RUNX3 and Retinoic Acid Receptor β DNA Methylation as Novel Targets for Gastric Cancer Therapy

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Abstract: Methylation of CpG islands in many tumor suppressor genes with or without changes in histone acetylation is an important mechanism of gene silencing during the development of different types of cancer. There are at least two types of CpG island methylator phenotypes in intestinal- and diffuse -types of gastric cancer. Hypermethylation of the p16 and of hMLH1 promoters is preferentially found in intestinal-type gastric carcinoma, while concordant hypermethylation of the CDH1 and RAR-β2 promoters is predominantly associated with diffuse-type gastric carcinoma. Loss of RUNX3 and pS2 expression by promoter methylation is a common event in both types of gastric carcinoma. These results suggest that CpG island methylator phenotype may be one of the major pathways responsible for the development of the two types of gastric cancer and that RUNX3 and RAR-β2 may provide potential targets for therapeutic intervention to treat gastric cancer. Reactivation of RUNX3 and RAR-β2 by demethylating agents [e.g., 5-aza-2-deoxycytidine (DAC)] and histone deacetylase (HDAC) inhibitors [e.g., suberoylanilide hydroxamic acid (SAHA)] may be clinical useful for gastric cancer therapy with retinoids. This article will provide an overview of the molecular machinery that underlies two types of gastric cancer and focus on HDAC inhibitors that are potentially effective anticancer agents, not only for breast and prostate cancers but also for gastric cancer.

Keywords: RUNX3, RAR-β, Gastric cancer, DNA methylation, Histone acetylation, HDAC inhibitor.

INTRODUCTION

Gastric cancer is the most common cancer worldwide and is second only to lung cancer as a cause of cancer mortality. Most recent world estimates indicate that 798,000 new cases are diagnosed and 628,00 deaths occur annually from gastric cancer [1]. The highest incidence is still observed in Japan because of the remarkable increase in the aged population over 60 years old [2].

Although most gastric cancers arise distally from the antrum and pylorus, about 20% involve the cardia and fundus and approximately 10% involve the stomach diffusely.

There are several histological classifications of gastric cancer. For example, Lauren (1965) divided gastric cancer into two types, intestinal and diffuse, whereas the Japan Research Society for Gastric Cancer (JRSGC, 1999) classified it into five common types [3, 4]. The JRSGC classification is similar to that of the World Health Organization. In this article, we will use a two-type classification: the intestinal or well-differentiated type and the diffuse or poorly differentiated type.

The genetic and epigenetic alterations found in gastric carcinoma differ depending on the histological type of gastric cancer, indicating that different genetic pathways exist for the intestinal and diffuse types of carcinomas [5-7]. In addition, cancer-stromal interactions mediated by growth factor/cytokine receptor systems, which plays a pivotal role in morphogenesis, angiogenesis, invasion and metastasis, also differ between the two types of gastric carcinoma [8, 9].

Meta-analysis of epidemiological studies and animal models show that both intestinal and diffuse types of gastric cancer are equally associated with *Helicobacter pylori (H. pylori)* infection [10]. Such an infection has been shown to produce reactive oxygen and nitrogen species, as well as activate NF-kB, followed by up-regulation of the interleukins IL-1, IL-6, IL-8, TNF-α and the arachidonic acid metabolizing enzyme cyclooxygenase-2 (COX-2) [11]. *H. pylori* strain, patient age, exogenous or endogenous carcinogens and genetic factors such as DNA polymorphism have been implicated in two distinct major genetic pathways for gastric tumorigenesis [12].

This article reviews the genetic pathways of the two types of gastric cancer and propose RUNX3 and RAR-β2 DNA methylation as novel targets for gastric cancer therapy.

GENETIC PATHWAYS OF THE TWO TYPES OF GASTRIC CANCER

Multiple genetic and epigenetic alterations in oncogenes, tumor suppressor genes, cell cycle regulators, cell adhesion molecules and DNA repair genes, as well as genetic instability and telomerase activation, are responsible for tumorigenesis and progression of gastric cancer (Figs. **1** and **2**). Among them, inactivation of various genes including p16, hMHL1, CDH1, RAR-β2, pS2 and RUNX3 by DNA methylation is involved in two distinct major genetic pathways of gastric cancer [12-15]. Hypermethylation of the p16 and of hMLH1 promoters is preferentially associated

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1573-3947/05 $50.00+.00

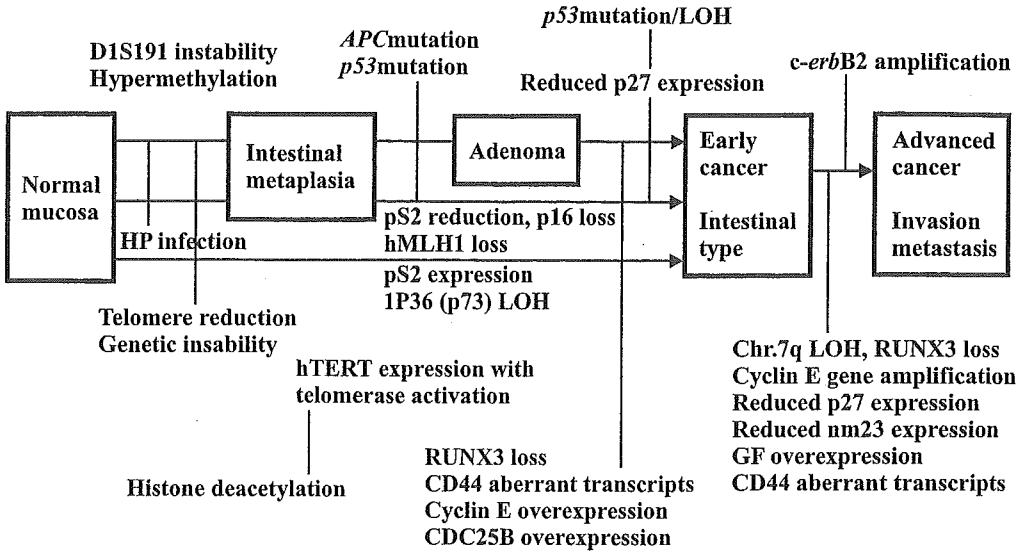

Fig. (1). Multiple genetic and epigenetic alterations during human gastric carcinogenesis (Intestinal type).

with well- differentiated or intestinal type gastric carcinoma, whereas concordant hypermethylation of the CDH1 and RAR-β2 promoters is predominantly detected in poorly differentiated or diffuse type gastric carcinoma. Loss or reduction of RUNX3 and pS2 expression by promoter methylation is a common event in both types of gastric carcinomas.

In addition to promoter methylation, acetylated histone H4 is reduced markedly in the majority of gastric carcinoma [12]. Histone H4 is progressively deacetylated from the early stage (precancerous lesions) to the late stage (invasion and metastastatic cancer) in the multi-step carcinogenesis process in the stomach. The similarity in the level of acetylated histone H4 in both the intestinal and diffuse types of gastric cancer indicate that histone H4 deacetylation may be involved in genesis of both types of gastric cancer.

In the multistep process of intestinal type carcinogenesis, the genetic changes can be divided into three pathways: an intestinal metaplasia→adenoma→carcinoma sequence, an intestinal metaplasia→carcinoma and carcinoma *de novo*. Infection with *H. pylori* may be a strong trigger for hyperplasia of hTERT-positive "stem cell" in intestinal metaplasia. Genetic instability and hyperplasia of hTERT positive stem cells precede replication error at the D1S191 locus, DNA hypermethylation at the D17S5 locus, pS2 loss, RAR-β2 loss, RUNX3 loss, abnormal transcripts of CD44 and p53 mutation. All of these changes accumulate in at least 30% of incomplete intestinal metaplasia and are common events in intestinal type gastric cancer. An adenoma→carcinoma sequence is found in about 20% of gastric adenoma with APC mutations. In addition to these events, p53 mutation and LOH, RUNX3 loss, reduced p27 expression, cyclin E over-expression and abnormal transcripts of c-met allow malignant transformation from the above precancerous lesions to intestinal type gastric carcinoma (Fig. 1). DCC loss, APC mutations, 1qLOH, p27 loss, reduced TGF-β receptor, reduced nm23 and c-erbB2 gene amplification play important roles in progression and metastasis. The *de-novo* gastric carcinogenesis involves LOH and abnormal expression of p73 gene that is responsible for the development of foveolar-type gastric cancer with pS2 expression.

On the other hand, LOH at chromosome 17p, mutation or LOH of p53, LUNX3 loss, and mutation or loss of E-cadherin are preferentially involved in the development of diffuse type gastric carcinoma. These genetic and epigenetic events occur simultaneously or in the relative short term in superficial gastritis induced by *H. pylori* infection. In addition to these alterations, gene amplification of K-sam and c-met, RUNX3 loss, 7qLOH, cyclin E gene amplification, p27 loss as well as nm23 loss are implicated in progression and metastasis, frequently associated with productive fibrosis. Mixed gastric carcinomas composed of intestinal and diffuse components exhibit some but not all of the molecular events described so far for each of the two types of gastric cancer (Fig. 2).

HISTONE ACETYLATION AND HDAC INHIBITORS

There is a functional link between DNA methylation and histone acetylation [16-18], as DNA methylation contributes to transcriptional silencing through several transcriptional repressive complexes including methyl-binding proteins (MBDs) and HDACs. In addition, DNA methyl transferase (DNTMT1) forms a repressive transcription complex with HDACs.

MBDs and HDACs cooperate *in vivo*, with a dominant effect of DNA methylation and histone acetylation, and repress expression of tumor suppressor genes such as p16 and p14 [18]. MBD2 is targeted for methylated regulatory regions and excludes the acetylated histone H3 and H4, resulting in a localized inactive chromatin [18]. We found that inactivation of various genes including p16, hMLH1, CDH1, RAR-β2, pS2 and RUNX3 by promoter methylation was frequently associated with gastric carcinoma [12-14]. These findings suggest that the expression of genes related

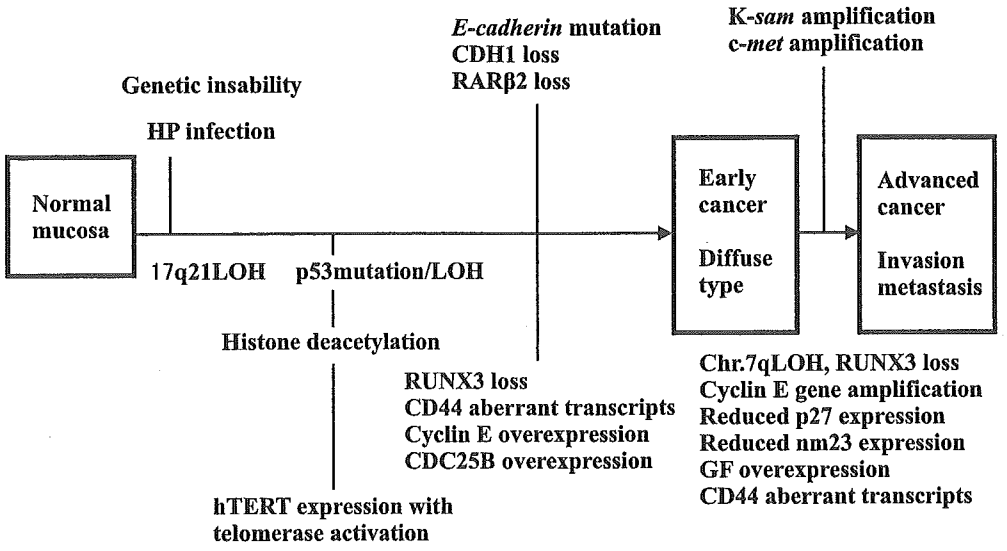

Fig. (2). Multiple genetic and epigenetic alterations during human gastoric carcinogenesis (Diffuse type).

to cell cycle, apoptosis, cell adhesion and DNA mismatch repair is suppressed via modification of histone acetylation in a manner linked to promoter methylation in gastric cancer. Moreover, low levels of histone acetylation frequently take place in the early stage of stomach carcinogenesis and confer tumor progression through chromatin remodeling. Alternatively, histone acetylation may provide a candidate anticancer target for gastric cancer.

