INK4 Family —A Promising Target for ‘Gene-Regulating Chemoprevention’ and ‘Molecular-Targeting Prevention’ of Cancer—

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Abstract

Inactivation of the p16^{INK4a} gene is one of the most frequent defects that contribute to oncogenesis in human cancer, since it is a tumor-suppressor gene. Therefore, functional restoration of p16^{INK4a} is one of the most effective methods for cancer prevention. We proposed the concept of ‘gene-regulating chemoprevention’ and ‘molecular-targeting prevention’ of cancer, which assumes that transcriptional regulation by drugs on tumor-suppressor genes or functionally similar genes to the tumor-suppressor genes contributes to the prevention of human malignancies. The p16^{INK4a} homologs p15^{INK4b}, p18^{INK4c} and p19^{INK4d} have been recently identified, and these four members constitute the INK4 family of proteins. All directly bind to cyclin D-cyclin dependent kinase (CDK) 4/6 and are therefore specific inhibitors of these complexes. We recently showed that histone deacetylase (HDAC) inhibitors, promising chemopreventive and chemotherapeutic agents, induce p15^{INK4b} and p19^{INK4d} gene expression and cause growth arrest, suggesting that both genes are important molecular targets for HDAC inhibitors. Furthermore, we found that 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which is widely used as a tumor promoter and protein kinase C activator, promotes human cancer cell growth through the down-regulation of p18^{INK4c} gene expression. This suggests that a mouse two-stage carcinogenesis model using TPA might partially represent the most common human carcinogenesis pathway related to RB. Our results suggest that the INK4 family consists of attractive and promising molecular targets for the ‘gene-regulating chemoprevention’ and ‘molecular-targeting prevention’ of cancer.

Key words: INK4 family, p15^{INK4b}, p16^{INK4a}, p18^{INK4c}, p19^{INK4d}, histone deacetylase inhibitors, TPA, protein kinase C, prevention

Introduction

Recent advances in cancer research have uncovered many molecular genetic abnormalities present in human malignancies (1). However, the overall mortality rates are unlikely to change unless cancer prevention is achieved (2). The key to the development of an effective preventive approach lies in the identification of genes and biochemical pathways involved in human tumorigenesis. The inactivation of p16^{INK4a} has been extensively reported for most human malignant tumors (3), and further

analysis has proven that p16^{INK4a} is an important molecule with a tumor suppressive function (3).

Importance of p16^{INK4a} gene inactivation in human tumorigenesis

p16^{INK4a} negatively regulates the cyclin D-cyclin dependent kinase (CDK) 4/6 complexes, thereby suppressing phosphorylation of the retinoblastoma (RB) tumor-suppressor gene product and inhibiting G1/S transition (3) (Fig. 1). Inactivation of this gene through gene deletions, point mutations or transcriptional silencing by methylation of the promoter is one of the most frequent defects that contribute to human oncogenesis, thereby establishing p16^{INK4a} as a tumor-suppressor gene product in many human tumors (4). p16^{INK4a}-deficient mice are susceptible to malignant tumors (5, 6), and germ-line mutational analysis indicates that mutations of this gene in humans are associated with familial syndromes such as malignant

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Fig. 1 CyclinD-CDK4/6 inhibition and RB activation by p15^{INK4b}, p16^{INK4a}, p18^{INK4c} and p19^{INK4d}.

Fig. 2 The effects of smoking and alcohol on p15^{INK4b}, p16^{INK4a}, p18^{INK4c} and p19^{INK4d}.

melanoma and pancreatic cancer (7). A recent study showed that inactivation of the p16^{INK4a} gene frequently occurs in oxidative stress-induced renal cell carcinoma in rats, suggesting a role for p16^{INK4a} in oxidative stress-induced carcinogenesis (8). Furthermore, it was found that there is a close relationship between smoking and methylation of the p16^{INK4a} gene promoter in lung cancer (Fig. 2) (9).

