6 by inhibiting the association between these CDKs and D-type cyclins. The p16-mediated inhibition of D-type cyclins controls early G1 cell cycle progression [4]. Although other INK4 family proteins such as p15 (CDKN2B) and p18 (CDKN2C) show similar biological features *in vitro*, the frequency of deregulation of p16 in tumors is significantly higher [5]. A 5′-CpG island in the promoter region of the p16 gene is hypermethylated in various types of cancers including non-small cell lung cancer (NSCLC), breast cancer and hepatocarcinoma (HCC) [6]. The status of hypermethylation correlates with the p16 protein level when measured by immunohistochemistry in clinical samples. For example, in one study of NSCLC, ~26% of 27 tumor samples examined showed hypermethylation in the promoter region of p16, leading to the down-regulation of p16 mRNA expression [7]. The observation of mutual inactivation of p16 and RB in NSCLC and SCLC also points to an important role for p16 in carcinogenesis as a tumor suppressor gene [8].

CDKN1B (p27) is a CIP/KIP family CDKI that binds to and regulates CDK2, 4 and 6. Upon UV irradiation or cell contact inhibition, the activated p27 inhibits D-type cyclins and CDKs, which arrests the cell cycle at the G1 phase. p27 reduction is also required for completion of the G1 phase and initiation of the S phase by sequential activation of E-type and A-type cyclin and CDK2 complexes. The activated CDK2 up-regulates transcription of genes which participate in DNA synthesis and subsequent processes that occur at S phase [9]. With respect to the association between p27 deregulation and cancer, p27 is frequently inactivated in various types of cancers, which implies a tumor suppressive function of the protein similar to p16. Unlike classical tumor suppressors such as p53 (TP53) and RB (RB1), genetic deletions or mutations of p27 are not frequently observed. However, multiple reports have demonstrated that the protein expression level of p27 is decreased or silenced in various types of cancers such as breast, colon, and lung cancer [10]. The mechanism of the decreased

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expression of p27 has also been investigated. The up-regulation of SKP2, which is the ubiquitin ligase E3 of p27, is one of the causative events of the decrease in p27 protein [11]. Despite detailed analysis of p27 from the perspective of its inhibition spectrum of CDKs and its post-translational regulation, p27-regulatory mRNA gene expression as an end point of the p27/CDK/RB pathway remains elusive.

Although both p27 and p16 are inactivated at high frequency in many types of cancers, and possess a common function involving down-regulation of E2F regulatory genes via activation of RB proteins, the impact of their expression levels on clinical prognosis differs [12]. p27 is widely regarded as an adverse prognostic indicator in a number of tumor types, while p16 seems not to be an independent risk factor. In a colon cancer study, the survival rate was poorer in p27-negative tumors (median, 69 months) compared with p27 positive tumors (median, 151 months), and p27 was an independent prognostic marker [13]. In HCC, p27 acts as an independent predictor of HCC recurrence among several cell cycle regulators such as RB, p21 (CDKN1A), cyclin D1 and p16 [14]. Breast cancer is one of the most extensively studied tumor types with respect to the relationship between p27 and prognosis. Reduced overall survival correlated significantly with down-regulation of p27 expression in a retrospective analysis with more than 2000 samples [15]. In contrast, limited studies have shown the implications of p16 as a prognostic predictor, although both p16 and p27 exhibit an inhibitory effect on cell cycle progression via reducing E2F target genes. The molecular mechanism by which p27 plays a distinct role from p16 as a prognostic factor remains elusive.

To date, a number of DNA microarray studies have clearly shown their effectiveness in analyzing the molecular mechanisms at work in the CDK/RB/E2F pathway. mRNA expression profiling of cultured cells with over-expression of exogenous E2F enabled identification of novel E2F regulatory genes [16–23]. Microarray analysis of U2OS cells with ectopic p16 expression or constitutively active RB expression has also led to the identification of p16/RB regulatory genes [24]. We have also performed molecular profiling of RB positive and negative matched pair cell lines, which resulted in the identification of ECT2 as a novel E2F regulatory gene [25]. These studies have demonstrated that global expression profiling is a powerful approach to decipher the CDK/RB/E2F pathway.