HDAC inhibitors cause growth arrest, differentiation and apoptosis in a wide variety of cancer cells. We previously reported that the HDAC inhibitor trichostatin A (TSA), inhibits cell growth and induces apoptosis of gastric carcinoma cell lines [19]. TSA induced the expression of p21, CBP (histone acetyltransferase, HAT), Bak and cyclin E, while it reduced the expression of E2F-1, E2F-4, HDAC1 and the phosphorylated form of Rb protein. Therefore, a balance of HAT and HDAC regulates the acetylation status of histone H4, which plays a key role in transcription by modifying chromatin structure in gastric cancer.

A number of structurally diverse HDAC inhibitors have been identified, many of which are natural products [20]. They are classified into several chemical structural modifiers including; 1) hydroxamic acids (such as TSA) [21], oxamflatin [22], and hydroxamic acid-based hybrid polar compounds like suberoylanilide hydroxamic acid (SAHA) [23] and proxamide [24]; 2) short –chain fatty acids (such as butyrate [25]; 3) cyclic tetrapeptides containing an AOE moiety; 4) cyclic tetrapeptides without AOE and the depsipeptide FR-901228 [26]; 5) benzamides (such as MS-275 [27]). The practical use of these chromatin modifiers has been limited so far by their toxicity, but the development of new, less toxic agents such as SAHA might solve this problem.

SAHA inhibits HDAC activity at or below micro molar concentrations, both *in vitro and in vivo* [28]. SAHA induces complete inhibition of prostate cancer cell growth in nude mice with little or no detectable toxicity. Interestingly, the accumulation of acetylated histones in peripheral mononuclear cells, as well as in tumor tissue, has been found in patients treated with as little as 75 mg/m^2 SAHA as a 2-hour infusion. Recently, the Memorial Sloan-Kettering Cancer Center group reported that no significant toxicity was observed in patients with refractory solid tumors receiving oral doses of 200 mg/day SAHA for 4 weeks [29]. A second hybrid polar hydroxamic acid-based HDAC inhibitor, pyroxamide, is in phase I clinical trail. In addition, FR-901228 is also one of the potent compounds to enter clinical trial and is now in phase II development [30].

The DNA methyltransferase inhibitors 5-aza-2'-deoxycytidine (DAC, decitabine) also exhibits antiproliferative and proapoptotic effects. DAC enhances the ability of all-trans-retinoic acid to suppress the proliferation of human breast cancer cells [31]. Apoptosis of lung cancer cell lines is enhanced by DAC [16]. Former clinical studies have indicated that DAC was too toxic for therapeutic purpose. However, recent studies have demonstrated that low doses of DAC, which are sufficient to reverse methylation, could have clinical activity [32]. Judging from epigenetic alterations in gastric cancer and antitumor activity of HDAC inhibitors, SAHA and other HDAC inhibitors are potentially effective anticancer agents, not only for breast and lung cancers but also for gastric cancer.

RUNX3 PROMOTOR METHYLATION

RUNX3, a Runt domain transcription factor involved in TGF-β signaling, is a candidate tumor suppressor gene localized on chromosome 1p36, in a region commonly deleted in a variety of human cancers, including gastric cancer. RUNX gene family is composed of three members, RUNX1/AML1, RUNX2 and RUNX3, which encode the DNA-binding (α) subunits of the Runt domain transcription factor polyomavirus enhancer-binding protein 2 (PEBP2)/core-binding factor (CBF), a heterodimeric transcription factor [33, 34]. All three RUNXs play important roles in both normal developmental processes and carcinogenesis. RUNX1, which is required for definitive hematopoiesis, is the target of chromosome translocations in

leukemia. RUNX2, which is essential for osteogenesis, is mutated in the human disease cleidocranial dysplasia. RUNX3 is necessary for the growth inhibition of gastric epithelial cells, neurogenesis of the dorsal root ganglia and T-cell differentiation. The gastric epithelium of RUNX3 knockout mice shows hyperplasia, reduced rate of apoptosis and reduced sensitivity to TGF-β1, suggesting that the tumor suppressor activity of RUNX3 operates downstream of the TGF-β signaling pathway [34]. In fact, RUNX3 protein has been found to bind the Samd2 and Smad3 proteins.

Recent studies on regulation of RUNX3 expression in human cancers demonstrated that it is silenced by hypermethylation of the promoter CpG islands in 63% of gastric cancer as well as in 73% of hepatocellular carcinomas, 70% of bile duct cancer, 75% of pancreas cancer, 62% of laryngeal cancer, 46% of lung cancer, 25% of breast cancer, 23% of prostate cancer, 12 % of endometrial cancer, 2.5% of uterine cervical cancer, and 5% of colon cancer [15, 33, 35-37]. RUNX3 gene methylation is especially frequent in cancers, which develop in tissues of a foregut origin. Interestingly, RUNX3 methylation is found in 8% of chronic gastritis, 28% of intestinal metaplasia, and 27% of gastric adenoma, but not in chronic hepatitis B. These findings suggest that RUNX3 is a target for epigenetic gene silencing already at early stages of gastric carcinogenesis.

Acetylation mediated by the transcriptional co-activator p300 is known to activate transcription either by modifying histones in chromatin to produce a trascriptionally active conformation, or by directly targeting transcriptional activators such as p53, GATA-1, and E2F [38-40]. p300 interacts with RUNX1 and stimulates RUNX1-dependent transcription, whereas HDAC-6 interacts with RUNX3 and represses RUNX3-dependent transcription [41, 42]. More recently, it has been reported that p300 posses histone acetyltransferase (HAT) activity that can acetylate lysine residues in RUNX3 [43]. The p300-dependent acetylation of three residues protects RUNX3 from ubiquitin ligase Smurf-mediated degradation. The extent of the acetylation is increased by the TGF-β signaling pathway and down-regulated by HDAC activities, indicating that the level of RUNX3 protein is controlled by the competitive acetylation and deacetylation of three lysine residues [43]. This finding sheds light on a new mechanism for the posttranslational regulation of RUNX3 expression.

In addition to changes in histone acetylation, methylation and hemizygous deletion were reported as mechanisms of RUNX3 gene silencing in the majority of gastric, bile duct and pancreatic cancer cell lines [33, 35]. Moreover, some of the cancer cell lines expressing RUNX3 gene also show partial methylation of RUNX3 without hemizygous deletion, indicating the presence of both methylated and unmethylated alleles [33].

Treatment with TSA or DAC or both agents restores RUNX3 mRNA in all of gastric, bile duct and pancreatic cancer cell lines that originally lacked RUNX3 expression [33, 35]. Moreover, exogenous expression of RUNX3 in a gastric cancer cell line with a silenced RUNX3 gene dramatically inhibits the tumor growth in nude mice [33]. Taken together, tumor growth in gastric cancer may potentially be controlled by reactivation of silenced RUNX3 using HDAC inhibitors.

RAR PROMOTER METHYLATION

The antiproliferative and differentiative effects of retinoids are mainly mediated through the actions of six unclear receptors of the steroid hormone receptor superfamily [44]. These include the three retinoic acid receptors (RAR-α, -β, and -γ) as well as the three retinoid X receptors (RXR-α, -β, and -γ). All six receptors act as retinoic acid-dependent transcriptional activators in their RAR-RXR heterodimeric forms. Defects in expression of several of these receptors have been observed during human carcinogenesis. In particular, the majority of lung, head and neck, breast and prostate cancers show loss of RAR-β by promoter methylation [45-48]. We found evidence for RAR-β promoter hypermethylation in 74% of diffuse type and 38% of intestinal type gastric cancers. Interestingly, hypermethylation of the RAR-β and CDH1 promoters occurred concordantly [13]. We have recently found that RAR-β2 promoter was methylated in 67%, 56%, and 53% of HNSCC tumors, HNSCC cell lines, and microdissected oral leukoplakia specimens, respectively. Significantly higher RAR-β2 hypermethylation was found in tumor tissue compared to adjacent normal tissue. These results indicate that RAR-β2 silencing by methylation is an early event in head and neck carcinogenesis [49, 50].

Our previous study showed that the overexpression of RAR-β induced growth arrest and apoptosis in oral and gastric cancer cell lines, associated with a decrease of HDAC1 and up-regulation of p300, CBP and acetylated histone H4 [48-51]. In addition, the overexpression of RAR-β reduced expression of DNA demethylase and DNMT3 β, suggesting that DNA demethylase and DMNT as well as histone-associated proteins might be consistent with inactivation of RAR-β [51]. Similar findings on the role of RAR-β in the inhibition of growth have been reported in other cell lines [52]. These *in vitro and in vivo* results support the hypothesis that RAR-β functions as a tumor suppressor and that the loss of RAR-β is implicated in human carcinogenesis.

The human RAR-β gene is located on chromosome 3p24, a region that is often experiencing LOH in different types of cancer and is thought to harbor tumor suppressor gene(s). RAR-β is expressed as three isoform: β1, β2, and β4 [53]. Human RAR-β1 arises from promoter P1, while human RAR-β2 and β4 are transcribed from promoter P2 containing a high affinity retinoic acid-responsive element RARE [54]. RAR-β2 is characterized by a CpG island that contains potential binding sites for transcription factors such as AP-1, AP-2 and SP-1 [55]. Expression of transfected RAR-β2 mRNA is greatly reduced in a number of the above-mentioned carcinomas. A growing body of evidence indicates that methylation of the promoter P2 causes silencing of the RAR-β gene and that the anticancer effect of retinoic acid is primarily mediated by RAR-β2 [56]. Expression of transfected RAR-β2 in RAR-β2 negative cancer cells restores retinoic acid-induced growth inhibition, associated with low tumorigenicity [57]. Moreover, expression of RAR-β2 antisense induced an increased frequency of carcinomas in transgenic mice [58]. Taken

together, reactivation of the RAR-β2 tumor suppressor gene may be a strategy to restore RAR-β2 anticancer effects in gastric cancer in which RAR-β2 promoter is epigenetically silenced.

RUNX3 AND RAR-β2 GENES AS POTENTIAL TARGETS FOR GASTRIC CANCER

Inactivation of RUNX3 by promoter methylation is the most important common event in both types of gastric cancer, while inactivation of RAR-β2 by promoter methylation occurs in more than 70% of the diffuse type gastric cancer which are frequently associated with young patients under 40 years old and poorer prognosis. Therefore, RUNX3 and RAR-β2 may provide potential targets for therapeutic intervention to treat gastric cancer.

Several regulatory genes that are silent in cancer cells can be reactivated by combination of the demethylating agent DAC and HDAC inhibitor TSA [59]. RUNX3 is indeed reactivated by the combination of DAC and TSA in gastric cancer cell lines in which RUNX3 is silent [33]. Moreover, in all of bile duct and pancreatic cancer cell lines not expressing RUNX3, the gene is reactivated by DAC alone or by the combination of DAC and TSA [35]. The intriguing studies suggest that reactivation of RAR-β2 by combination of TSA and retinoic acid brings about significant growth inhibition of a variety of carcinoma cells both *in vivo and in vitro* [60]. Induction of RAR-β2 acetylation by TSA can restore RAR-β2 transcription from both unmethylated and methylated RAR-β2 promoters in RAR-β2 negative carcinoma cells [56]. We also found reactivation of RAR-β by TSA in gastric cancer cell line not expressing RAR-β [44].