‘Gene-regulating chemoprevention’ and ‘molecular-targeting prevention’ of cancer

Due to the significant role of p16^{INK4a} in human carcinogenesis, functional restoration of p16^{INK4a} is useful for cancer prevention. We previously suggested that transcription regulating agents of tumor-suppressor genes, downstream target genes or functionally similar genes of tumor-suppressor genes may be useful for the prevention of tumors, which we therefore termed ‘gene-regulating chemoprevention’ and ‘molecular-targeting prevention’ of cancer (10, 11). For the case of hypermethylation of the promoter region for silencing the expression of tumor-suppressor genes, treatment with demethylating agents should restore the expression. If tumor-suppressor genes themselves are inactivated by gene deletions or point mutations, activation of the expressions of downstream target genes of a tumor-suppressor gene or functionally similar genes of the tumor-suppressor gene may compensate for the loss of function.

Identification of p15^{INK4b}, p18^{INK4c} and p19^{INK4d} genes as p16^{INK4a} homologs

The p16^{INK4a} homologs p15^{INK4b}, p18^{INK4c} and p19^{INK4d} were recently identified (3), and these four members constitute the INK4 family of proteins. Members of this family negatively regulate the cyclin D-CDK4/6 complexes that promote G1/S transition by phosphorylating the RB tumor-suppressor gene product (3) (Fig. 1). There is a close association between chronic alcohol consumption and increased risk of cancer of the upper aerodigestive tract. A recent study elucidated that ethanol decreases p18^{INK4c} and p19^{INK4d} expression at the protein level in a head and neck squamous cell carcinoma cell line (12) (Fig. 2). Furthermore, it was found that there is a close relationship between smoking and methylation of the p15^{INK4b} gene (13) (Fig. 2). However, in contrast to p16^{INK4a}, extensive studies elucidated that genetic alterations of p15^{INK4b}, p18^{INK4c} and p19^{INK4d} genes are rare events in human cancer.

A possible molecular basis for these biochemically indistinguishable molecules being able to carry out distinct tumor-suppressive functions lies in differences in their transcriptional regulation. The INK4 proteins are expressed in distinct and tissue-specific patterns (14), and their transcription responds differentially to diverse stimuli. Induction of p15^{INK4b} in response to transforming growth factor β , or up-regulation of p16^{INK4a} with increasing population doublings or oncogenic ras are representative examples of such differential transcriptional regulation (15, 16).

p15^{INK4b}, p18^{INK4c} and p19^{INK4d} possess functions similar to that of p16^{INK4a}. Therefore, activation of p15^{INK4b}, p18^{INK4c} and p19^{INK4d} genes by drugs may counterbalance the loss of function of this gene. Based on this concept, we recently showed that histone deacetylase (HDAC) inhibitors induce p15^{INK4b} and p19^{INK4d} gene expression and cause growth arrest in p16^{INK4a}-inactivated human cancer cells (17, 18). We also found that 12-*O*-tetradecanoylphorbol-13-acetate (TPA), widely used as a protein kinase C (PKC) activator, promotes human cancer cell growth through the down-regulation of p18^{INK4c} gene expression. This suggests that a mouse two-stage carcinogenesis

model using TPA might partially represent the most common human carcinogenesis pathway related to RB (19). Collectively, these results indicate that INK4 family members are important molecular targets for cancer prevention. We describe these findings together with recent progress in this mini-review.

p15^{INK4b} and p19^{INK4d} genes as important molecular targets for histone deacetylase (HDAC) inhibitors

HDAC inhibitors induce growth arrest, differentiation and apoptotic cell death in cancer cells (20), and also inhibit the growth of cancer cells in animal models (21). During the last decade, a number of HDAC inhibitors have been identified (22). Clinical applications have been started in a phase II study which is testing depsipeptide, a HDAC inhibitor, on patients with T-cell lymphoma (23). Butyrate, a short chain fatty acid, represents one class of HDAC inhibitors, and is an important molecule for preventing colorectal cancer (24). It is one of the most abundant short chain fatty acids found in the large intestine and is generated by bacterial fermentation of dietary fibers (24).