In the present study, we performed microarray analysis of cultured cells over-expressing either p27 or p16 in order to investigate their common and distinct features. While E2F-target genes were enriched among the genes that were commonly changed by both CDKIs, significant enrichment of NF-Y-target genes was unexpectedly observed in the gene set specifically down-regulated by p27 expression. The prediction of prognosis using the NF-Y-target genes indicated that samples with up-regulated expression of the NF-Y-target genes regulated by p27 showed poor prognosis in multiple cancers, which could explain the molecular mechanism underlying the distinct features of p27 and p16 as prognostic factors.

Results

Identification of the genes commonly regulated by both p16 and p27 induction

To identify p16 and p27 target genes by microarray analysis, human colorectal HCT116 cells were infected with adenovirus expression vectors containing p16, p27, or the control lacZ gene at a titer of 200 multiplicity of infection (moi). Before analysis, expression levels of the CDK inhibitors in the cells were confirmed with RT-PCR and Western blotting. Similar expression levels were observed for both mRNA and protein of p16 and p27 (Fig. 1a and b). Cell growth was arrested in both cells expressing p16 and p27. Cell cycle distribution

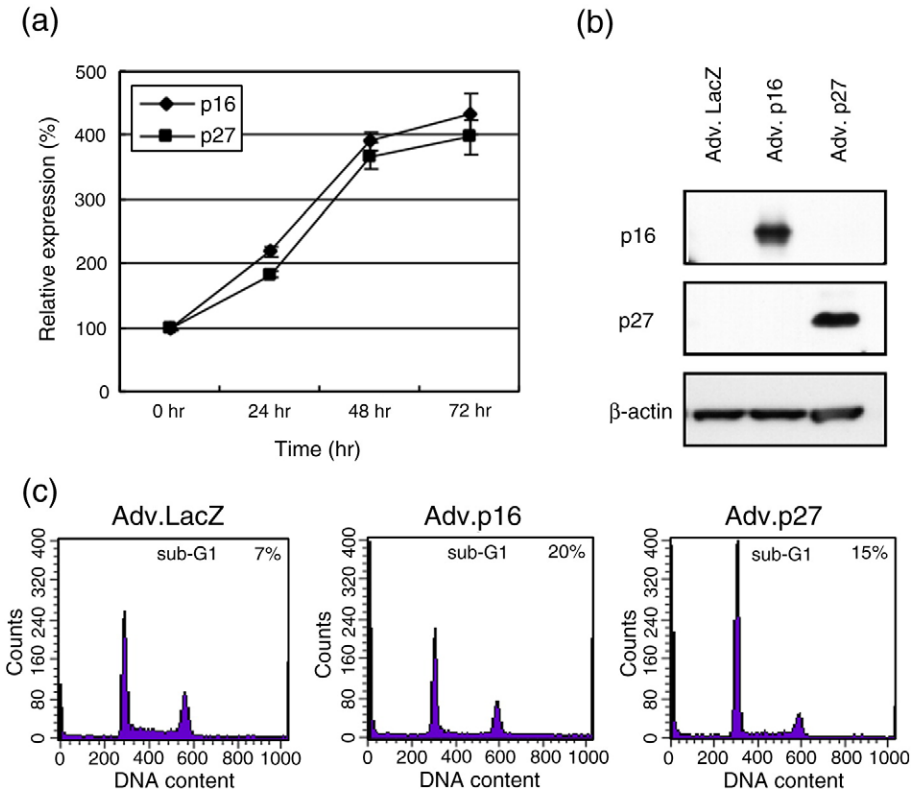

Fig. 1. Expression levels of p16 and p27, and cell cycle distribution post adenoviral infection containing p16 and p27. (a) mRNA expression level of p16 and p27. Human colon carcinoma HCT116 cells were infected with p16-, p27- or control lacZ-expressing adenovirus. Expression level of p16 and p27 mRNA was measured by quantitative RT-PCR analysis and shown as relative expression level to control. (b) Protein expression of p16 and p27. HCT116 cells were infected with p16-, p27- or lacZ-expressing adenovirus. After 72 h infection, whole cell lysates were subjected to Western blotting against anti-p16, anti-p27 or anti-β-actin antibodies. (c) Cell cycle distribution and subG1 induction. Cells treated as described in *b* were labeled with propidium iodide to determine the DNA content, and cell cycle distribution of the stained cells was analyzed by flow cytometry.