RAR-β2 methylation in HNSCC cell lines was correlated with loss of RAR-β2 expression. The demethylating agent DAC restored RAR-β2 inducibility by retinoic acid in some of the cell lines with a methylated RAR-β2 promoter. In some cell lines, this effect was associated with increased growth inhibition after combined treatment with DAC and retinoic acid. These results indicate that DAC can restore RAR-β2 inducibility by retinoic acid in most cell lines, and that the combination of DAC and retinoic acid is more effective in growth inhibition than each agent alone [49].

These results overall suggest that reactivation of RAR-β2 and RUNX3 by HDAC inhibitors such as SAHA may be clinical useful for gastric cancer and HNSCC therapy in combination with retinoids.

ABBREVIATIONS

COX2 = Cylooxygenase-2
DAC = 5-Aza-2-deoxytidine
DNTMT1 = DNA methyltransferase
HAT = Histone acethyltransferse
HDAC = Histone deactylase
hTERT = Human telomerase reverse transcriptase
HNSCC = Head and neck squamous cell carcinoma
H. pylori = Helicobacter pylori
LOH = Loss of heterozygosity
MBDs = Methyl-binding proteins
PEBP2 = Polyomaviurs enhancer-binding protein 2
RAR = Retinioc acid receptor
SAHA = Suberoylanilide pylori hydroamic acid
TGF = Tansforming growth factor
TNF = Tumor necrosis factor
TSA = Trichostatin A

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Received: Septmber 29, 2004 Accepted: October 12, 2004

Cancer Immunol Immunother (2005) 54: 729–740
DOI 10.1007/s00262-004-0645-2

ORIGINAL ARTICLE

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G1P3, an interferon inducible gene 6-16, is expressed in gastric cancers and inhibits mitochondrial-mediated apoptosis in gastric cancer cell line TMK-1 cell

Received: 13 August 2004 / Accepted: 17 November 2004 / Published online: 1 February 2005

Abstract Expression of an interferon inducible gene 6-16, G1P3, increases not only in type I interferon-treated cells but also in human senescent fibroblasts. However, the function of 6-16 protein is unknown. Here we report that 6-16 is 34 kDa glycosylated protein and localized at mitochondria. Interestingly, 6-16 is expressed at high levels in gastric cancer cell lines and tissues. One of exceptional gastric cancer cell line, TMK-1, which do not express detectable 6-16, is sensitive to apoptosis induced by cycloheximide (CHX), 5-fluorouracil (5-FU) and serum-deprivation. Ectopic expression of 6-16 gene restored the induction of apoptosis and inhibited caspase-3 activity in TMK-1 cells. Thus 6-16 protein has anti-apoptotic function through inhibiting caspas-3. This anti-apoptotic function is expressed through inhibition of the depolarization of mitochondrial membrane potential and release of cytochrome c. By two-hybrid screening, we found that 6-16 protein interacts with calcium and integrin binding protein, CIB/KIP/Calmyrin (CIB), which interacts with presenilin 2, a protein involved in Alzheimer's disease. These protein interactions possibly play a pivotal role in the regulation of apoptosis, for which further detailed analyses are need. These results overall indicate that 6-16 protein may have function as a cell survival protein by inhibiting mitochondrial-mediated apoptosis.

Keywords Interferon inducible protein 6-16 · G1P3 · Mitochondria · Apoptosis · CIB · Survival gene · Bcl-2

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Introduction

Interferon inducible gene 6-16, G1P3, was first identified as one of the genes that are induced by interferon α and β [1]. It locates on chromosome 1p35 [2] and produces three types of mRNA by differential splicing between exon2 and exon3 [3]. The most abundant mRNA (B type) encodes a 14 kDa hydrophobic protein of 130 amino acids. Although extensive studies on the upstream regulatory region of 6-16 gene are well reported [4], little is known that the function or role of 6-16 protein in interferon treated cells. We isolated several cDNA clones that are expressed at higher level in senescent human fibroblasts than in young counterparts and found that one of these cDNA clones is 6-16 [5]. One of the characteristics of senescent human fibroblasts is that the cells do not proliferate but are viable for relatively long period of time, usually for more than a year in a

culture dish, without apparent apoptosis. SV40 large T antigen overcomes normal senescent pathway by inactivating some proteins, which are associated with cell proliferation, such as tumor suppressor gene p53 and pRB proteins. Interestingly, 6-16 is still expressed in SV40-transformed cells after extending their life-span [6]. 6-16 expression may be independent from p53 pathway and/or pRB pathway. Interestingly, we found that human senescent fibroblasts express 6-16 by producing interferon-β by autocrine mechanism. Interferon regulatory factor 1, IRF-1, is a major regulator of the interferon signaling pathway. Treatment of anti-IRF-1 antibody to human senescent cells or life-extended SV40-transformed fibroblasts resulted in down-regulation of 6-16 expression [6]. It is still unknown how senescent cells are protected from apoptosis, because senescent cells could be attached to the dish and are viable for more than 1 year with just a medium change; hence, it is believed that some anti-apoptotic gene may be protecting the senescent cells from apoptosis.

Attenuation of apoptosis appears recession for establishment and maintenance of transformed phenotype [7]. We found that 6-16 gene is expressed at a high level in immortalized cells and in gastrointestinal tumor cells. These data suggest that an increase in expression of 6-16 is associated with attenuation of apoptosis.

Mitochondria is an important organelle for the control of apoptosis, in addition to the role as the center of energy metabolism, and influence the commitment of cell death by regulating the mitochondrial permeability and membrane potential [8–11]. Bcl-2, an anti-apoptotic protein, is known to be located on mitochondria and expressed at high level in some tumor cells and tissues [11]. We report here that 6-16 is relatively expressed in cancer cells and tissues, and 6-16 is a novel anti-apoptotic protein located in the mitochondria and can be a new target for cancer chemotherapy and mitochondrial diseases.

Materials and methods

Northern blotting and RT-PCR

RNA isolation and Northern blotting were performed as described previously [6]. All 6-16 splice valiants are recognized by the probe for Northern analysis. RT-PCR was performed according to the protocol with the Thermoscript one-step RT-PCR system (Invitrogen, USA). For the detection of B and C type of 6-16 spliced valiants, we used 5′-GGGTGGAGGCAGGTGAGAATGCGG-3′ as the forward primer and 5′-TGACCTTCATGGCCGTCGGAGGAG-3′ as the reverse primer. Samples were incubated at 50°C for 30 min and denatured at 94°C for 2 min, and then cycled 32 times with 30 s at 94°C and 30 s at 62°C followed by a final extension step of 5 min at 68°C. GAPDH was used for internal control for validating RNA amounts as described before [12].

In situ mRNA hybridization analysis

In situ mRNA hybridization (ISH) was performed as described previously [13] with minor modification. Briefly, an interferon inducible gene 6-16-specific oligonucleotide probe was designed complementary to the 5′-end of human 6-16 mRNA transcript (GenBank NM022873). The DNA oligonucleotide sequence 5′-CGCCGCCCCCATTCAGGA-3′ was of the antisense orientation and hence complementary to 6-16 mRNA. To verify the integrity and lack of degradation of mRNA in each sample, we used a d(T) oligonucleotide. All DNA probes were synthesized with six biotin molecules (hyperbiotinylated) at the 3′-end via direct coupling using standard phosphoramidite chemistry (Research Genetics, Huntsville). ISH was carried out using the Microprobe manual staining system (Fisher Scientific, Pittsburgh). A positive reaction in this assay stained red. Control for endogenous alkaline phosphatase included treatment of sample in the absence of the biotinylated probe and use of chromogen alone. To check the specificity of the hybridization signal, the following controls were used: RNAse pretreatment of tissue sections, substitution of the antisense probe with a biotin-labeled sense probe, and competition assay with unlabeled antisense probes. No or markedly decreased signals were obtained after either of these treatments.

Antibody

Rabbit polyclonal antibody against human 6-16 was raised against synthetic peptide (YATHKYLDSEEDEE) corresponding to amino acid residues 117–130 of human 6-16, and was purified by MAbTrap GII affinity chromatography kit (Amersham Bioscience, USA). This antibody was sufficient for immunoblotting but insufficient for immunoprecipitation and immunostaining of 6-16 protein. Another rabbit polyclonal anti-human 6-16 antibody (OT904-1A) was also prepared against synthesis peptides (VEAGKKKCSESSDSG) corresponding to amino acid residues 21–35 of human 6-16. This antibody was sufficient for immunostaining. Mouse monoclonal anti-human CIB antibody was kindly provided by Leslie V. Parise. Anti-cytochrome c antibody (clone 7H8.2C12 which recognizes the denatured form of human, mouse and rat cytochrome c; BD Pharmingen, USA), monoclonal anti-human Bcl-2 antibody (clone124, Upstate Biotechnology, USA) and polyclonal anti-Bax antibody (Upstate Biotechnology) were used for immunoblot and immunoprecipitation. Anti-cytochrome c antibody (clone 6H2.B4 which recognizes the native form of human, mouse and rat cytochrome c; BD Pharmingen) was used for immunostaining. Polyclonal anti-GST rabbit antibody (New England Biolabs, USA) and polyclonal anti-MBP antibody (kindly provided by Dr. M. Nakata, Sumitomo Electric Industries, Japan) were used for immunoblot.

Cell culture, PI staining, caspase-3 activity assay, and mitochondrial membrane potential assay

The 0.8 kb fragment encoding full length 6-16 was inserted in the pCXN vector, which was digested with Xho I and blunted with klenow fragment. To establish stable transfected TMK-1 cells expressing 6-16 (TMK-1-6-16), TMK-1 cells were transfected with pCXN/6-16 vector and were selected for neomycin resistance with 200 μg/ml Geneticin (Invitrogen, USA). Gastric cancer cell lines, TMK-1, TMK-1-6-16 and MKN-28 were cultured at 37°C and 5% CO_2 in RPMI 1640 (Invitrogen) supplemented with 10% FCS and Antibiotic-Antimycotic (Invitrogen). A normal human fetal fibroblast strain, TIG-3, was cultured in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% FCS and Antibiotic-Antimycotic (Invitrogen).

Apoptosis was induced by actinomycin D (10, 20 and 40 μg/ml) for 24 h, cycloheximide (10, 30 and 100 μM) for 6 h, H_2O_2 (0.5, 5 and 50 μM) for 6 h, etoposide (200, 400 and 600 μM) for 24 h, bleomycin (100, 200 and 400 μg/ml) for 24 h, 5-FU (100, 200 and 400 μg/ml) for 24 h, aphidicolin (100, 200 and 400 μM) for 24 h or serum-deprivation for 60 h. Cells were collected and stained with PI-RNase solution (BD Biosciences, USA), and were analyzed for DNA content by Flow cytometry on a FACS Calibur. For DNA ladder analysis, apoptotic DNA was extracted by lysis buffer (50 mM Tris-HCl pH7.5/ 20 mM EDTA/1% NP40), and run on 2% agarose gel.

For Caspase-3 activity analysis, cells were grown in six-well plates overnight, and were treated with CHX (10 and 30 μM) for 6 h or with 5-FU (80 μg/ml) for 24 h. Cells were collected and incubated with PhiPhiLux substrate buffer (OncoImmunin Inc., Gaithersbury, MD, USA) for 60 min at 37°C in 5% CO_2 incubator, and were analyzed by Flow cytometry on a FACS Calibur.