We previously discovered that the HDAC inhibitors butyrate and trichostatin A (TSA) inhibit cellular proliferation and induce expression of the p21^{WAF1} gene through its promoter in a p53-independent manner (25, 26). p21^{WAF1} is a p53 target gene which negatively regulates the cyclin A/E-CDK2 complexes, thereby suppressing phosphorylation of the RB tumor-suppressor gene product and inhibiting G1/S transition (27). However, several recent reports suggested the existence of a p21^{WAF1}-independent pathway which induces growth arrest by HDAC inhibitors. Butyrate induces growth arrest during the G1 phase in p21 (−/−) mice fibroblasts (28). In addition, the growth of HCT116 p21 (−/−) is also inhibited by TSA (29).

Therefore, we examined whether members of INK4 family are involved in the G1 phase arrest induced by HDAC inhibitors. Recently, we showed that TSA and butyrate induce

p15^{INK4b} gene expression in p16^{INK4a}-inactivated human immortalized keratinocyte HaCaT cells (17) (Fig. 3). Our results indicated that HDAC inhibitors stimulate p15^{INK4b} promoter activity and up-regulate the expression of p15^{INK4b} mRNA and protein, and that hyperphosphorylated form of the RB protein is converted into a hypophosphorylated form in HaCaT cells. Furthermore, the overexpression of p15^{INK4b} completely suppresses the colony formation of HaCaT cells. Thus, HDAC inhibitors activate expression of the p15^{INK4b} gene through its promoter, and this activation may contribute to growth arrest induced by these molecules (17).

We determined that TSA and butyrate also induce expression of the p19^{INK4d} gene in p16^{INK4a}-deleted human T cell leukemia cell line Jurkat (18) (Fig. 3). HDAC inhibitors enhance p19^{INK4d} promoter activity and up-regulate the p19^{INK4d} mRNA and protein levels, resulting in hyperphosphorylated form of the RB protein being converted into a hypophosphorylated form. Deletion and mutation analysis indicated that one of the major TSA-responsive elements is the Sp1 binding site in the p19^{INK4d} promoter. The Sp family of transcription factors (Sp1, Sp2, Sp3 and Sp4) is known to bind to the same Sp1 sites (30). We then found that Sp1 and Sp3 interact with the Sp1 binding site in the p19^{INK4d} promoter by electrophoretic mobility-shift assay (18). So far, eight HDACs have been described in mammalian cells (22). Recently, Sp1 and Sp3 have been reported to be associated with HDAC2 in several promoters (31, 32). We then showed that HDAC2 was present in the p19^{INK4d} proximal promoter region in the absence but not the presence of TSA, using chromatin immunoprecipitation assay. These results suggest that treatment with TSA transcriptionally activates the p19^{INK4d} gene by releasing HDAC2 from the histone-DNA complex at the p19^{INK4d} promoter. Moreover, mouse embryo fibroblasts that lack the p19^{INK4d} gene are resistant to the growth inhibitory effects of TSA compared to their wild-type counterparts, suggesting that p19^{INK4d} is an important molecular target of HDAC inhibitor-induced growth arrest (18).

Since the p15^{INK4b} and p19^{INK4d} genes are rarely mutated in human malignancies (3), the actions of HDAC inhibitors on these genes are important from the standpoint of the ‘gene-regulating chemoprevention’ and ‘molecular-targeting prevention’ of cancer. The induction of these genes by HDAC inhibitors enables them to function as a replacement for p16^{INK4a} in p16^{INK4a}-inactivated cancer cells. We are now investigating whether HDAC inhibitors also affect p18^{INK4c} gene expression.

p18^{INK4c} gene as an important molecular target for 12-O-tetradecanoylphorbol-13-acetate (TPA)

As described above, inactivation of the p16^{INK4a} gene is one of the most frequent defects in human malignant tumors (4). On the other hand, genetic alteration of the p18^{INK4c} gene is a rare event in human tumors (3). From these findings, it was suggested that p16^{INK4a}, and not p18^{INK4c}, plays a role in tumor suppression in humans. However, p18^{INK4c}-deficient mice exhibit frequent development of a wide spectrum of tumors, establishing p18^{INK4c} as a tumor-suppressor gene at least in mice (33, 34). Furthermore, it was recently reported that treatment of p18^{INK4c} null and heterozygous mice with a chemical carcinogen

Fig. 3 Induction of the p15^{INK4b} and p19^{INK4d} genes by butyrate or HDAC inhibitors in p16^{INK4a}-inactivated cancer cells.