For mitochondrial membrane potential analysis, cells were grown in six well plate overnight, and were treated with CHX (10 and 30 μM) or 5-FU (50 and 100 μg/ml) for an hour. Cells were collected and incubated with Mitosensor reagent buffer (Clontech) for 30 min at 37°C in CO_2 incubator, and were analysis by Flow cytometry on a FACS Calibur.

Immunofluorescence analysis

Cells grown on eight-well Lab-Tek chamber slides (Nalge Nunc International, Napaperville, IL) were fixed in 4% paraformaldehyde / PBS (pH 7.4) for 20 min and permeabilized in blocking buffer (0.2% Triton X-100/ 3% BSA/PBS) for 30 min. The cells were incubated for 1 h at room temperature with mouse anti cytochrome c monoclonal antibody (6H2.B4) diluted 1:100, or with rabbit anti-human 6-16 polyclonal antibody (OT904-1A) diluted 1:100 in blocking buffer. The cells were washed four times in PBS and incubated with Alexa FluorTM 488 goat anti-mouse IgG conjugate (Molecular Probes, USA) or with Alexa FluorTM 488 goat anti-rabbit IgG conjugate (Molecular Probes) diluted 1:200 in blocking buffer for an hour at room temperature. Immunofluorescent staining was analyzed using a confocal laser-scanning microscope (LSM510, Carl Zeiss, Germany).

Mitochondria were labeled in intact cells with Mito-Tracker CM-H_2XRos (Molecular Probes). Cells were incubated in the MitoTracker medium (final concentration 500 nM) for 45 min before finishing to treat with CHX or 5-FU. Then, the cells were fixed, permeabilized and double-labeled with anti-cytochrome c antibody (6H2.B4) as described above.

Cell fractionation, immunoprecipitation and immunoblot analysis

For isolation of cellular fraction, cells were suspended in sucrose-supplemented extraction buffer (SCEB, 300 mM sucrose, 10 mM HEPES pH 7.4, 50 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$, 1 mM DTT, protease cocktail), left on ice for 30 min, and homogenized by 70 strokes in an ice-cold Dounce homogenizer. Unbroken cells and nuclei were pelleted by centrifugation for 10 min at 2,000×*g*, and the supernatant was further spun at 13,000×*g* for 20 min to separate mitochondria-rich fraction from cytosol fraction. The pellet was resuspended in 50 μl of SCEB (mitochondria fraction), and the supernatant was cytosolic fraction.

For co-immunoprecipitation experiments, cells were lysed in NP-40 lysis buffer (50 mM Tris-HCl, pH7.4, 120 mM NaCl, 1% NP-40) containing protease inhibitor cocktail (Roche, USA). Antigen-antibody reaction was performed by incubating 0.5 ml (500 μg protein) of the cell extract with rabbit anti-human 6-16 antibody (117–130), mouse anti-CIB antibody, rabbit anti-Bax antibody or mouse anti-Bcl-2 antibody overnight at 4°C. The immunocomplex were incubated with 50 μl protein-G-Sepharose for 3 h at 4°C, and the beads were washed three times with NP-40 lysis buffer and boiled in Laemmli buffer.

For immunoblot analysis, the proteins were separated on 13% or 15% SDS-PAGE, and transferred to Immobilon-P (Millipore) and immunoblotted with anti-6-16 antibody (117–130), anti-CIB antibody, anti-Bcl-2 antibody, anti-Bax antibody or anti-cytochrome c antibody (7H8.2C12) diluted 1:500. The signal was detected using ECL-Plus (Amersham Bioscience).

Yeast two-hybrid assay

Dr Y. Takai (Osaka University, Suita, Japan) kindly supplied yeast L-40 strain and pBTM116/HA for yeast two-hybrid screening. A strain of L40 carrying pBTM116/6-16 was transformed with pGADGH HeLa cDNA library (Clontech). Approximately 1×10^6 transformants were screened for the growth on SD medium

plated lacking Trp, Leu, and His as evidenced by transactivation of a LexA-HIS3 reporter gene and His prototrophy. His+ colonies were scored for β-galactosidase activity. Plasmids harboring cDNAs were recovered from positive colonies and introduced by electroporation into *E. coli* HB101 on the M9 plate lacking Leu. Then the plasmids were recovered from HB101 and transformed again into L40 containing pBTM116HA/6-16. The nucleotide sequences of plasmid DNAs were determined.

Interaction of proteins in vitro

6-16-pGEX-2T (Amersham Bioscience) or CIB-pMAL-C2 (New England Biolabs) expression vectors were constructed to produce GST-6-16 and MBP-CIB proteins. GST-6-16 and MBP-CIB proteins were purified from *E. coli* transformed with their expression vectors treated with 0.1 M IPTG by using amylose resin (New England Biolabs) or glutatione Sepharose 4B (Amersham Bioscience). GST-6-16 protein (20 pmol) was incubated with MBP-CIB protein (40 pmol) in 40 μl of reaction buffer (20 mM Tris-HCl pH7.5, 1 mM DTT and 0.05% CHAPS) for 1 h at 4°C. After glutathione Sepharose 4B (Amersham Bioscience) was added and further incubated for 1 h, the precipitates were washed three times and subjected to immunoblot analysis using anti-MBP and anti-GST rabbit polyclonal antibody.

Results

6-16 was expressed in gastric cancer cells and tissues

We examined expression levels of 6-16 mRNA in eight gastric cancer cell lines (MKN-1, MKN-7, MKN-28, MKN-45, MKN-74, TMK-1, HSC-39 and KATO III) by Northern blot analysis. Seven out of eight gastric

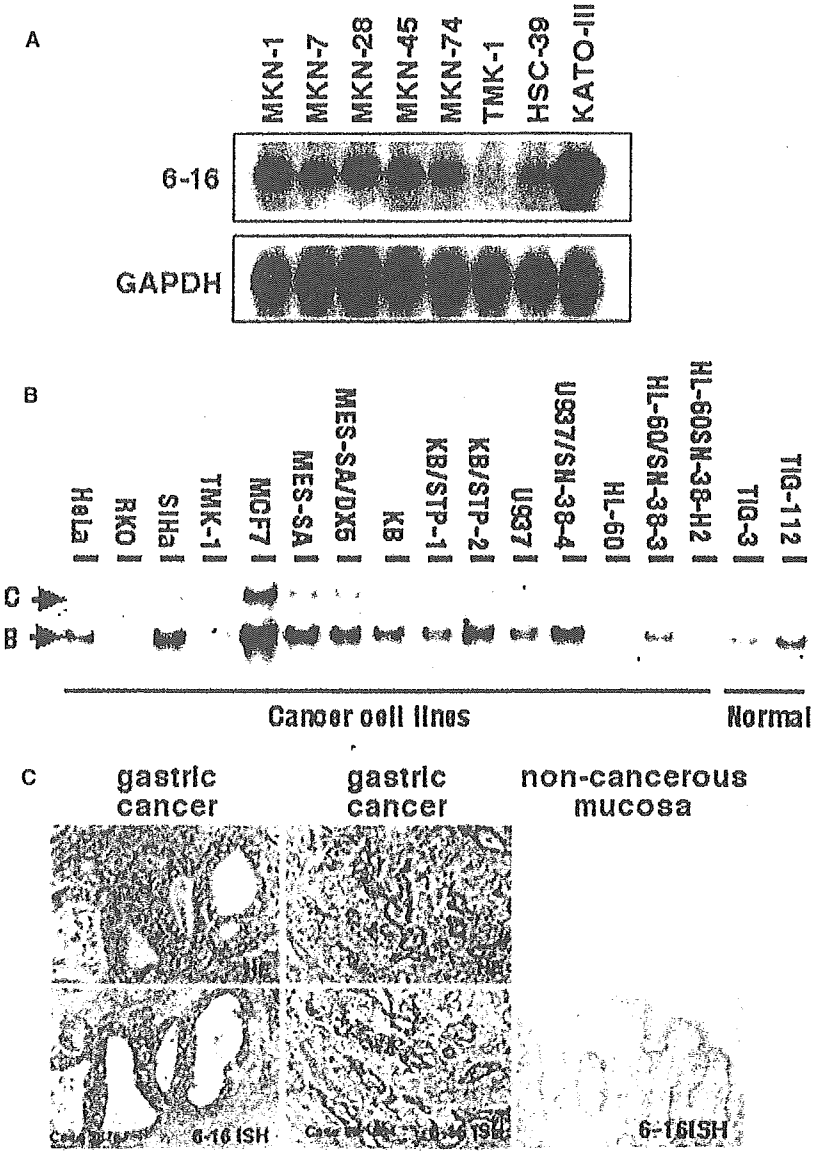


Fig. 1 Expression of 6-16 mRNA in gastric cancer cells and carcinoma tissues. **a** Expression of 6-16 mRNA was detected by Northern blot analysis with 10 μg total RNA in eight gastric cancer cell lines. Hybridization of a G3PDH (control) probe to the same filter membrane is depicted in the *lower panel*. **b** Expression of 6-16 mRNA was detected by RT-PCR analysis. 0.2 μg of RNA were used for RT-PCR reaction. PCR products were run on 6% acrylamide gel electrophoresis and staining with CYBR Green I nucleic acid staining. **c** Surgically resected adenocarcinomas of the stomach were examined on in situ hybridization using the microprobe manual staining system (Fisher Scientific). 6-16-specific anti-sense oligonucleotide DNA probe (5′-GCA CGC CGC CCC CAT TCA GGA TCG CAG-3′) was designed. *lower panel* 6-16 in situ hybridization. *upper panel* hematoxylin-eosin (HE) staining

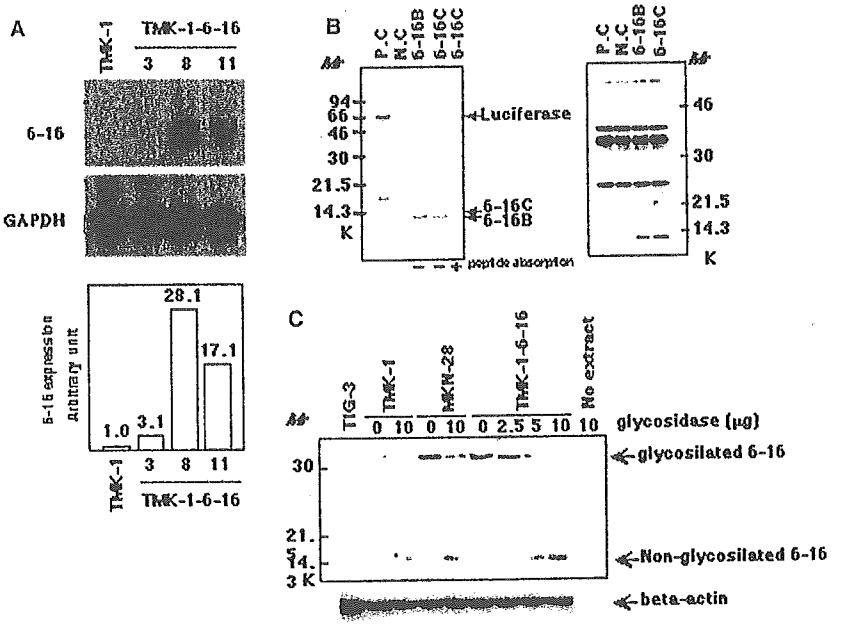
carcinoma cell lines expressed 6-16 mRNA, while TMK-1 cells showed very low levels (Fig. 1a). p53 mutation (codon 173, GTG to ATG) was found in TMK-1 cells [14]. Other gastric cell lines also have p53 mutation excluding MKN-45 [14]. However, there is no correlation between p53 mutation status and 6-16 expression levels. TMK-1 cell is sensitive to apoptosis compared with other gastric cancer cell line used in Fig. 1a. In the other cancer cell lines that were frequently used for apoptosis research, colorectal cancer cell line RKO, which is sensitive to apoptosis, express low levels of 6-16. In contrast, high levels of 6-16 expression was found in breast cancer cell line MCF-7 cell, which is resistant to apoptosis (Fig. 1b). Interestingly, HL-60 cell line does not have a significant level of 6-16 expression, but anti-cancer drug resistant clone of HL-60 cells do have high levels of 6-16 expression (Fig. 1b). Human uterine sarcoma cell line, NES-SA and the multiple drug-resistant uterine sarcoma cell line, MES-SA/DX7 also express high levels of 6-16, but there is significant difference between these two cell lines. Taken together, there is good correlation between 6-16 expression levels and resistant to apoptosis. We next studied the expression of 6-16 mRNA in primary gastric carcinomas by in situ mRNA hybridization (ISH) (Fig. 1c). In almost all of these tumors, higher expression of 6-16 mRNA was shown in cytoplasm of the tumor cells in comparison with the corresponding non-neoplastic mucosas. We also recognized weak signal in stromal fibroblast cells and fundic gland cells, but not in muscular tissues of the gastrointestinal tracts (Fig. 1c).

6-16 is 32 kDa glycosylated protein

To elucidate the function of 6-16, we established 6-16 expressing TMK-1 cells by transfection of 6-16 cDNA. After selection with G418, we isolated several clones and examined the expression of 6-16. Among them, clone 8 expresses the highest level of 6-16 mRNA (Fig. 2a). Isolated clones in order of 6-16 expression levels are No. 8, 11 and 3. Clone 11 also expresses 6-16, but slightly lower than clone 8. Expression of 6-16 in clone 3 is lowest in these clones. We used these three types of clones to examine the function of 6-16. Unless otherwise indicated, TMK-1-6-16 clone 8 is referred to as TMK-1-6-16, and is used for further examination in comparison with the parental TMK-1.

To analyze 6-16 protein expression, we generated 6-16 polyclonal antibody that was raised against synthetic peptide (YATHKYLDSEEDEE) corresponding to amino acid residues 117–130 of human 6-16 and was purified by MAbTrap GII affinity chromatography. Theoretical molecular weight of 6-16 is about 14 kDa protein by using ExPASy molecular biology database server. By using in vitro transcription/translation

Fig. 2 Localization of 6-16 at mitochondria. a Total RNA was isolated from each cell line, and 10 μg of each was used for Northern blot analysis. Semi-quantitative analysis of 6-16 mRNA level of autoradiographs was performed using the public domain NIH Image program. The units are arbitrary, and were calculated based on the expression of 6-16 mRNA in TMK-1 cells as 1.0. Hybridization of a G3PDH (control) probe to the same filter membrane is depicted in the *lower panel*. b In vitro transcription/translation products were synthesized from luciferase control (positive control: P.C), pZero/Kan (negative control: N.C) and 6-16-pZero/Kan expression vector by using TNT coupled Wheat Germ Extract System (Promega). Incorporation products of ^{35}S-methionine were detected by autoradiography after separating by 15% SDS-PAGE (*left panel*). Non-radioactive products were detected by immunoblot analysis with anti-6-16 antibody (*right panel*). C, Extracts from TIG-3, TMK-1, TMK-1-6-16 and MKN-28 cells were incubated with different concentrations of glycosidase at 37°C for 48 h, and detected by immunoblot analysis with anti-6-16 antibody



experiments from 6-16 cDNA, 14 kDa protein was detected both by autoradiography of ^{35}S-methionine-incorporated products and immunoblotting using a specific antibody (data not shown). By using cell extract from human culture cell, we could not detect 14 kDa 6-16 protein by Western blotting analysis, but 32 kDa protein was detected in 6-16 highly expressing cells such as TMK-1-6-16 and MKN-28. TIG-3 and TMK-1 cells are low or no 6-16 expression by immunoblotting analysis (Fig. 2b). Thus, it is speculated that 6-16 has some protein modifications such as phosphorylation or glycosilation. By the ProDom database analysis (motif), 6-16 protein has multiple possible glycosylation sites including 15 serine residues and 5 threonine residues. To clarify this possibility that 6-16 protein is glycosilated, we treated glycosidase to the cell lysates that were isolated from TMK-1 and TNK-1-6-16 cells, and performed SDS-PAGE and immunoblotting. By digestion with glycosidase, the shift of 32 kDa protein band to 14 kDa band was observed with increasing enzyme concentration, indicating an extensive glycosylation of the native 6-16 protein (32 kDa) in cells (Fig. 2b). These results suggest that 6-16 protein is 34 kDa glycosilated protein.

Apoptosis was attenuated in cells expressing 6-16 protein at high level

6-16 protein is expressed at high levels in both tumor cells and senescent cells. As shown in Fig.1, most of the gastric cancer cell lines expressed 6-16 at high levels except TMK-1 cell. So, we examined whether overexpression of 6-16 protein has an effect on the apoptosis. Out of eight inducers of apoptosis field such as actinomycin D, cycloheximide (CHX), H_2O_2, etoposide, bleomycin, 5-fluoro-2′-deoxyuridine(5-FU), aphidicolin or serum-deprivation, only CHX, 5-FU and serum-deprivation induced apoptosis in TMK-1. However, in TMK-1-6-16 cells which are overexpressing 6-16 protein, apoptosis induced by CHX or 5-FU were significantly inhibited by subG0 analysis using Flow cytometry (Fig. 3a). The other five inducers of apoptosis did not induce apoptosis in either TMK-1 and TMK-1-6-16 cells (data not shown). The increase in subG0 fraction was observed in the dose-dependent manner after CHX or 5-FU treatment in TMK-1, but not in TMK-1-6-16 cells (Fig. 3a). TMK-1-6-16 (clone 3) and TMK-1-6-16 (clone 11) expressed less 6-16 (Fig. 1b) and was less resistant to CHX or 5-FU induced apoptosis than TMK-1-6-16

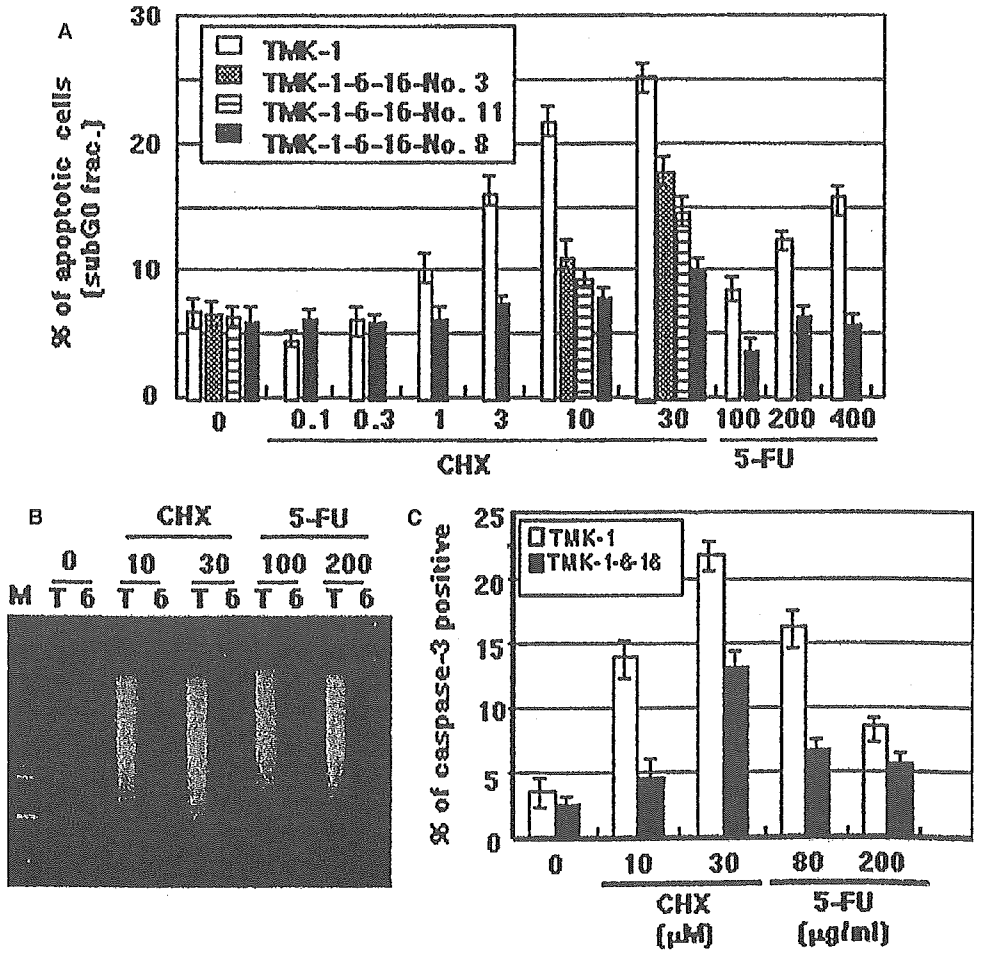


Fig. 3 6-16 inhibits apoptosis and caspase-3 activity by CHX and 5-FU. a TMK-1 and TMK-1-6-16 cells were treated with CHX for 6 h or with 5-FU for 24 h. Cells were collected and stained with PI-RNase solution, and were analyzed for DNA content by Flow cytometry. Percentage of cells with subG0 DNA content was calculated. b TMK-1 (T) and TMK1-6-16 (6) cells were treated with CHX (10 and 30 μM) for 6 h or with 5-FU (100 and 200 μg/ml). NP-40 (1%) extracted DNA was purified and run on 2% agarose gel. M indicates 100 bp DNA ladder of size marker. c TMK-1 and TMK-1-6-16 cells were treated with CHX (10 and 30 μM) for 6 h or with 5-FU (80 μg/ml) for 24 h. Cells were collected and were measured for caspase-3 activity by PhiPhilux G1D2 Kit by using Flow cytometry. Cells with FL-1H amp gain over 180 were referred to as caspase-3 positive cells