Fig. 4 Suppression of the p18^{INK4c} gene by TPA in a PKC-dependent fashion.

causes tumorigenesis at an accelerated rate (35). The remaining wild-type allele of p18^{INK4c} is normal in tumors derived from heterozygotes, suggesting that p18^{INK4c} is a haploinsufficient tumor suppressor. This suggests that a quantitative decrease in p18^{INK4c} gene expression may contribute to human tumorigenesis, which lacks genetic alterations of the p18^{INK4c} gene. However, little is known about the regulatory mechanism responsible for human p18^{INK4c} gene expression.

TPA is a typical tumor promoter in a two-stage carcinogenesis model in mice (36), activation of PKC by TPA is a pivotal event in TPA-mediated tumor promotion (36). On the other hand, most human tumors have genetic or epigenetic alterations in their components of the RB pathway (4, 37). It is unknown whether any connection between the TPA-PKC pathway and RB pathway exists. We recently disclosed that p18^{INK4c}, a component of the RB pathway, is a target molecule of TPA (19).

We showed that TPA suppresses the expression of p18^{INK4c} through its promoter, accompanied by the induction of human cancer cell growth (19) (Fig. 4). The reduction of p18^{INK4c} using small interfering RNA (siRNA) also enhances cell growth, suggesting that it is a critical target of TPA. Furthermore, Ro 31-8425, a potent and highly specific PKC inhibitor, abrogates the suppressive effect of TPA on p18^{INK4c} gene expression. However, the expression of dominant-negative c-Jun (TAM-67) does not inhibit the effect of TPA on the p18^{INK4c}. Taken together, our results suggest that activation of PKC promotes

human cancer cell growth through down-regulation of p18^{INK4c} in an AP-1 activation-independent manner (19).

The observation that activation of PKC promotes human cancer cell growth through the suppression of human p18^{INK4c} supports the hypothesis that p18^{INK4c} also plays a role in human tumorigenesis. Situated at the crossroads of many signal transduction pathways, PKCs are activated by upstream signaling molecules such as growth factor receptors (for example, platelet-derived growth factor receptor), and are able to activate downstream signaling molecules such as the proto-oncogene Raf-1 (38, 39). In addition, PKC activity is increased in some human tumors compared with their normal counterparts (40–42). These results suggest that the accelerated cellular proliferation of some human tumors caused by enhanced PKC activity may involve the suppression of p18^{INK4c}, which is a ubiquitously expressed cyclin-dependent kinase inhibitor.

Future aspects of the INK4 family—promising targets for the ‘gene-regulating chemoprevention’ and ‘molecular-targeting prevention’ of cancer—

Epidemiologic studies have provided consistent evidence that people who consume higher levels of fruits and vegetables are at decreased risk for various cancers (43). Furthermore, certain dietary constituents found in the everyday diet exhibit inhibitory effects on carcinogenesis in animal models (44). We determined that p15^{INK4b} and p19^{INK4d} are important molecular targets of butyrate (17, 18), which is generated by the bacterial fermentation of dietary fibers (24). Therefore, the anticarcinogenic dietary factors may induce INK4 family gene expression as well as butyrate. We also elucidated that TPA, widely used as a tumor promoter and PKC activator, suppresses p18^{INK4c} gene expression, thereby promoting human cancer cell growth (19). This suggests that a mouse two-stage carcinogenesis model with TPA may reflect in some part human carcinogenesis related to the RB pathway. We are now planning to use this *in vitro* experimental system to screen for potential anticarcinogenic substances instead of using a two-stage carcinogenesis model in mice. Furthermore, we intend to examine whether the selected substances inhibit the progression from liver cirrhosis to hepatocarcinoma in humans in the future.

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