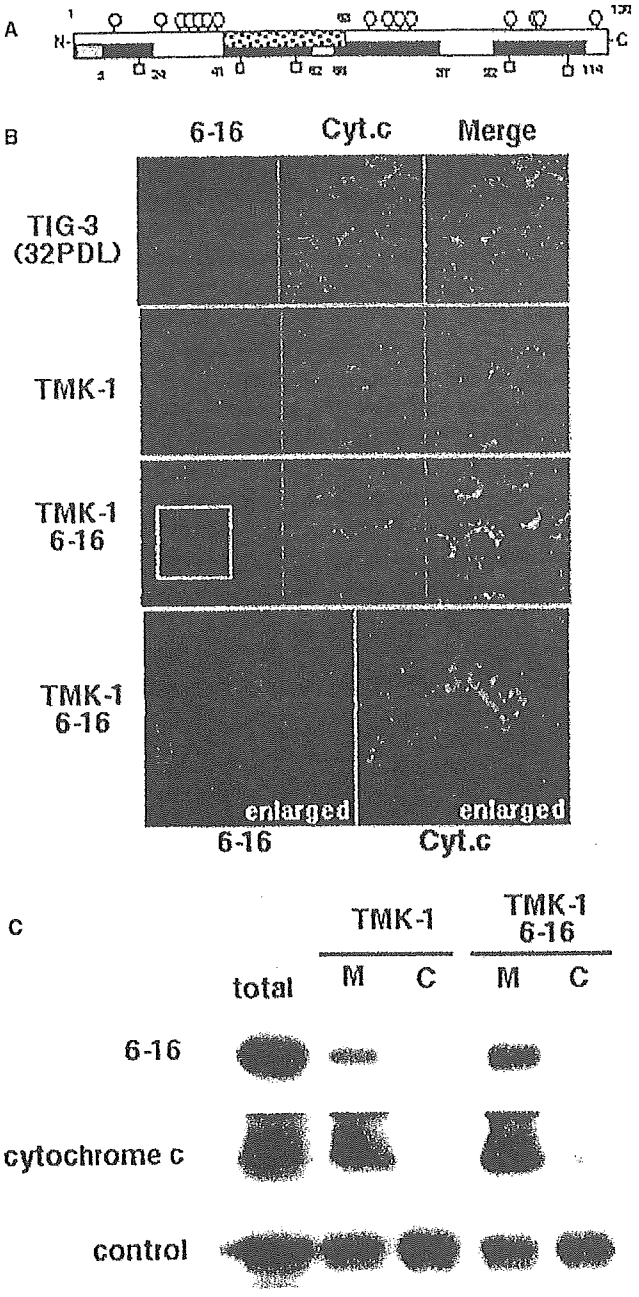


Fig. 4 6-16 co-localized with cytochrome c at mitochondria. **a** 6-16 protein has possible glycosylation sites (15 serine residues and 5 threonine residues) and possible mitochondria localization site [one intramitochondrial target sequence, one APOLAR (apolar signal of intramitochondria sorting) signal domain and four mitochondrial helices domains]. *Open circle* serine residues, *square* threonine residues, *black bar* transmembrane helices domain, *gray bar* intramitochondrial target sequence, *dotted bar* APOLAR signal domain. **b** TIG-3 (32PDLs), TMK-1 and TMK-1-6-16 cells were fixed with 4% paraformaldehyde, and permeabilized in 0.3% Triton-X100. 6-16 (red) and cytochrome c (green) were detected by immunofluorescence. Co-localization was seen by merged image of the green and red signals (yellow). **c** Localization of 6-16 protein was detected by immunoblot analysis of whole-cell lysates (total), mitochondria fraction (M) and cytosolic fraction (C). All fractions were adjusted to 20 μg proteins and analyzed by immunoblotting with anti-6-16 antibody or anti-cytochrome c antibody

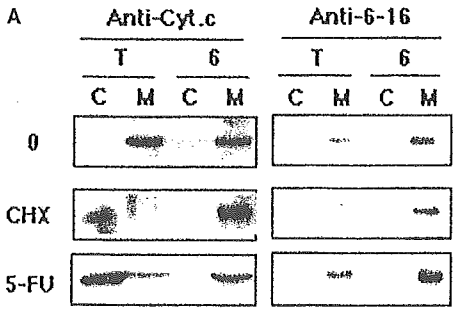
(Fig. 3a). MCF-7 expressed 6-16 at high levels and was well known to apoptosis-resistant cancer cell line (Fig. 1b). In contrast to MCF-7, RKO that is well known to apoptosis sensitive cancer cell line expressed 6-16 at low levels (Fig. 1b). In addition, TMK-1 is most sensitive cancer cell line in gastric cancer cell line which showed in Fig. 1a (data not shown). Taken together, there is good correlation between 6-16 expression levels and resistance of apoptosis. The amount of DNA extracted in NP-40 buffer, called DNA ladder, was increased in TMK-1 cells after CHX or 5-FU treatment, but not in TMK-1-6-16 (Fig. 3b). The extracted DNA showed a characteristic ladder found in DNA from apoptotic cells (Fig. 3b). DNA ladder formation is dose dependent manner (data not shown). These results indicate that 6-16 protein attenuates apoptosis induced by CHX or 5-FU in TMK-1 cell.

6-16 localized at mitochondria and co-localized with cytochrome-c

The PSORT II and TMpred database analysis have shown that 6-16 protein has transmembrane helices, a mitochondria targeting sequence and an intramitochondrial sorting signal(Fig. 4a). It was quite interesting that, from database analysis on amino acid sequence, 6-16 protein has four transmembrane domains, a mitochondrial targeting sequence and an intra-mitochondrial sorting signal (Fig. 4a). So, it is possible that 6-16 is localized at mitochondria. To check this possibility, we have examined subcellular localization of 6-16. These data led us to examine intracellular localization and possible role on apoptosis of 6-16 protein. By immunofluorescence, 6-16 protein was co-localized with cytochrome c in both TMK-1 and TMK1-6-16 cells (Fig. 4b). Essentially, the same results were obtained using a colon cancer cell line, RKO, and a breast cancer cell line, MCF-7 (data not shown). 6-16 protein was not detected in normal human fibroblast, TIG-3, at 32 PDL (Fig. 4b) but was weakly detected at 75 PDL (data not shown), consistent with the previous results that the expression of 6-16 mRNA increased with cellular senescence [6]. Mitochondrial localization of 6-16 protein, as well as cytochrome c, was confirmed by immunoblotting of subcellular fractionations (Fig. 4c). Taken together with these data, 6-16 is mainly localized in mitochondria and co-localized with cytochrome c.

6-16 protein inhibits cytochrome c release and reduction of mitochondrial membrane potential

To search for the mechanism of anti-apoptotic function of 6-16, we first examined caspase-3 activity. The percentage of cells positive for caspase-3 activity determined by Flow cytometry was remarkably increased in TMK-1 cells after the treatment with CHX and 5-FU


A
Anti-Cyt.c
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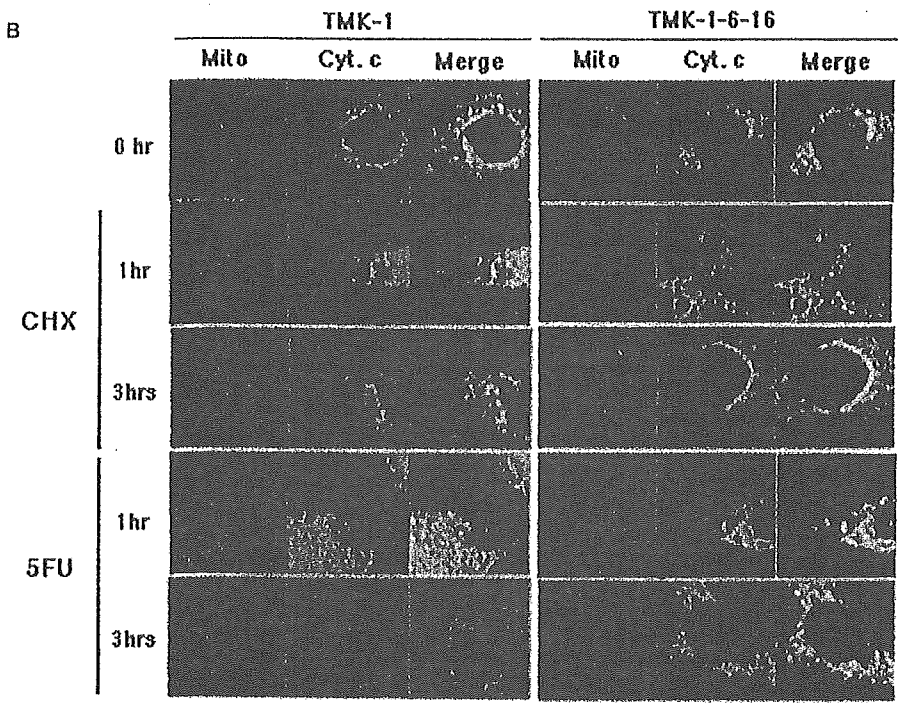
B
TMK-1
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Mito
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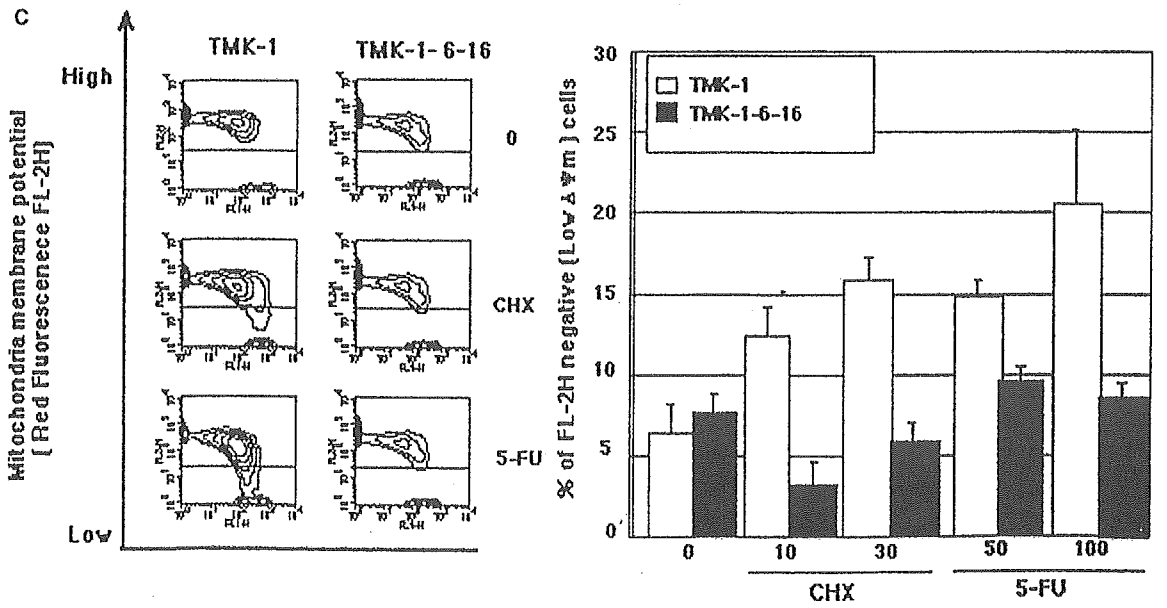
C
TMK-1
TMK-1-6-16
High
Low
Mitochondria membrane potential
[Red Fluorescenece FL-2H]
0
CHX
5-FU
TMK-1
TMK-1-6-16
% of FL-2H negative [Low ΔΨm] cells
0
5
10
15
20
25
30
0
10
30
50
100
CHX
5-FU

◀

Fig. 5 6-16 protein inhibits release of cytochrome c and reduction of mitochondrial membrane potential. **a** For immunofluorescence, cells were treated with CHX (10 μM) and 5-FU (200 μg/ml), labeled with MitoTracker CM-H_2XRos (Red) for 45 min, and fixed with 4% paraformaldehyde, permeabilized in 0.3% Triton-X100, and stained with anti-cytochrome c antibody (green). **b** TMK-1 and TMK-1-6-16 cells were treated with CHX (10 μM) for 6 h or with 5-FU (80 μg/ml) for 24 h. Cells were collected and separated to mitochondria (M) and cytosolic (C) fractions. Twenty micrograms of protein was used for immunoblot analysis with anti-cytochrome c antibody or anti-6-16 antibody. **c** TMK-1 and TMK-1-6-16 cells were treated with CHX (10 and 30 μM), 5-FU (50 and 100 μg/ml) for an hour. Cells were collected and incubated with Mitosensor reagent buffer (Clontech) for 30 min at 37°C in CO_2 incubator, and Δ Ψ m was analyzed by Flow cytometry (*left panel*). Cells with fluorescence intensity below 30 were referred to as FL-2H negative cells (*right panel*). *Error bars* represent standard deviations from three independent samples

(Fig. 3c). The increase was seen in TMK-1-6-16 only at high concentration of CHX at 30 μg/ml. These results suggested that 6-16 inhibited the caspase-3 dependent apoptotic pathway.

The apoptosis related-proteins (e.g. cytochrome c, caspase-2, −3, −9, Hsp10, Smac/DIABLO and AIF) are released from mitochondria into the cytosol during apoptosis [10, 15], and the activation of the caspase cascade is dependent on the release of cytochrome c from mitochondria [10, 16, 17]. Immunoblot analysis demonstrated that in TMK-1 cells, cytochrome c in mitochondria was released into cytosolic after treatment with CHX or 5-FU (Fig. 5a). In TMK-1-6-16 cells, however, cytochrome c was detected in mitochondria after either treatment (Fig. 5a). 6-16 protein remained in mitochondria fraction in both TMK-1 and TMK-1-6-16 cells after treatment with either CHX or 5-FU (Fig. 5a). Release of cytochrome c was also monitored by immunofluorescence staining. In non-apoptotic TMK-1 cells, the staining patterns of mitochondria (red) and cytochrome c (green) were completely overlapped (merge: yellow) (Fig. 5b). After treatment with CHX or 5-FU, the signals of cytochrome c were distinct from that of mitochondria in TMK-1 cells (Fig. 5b left panel), whereas these two signals always overlapped in TMK-1-6-16 cells (Fig. 5b right panel). When TMK-1 cells were treated with CHX or 5-FU for an hour, cytochrome c was diffusely observed in cytosol. After 3 h, cytochrome c signal appeared in granules or grains in cytosol distinct from mitochondria, indicating that cytochrome c in cytosol might interact with the protein complex including Apaf-1/pro-caspase-9 protein [16].

As the release of cytochrome c from mitochondria is well associated with depolarization of mitochondrial membrane potential (Δ Ψ m) [8–10], we next measured mitochondrial membrane potential at single cell level by Flow cytometry after staining cells with JC-1 or Mito-Sensor reagent [18, 19]. In TMK-1 cells, a cell population with decreased Δ Ψ m (depolarization) appeared after the treatment with CHX or 5-FU, but not in TMK-1-6-16 cells (Fig. 5c). These results overall suggested that 6-16 protein inhibits cytochrome c release from mitochondria by inhibiting depolarization of mitochondrial membrane potential (Δ Ψ m), resulting in attenuation of apoptosis.

6-16 interacts with calcium integrin binding protein, CIB

In order to further understand the anti-apoptotic mechanism of 6-16 protein, we screened 6-16 interacting protein by yeast two-hybrid methods. The fusion protein of full length 6-16 and GAL4 (GAL4-6-16) was used as bait. By the screening of 1×10^6 colonies of HeLa cDNA library, 41 positive clones (His+ and LacZ+) were identified. Among them, twenty-seven cDNA clones encoded CIB/KIP/calmyrin (we refer to it as CIB in this paper) (Genbank: U822226, U85611). CIB (calcium and integrin binding protein) [20] was also reported from independent groups as DNA-PK interaction protein (KIP) [21] and calcium-binding myristoylated protein with homology to calcineurin (calmyrin) [22]. Twelve cDNA clones encoded γ-subunit of the eukaryotic cytosolic chaperonin-containing protein, TCP-1 (CCTγ) (EMBL:X74801), one cDNA clone encoded calcium binding protein related S-100 (CAPL) protein (GenBank : M80563) and one was an unknown gene.

We next examined the association of 6-16 and CIB proteins in vitro. For this purpose, we prepared GST-tagged 6-16 protein (GST-6-16) and MBP-tagged CIB protein (MBP-CIB) by purification from *E. coli* transformed with each construct and assayed for in vitro

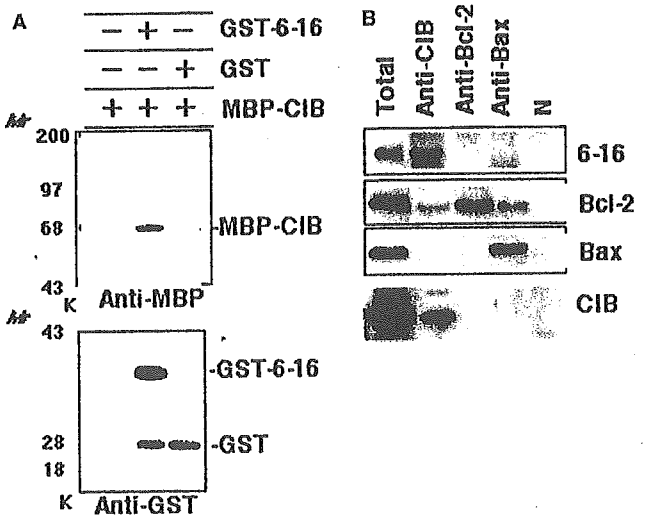

Fig. 6 CIB protein binds to 6-16 and Bcl-2 protein. **a** GST-6-16 and MBP-CIB proteins were purified from *E. coli*, and were incubated in vitro, precipitated with glutathione sepharose 4B and detected by immunoblot analysis with anti-GST antibody (*lower panel*) or anti-MBP antibody (*upper panel*). **b** MKN-28 cell extracts were immunoprecipitated with antibody against CIB, Bcl-2 or Bax (*top margin of the panel*), and subjected to immunoblot analysis with anti-6-16 antibody, anti-Bcl-2 antibody or anti-Bax antibody (*right margin of the panel*)

binding. As shown in Fig. 6a, GST-6-16 and MBP-CIB proteins co-immunoprecipitated in vitro, and 6-16 interacted with CIB using by immunoprecipitation with MKN-28 cell extracts in vivo (Fig. 6b).

We demonstrated that 6-16 protein was localized at mitochondria and attenuated apoptosis by controlling $\Delta \Psi$ m and cytochrome c release. The Bcl-2 family proteins are known to control $\Delta \Psi \mu$ and cytochrome c release via VDAC. We then hypothesized that 6-16 and/or CIB proteins might interact with Bcl-2 family proteins. For this purpose, we searched for cell lines that expressed both 6-16 and CIB at high level and found a gastric cancer cell line MKN-28, which also expressed Bcl-2 at high level (Fig. 5b). By immunoprecipitation with anti-CIB antibody, anti-Bcl-2 antibody or anti-Bax antibody followed by immunoblotting, we found that 6-16 protein interacted with CIB, but not with Bcl-2 or Bax (Fig. 6b). CIB protein interacted with 6-16 and weakly with Bcl-2, but not with Bax (Fig. 6b). Bcl-2 interacted weakly with CIB and Bax (Fig. 6b).

Discussion

In this study, we found that 6-16 protein inhibits apoptosis induced by 5-FU or CHX in gastric cancer cell line TMK-1 cells through the mitochondrial pathway. There are four different inhibition categories of apoptosis. First, in the death receptor pathway (e.g. Fas, TNF and TRAIL), RIP (receptor-interacting protein), c-FLIP (cellular-Flice-like inhibitory protein) and FAP-1 (Fas-associated phosphatese-1) inhibit death signal from the death receptors nearly located at the plasma membrane [23, 24]. Second, in the mitochondrial pathway, anti-apoptotic Bcl-2 family proteins prevent mitochondrial membrane permeabilization to inhibit mitochondrial membrane potential change and cytochrome c release [25]. Third, IAPs (inhibition of apoptosis proteins), including XIAP, cIAP-1 and cIAP-2, selectively inhibit the activity and activation of various caspases [17, 24]. Heat shock proteins (e.g. Hsp10, 27, 60, 70 and 90) also can promote or inhibit caspase activation by altering the conformation of various proteins. Finally, in degradation of chromosomal DNA during apoptosis, ICAD inhibit CAD activity as CAD/ICAD complex [26, 27]. Among these four categories, we found that 6-16 protein inhibits mitochondria-mediated apoptosis as well as anti-apoptotic Bcl-2 family proteins. BH4 domain of anti-apoptotic Bcl-2 family members closes VDAC and inhibits apoptotic mitochondrial changes and cell death [28, 29]. However, 6-16 protein does not have a BH-4 domain by protein domain homology analysis, suggesting that it may function via novel anti-apoptotic mechanisms different from Bcl-2 family proteins. It is important to understand the mechanism of up-regulation of 6-16 expression in cancer cells. Previously, we have found that 6-16 expression was up-regulated in senescent cells through beta interferon signaling pathway, because 6-16 expression was blocked by anti-INF-beta treatment to cultured senescent cells. It is possible that cancer cells may produce beta interferon and induce 6-16 expression by autocrine mechanism, but further examination are need to conclude this possibility.

Cancer and senescence may be viewed as a balance between proliferation and cell death. Many anticancer drugs are designed to induce apoptosis via cytochrome c/Apaf-1/caspase-9 (apoptosome) pathway [16, 24], and the mitochondria play a crucial role for the regulation of tumorigenesis and senescence. Apoptosis induced by CHX is mediated by the apoptosome pathway, whereas 5-FU induced apoptosis is owing to both the Fas/FasL pathway and the apoptosome pathway [24]. 5-FU treatment results in a p53-dependent increase in expression of FasL in human colon cancer cell lines, and apoptosis occurs through Fas/FasL pathway. One of the Fas/FasL pathways also acts to mitochondria after mediating BID and caspase-8. As Fas is expressed in TMK-1 cells (unpublished data), 6-16 protein also inhibits at the mitochondria the apoptosis signal via both the Fas/FasL pathway and the apoptosome pathway in gastric cancer cell line, TMK-1 cells. Therefore, because 6-16 is strongly expressed in almost all the gastrointestinal cancer cells, it is a possibly that 6-16 protein at mitochondria is involved in resistance to anticancer drugs. Free radicals, apoptosis inducers, are known to induce cellular senescence and increase with cellular senescence. 6-16 expression increased with senescence and may also inhibit apoptosis by free radical and maintain cell viability in senescence cells.

Interferon (IFN) has antiviral activity and is also known to induce apoptosis. When cells are infected with virus, 6-16 is expressed after induction of IFN. Then, an increase in 6-16 expression may cause resistance against apoptosis after viral infection. If so, IFN has apparently conflicting dual functions of both induction and inhibition of apoptosis.

We identified CIB as a 6-16 interacting protein through yeast two-hybrid screening methods. CIB protein was reported to interact with cytoplasmic domain of integrin αIIbß3 [20], eukaryotic DNA-dependent protein kinase DNA-Pkcs [21], presenilin 2 (PS2) [22], and the polo-like protein kinases Fnk and Snk [30]. The structural properties of CIB indicate that it is a hydrophilic calcium-binding protein with two EF-hand motifs corresponding to the two C-terminal Ca^{2+} binding domains, most similar to calcineurin B (58% similarity) and calmodulin (56% similarity) [20]. Calcineurin is found to dephosphorylate BAD, a pro-apoptotic member of the Bcl-2 family, thus enhancing BAD heterodimerization with Bcl-X_L and promoting apoptosis. Therefore, CIB might possess protein phosphatase activity like calcineurin or other BH3 only group of Bcl-2 family proteins to promote apoptosis. It is of interest that CIB interacts with 6-16, but further examinations are needed to conclude the involvement in the anti-apoptotic activity.

Intracellular Ca^{2+} concentration changes are important as early events in apoptosis, and maintenance of both mitochondrial and ER Ca^{2+} pool is necessary for cell survival [31, 32]. CIB (calmyrin) was found to form the complexes including presenilin, ß- and delta-catenin, p0071, amyloid ß-protein precursor, filamin/Fh-1, Notch, GSK3ß, Rab11, QM/Jif-1 and Bcl-X_L. Moreover, Presenilin 2 interacts with Sorcin, which is a penta-EF-hand Ca^{2+} -binding protein that modulates the ryanodine receptor (RyR) intracellular channel [33]. Presenilin 1 and 2 are well known for their role in Alzheimer's disease, which is associated with accumulation of ß-amyloid (amyloidogenic Aß42 peptide), abnormality of the mechanism in ER (endoplasmic reticulum) and increased rate of mitochondria-mediated apoptosis in selected areas of the brain [34]. CIB interacted with PS2, and overexpression of CIB and/or PS2 promotes cell death in vitro [22]. In fact, transfection of CIB into TMK-1 and MKN-28 cells also induced apoptosis (unpublished data). 6-16 and CIB may regulate not only mitochondria channels but also Ca^{2+} channels in ER (endoplasmic reticulum). CIB co-localizes and interacts with PS2 localized in the ER [22], and 6-16 possibly localizes at ER membrane because 6-16 has ER membrane retention signal of XXRR-like motif in the N-terminus revealed by PSORT search. Bcl-2 protein is also reported to localize not only at mitochondrial membrane but also at ER and nuclear membrane, and Bcl-2 modulates both mitochondrial and ER Ca^{2+} concentration [31, 32].

Our results indicate that 6-16 and CIB may play a critical role in the regulation of apoptosis via controlling mitochondrial and ER channels through the interaction with Bcl-2 family proteins. Therefore, 6-16 may function as a cell survival protein and CIB as cell death protein. Further examination is needed for understanding the function of interaction of 6-16 protein and CIB protein. However, these data provide a new mechanism in protecting apoptosis. It is possible that the IFN-inducible gene, 6-16 is a new target for cancer therapy and mitochondrial diseases.

Acknowledgements Yeast L-40 strain and pBTM116/HA for yeast two-hybrid screening were kindly supplied by Dr Y Takai (Osaka University, Japan). Anti-GST rabbit pAb was kindly provided by Dr. M. Nakata (Sumitomo Electric Industries, Japan). Anti-human CIB/KIP/calmyrin antibody was kindly provided by Leslie V. Parise (North Carolina University, USA). A mammalian expression plasmid, pCXN was kindly provided by Dr J. Miyazaki (Osaka University, Japan). This work was supported by Grant-in Aid from the Ministry of Education, Science, Sports and Culture of Japan.

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Journal of Pathology
J Pathol 2005; **205**: 65–73
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/path.1684

Original Paper

Histone H3 acetylation is associated with reduced $p21^{WAF1/CIP1}$ expression by gastric carcinoma

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Abstract

Histone acetylation appears to play an important role in transcriptional regulation. Inactivation of chromatin by histone deacetylation is involved in the transcriptional repression of several tumour suppressor genes, including $p21^{WAF1/CIP1}$. However, the *in vivo* status of histone acetylation in human cancers, including gastric carcinoma, is not well understood. This study shows that histone H3 in the $p21^{WAF1/CIP1}$ promoter region is hypoacetylated and that this hypoacetylation is associated with reduced $p21^{WAF1/CIP1}$ expression in gastric carcinoma specimens. Chromatin immunoprecipitation assays revealed that histone H3 was hypoacetylated in the $p21^{WAF1/CIP1}$ promoter and coding regions in 10 (34.5%) and 10 (34.5%) of 29 gastric carcinoma specimens, respectively. Hypoacetylation of histone H4 in the $p21^{WAF1/CIP1}$ promoter and coding regions was observed in 6 (20.7%) and 16 (55.2%) of 29 gastric carcinoma specimens, respectively. $p21^{WAF1/CIP1}$ mRNA levels were associated with histone H3 acetylation status in the $p21^{WAF1/CIP1}$ promoter region ($p = 0.047$) but not $p53$ mutation status ($p = 0.460$). In gastric carcinoma cell lines, expression of $p21^{WAF1/CIP1}$ protein was induced by trichostatin A, a histone deacetylase inhibitor. This induction was associated with hyperacetylation of histone H3 in the $p21^{WAF1/CIP1}$ promoter region. Hyperacetylation of histone H4 in the $p21^{WAF1/CIP1}$ promoter region did not appear to be associated with increased expression. Induction of $p21^{WAF1/CIP1}$ protein expression was associated with hyperacetylation of histones H3 and H4 in the $p21^{WAF1/CIP1}$ coding region. Expression of a dominant-negative mutant of p53 reduced expression of $p21^{WAF1/CIP1}$ protein. Histone H4 acetylation in both the promoter and coding regions of the $p21^{WAF1/CIP1}$ gene in cells expressing dominant-negative p53 was less than half of that in cells expressing wild-type p53, whereas histone H3 acetylation in both the promoter and coding regions was slightly reduced (by approximately 20%) in cells expressing the dominant-negative p53. These findings provide evidence that alteration of histone acetylation occurs in human cancer tissue specimens such as those from gastric carcinoma.

Received: 6 April 2004
Revised: 22 September 2004
Accepted: 26 September 2004

Keywords: **histone acetylation; histone H3; histone H4; chromatin immunoprecipitation; p53; gastric carcinoma; $p21^{WAF1/CIP1}$**

Introduction

A variety of genetic and epigenetic alterations are associated with gastric carcinoma (GC) [1,2]. We have reported reduced expression of $p21^{WAF1/CIP1}$ in 34% of GC tissues [3]. $p21^{WAF1/CIP1}$ was identified through its activation by p53 [4], association with cyclin/cyclin-dependent kinase complexes [5], and increased expression during senescence [6]. Although $p21^{WAF1/CIP1}$ is activated in a p53-dependent manner in response to DNA damage to ensure cell-cycle arrest and DNA repair, various agents that promote differentiation can increase $p21^{WAF1/CIP1}$ expression in a p53-independent manner. We have also reported that $p21^{WAF1/CIP1}$ expression is induced by 9-*cis*-retinoic acid [7] and inhibition of telomerase [8], but we found no correlation between expression of $p21^{WAF1/CIP1}$ and abnormal accumulation of p53 in GC tissues [3].

Changes in DNA methylation patterns, such as hypermethylation of CpG islands, are observed frequently in human cancers [9]. Hypermethylation of CpG islands in promoters is associated with the silencing of some tumour suppressor genes [10]. Methylation and inactivation of various genes have been reported in GC [11,12]. Although hypermethylation of the $p21^{WAF1/CIP1}$ promoter occurs in acute lymphoblastic leukaemia [13], the $p21^{WAF1/CIP1}$ promoter is not hypermethylated in GC [14].

Several lines of evidence suggest that histone acetylation plays an important role in transcriptional

regulation [15]. There appears to be a positive correlation between the level of histone acetylation at specific loci and transcriptional activity, and the recruitment of histone acetyltransferases and hyperacetylation of histones in promoter regions often correlate with transcriptional activation [16,17]. Histone hyperacetylation is thought to relax the chromatin structure and allow transcription factors to access promoter sequences [18,19]. Some genes, including $p21^{WAF1/CIP1}$ [20] and *hTERT* [21], are thought to be regulated by histone acetylation. We have reported that trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, induces $p21^{WAF1/CIP1}$ expression in GC cell lines [22].

Taken together, the currently available data suggest that reduced expression of $p21^{WAF1/CIP1}$ in GC tissues may be due to aberrant histone acetylation and not p53. Little is known, however, about the *in vivo* histone acetylation status in human cancers, including GC. To date, there are no reports of changes in promoter acetylation in human cancer specimens. Thus, we investigated the histone acetylation status of the $p21^{WAF1/CIP1}$ promoter region by means of chromatin immunoprecipitation (ChIP) assays with antibodies against the acetylated forms of histones H3 and H4. Because a recent study in yeast suggested that hypoacetylation of histones in coding regions is important for transcriptional inhibition [23], we investigated the histone acetylation status in the coding region of $p21^{WAF1/CIP1}$. We show for the first time that histone acetylation is altered in GC tissue specimens and that this can reduce $p21^{WAF1/CIP1}$ expression in a p53-independent manner.

Materials and methods

Tissue samples

Twenty-nine GC tissue specimens from 29 patients were studied. The tissue specimens were obtained from Hiroshima University Hospital and affiliated hospitals. Tumours and corresponding non-neoplastic mucosae were removed surgically, frozen immediately in liquid nitrogen, and stored at −80 °C until use. All GCs were located in the middle third of the stomach. Tissues were embedded in OCT compound (Sakura Finetechnical Co, Ltd, Tokyo, Japan) and frozen sections were prepared. After we had confirmed microscopically that the tumour specimens consisted mainly (> 50%, on a nuclear basis) of carcinoma tissue and that non-neoplastic mucosa did not show any tumour cell invasion or significant inflammatory changes, samples from embedded tissues were used for ChIP assay, RNA extraction, and genomic DNA extraction. Histological classification of GC was performed according to the Lauren classification system [24]. Tumour staging was carried out according to the TNM stage grouping [25]. Because written informed consent was not obtained, all samples were cleared of any identifying information, for strict privacy protection, before histone acetylation status was analysed. This procedure is in accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government and was approved by the Ethics Review Committee of the Hiroshima University School of Medicine.

Cell culture and drug treatment

Eight cell lines derived from human GCs were used. The TMK-1 cell line was established in our laboratory from a poorly differentiated adenocarcinoma [26]. Five GC cell lines of the MKN series (MKN-1, adenosquamous cell carcinoma; MKN-7, MKN-28, and MKN-74, well-differentiated adenocarcinomas; and MKN-45, poorly differentiated adenocarcinoma) were kindly provided by Dr T Suzuki. The KATO-III and HSC-39 cell lines, which were established from signet ring cell carcinomas, were kindly provided by Dr M Sekiguchi and by Dr K Yanagihara [27], respectively. All cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical Co, Ltd, Tokyo, Japan) containing 10% fetal bovine serum (FBS; BioWhittaker, Walkersville, ME, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. To analyse transcriptional activation of the $p21^{WAF1/CIP1}$ gene, MKN-28, MKN-74, and KATO-III cells were incubated for 5 days with 1 μM 5-aza-2′-deoxycytidine (Aza-dC; Sigma Chemical Co, St Louis, MO, USA) or for 24 h with 300 nM TSA (Wako, Tokyo, Japan).

Stable transfection

pCMV-p53mt135 expression vector (CLONTECH, Palo Alto, CA, USA) was transfected into MKN-74 cells with FuGENE6 (Roche Diagnostics, Mannheim, Germany). pCMV-p53mt135 expresses a dominant-negative mutant of p53. The *p53* and *p53mt135* genes differ by a G-to-A transition at nucleotide 1017. Stable transfectants were selected with 2 weeks of culture with 80 μg/ml G418 (Invitrogen Corp, Carlsbad, CA, USA). Clone number 5 expressed $p21^{WAF1/CIP1}$ protein at a level lower than that of the mock transfectant (see the Results section) and was used for further analyses of the dominant-negative mutant.

ChIP assay

The ChIP assay was performed as described previously [28]. Polymerase chain reaction (PCR) analysis of immunoprecipitated DNA was performed using primers specific for the 5′ upstream region of the *ACTB* gene. PCR product (15 μl) was loaded onto 8% non-denaturing polyacrylamide gels, separated by electrophoresis, stained with ethidium bromide, and visualized under UV light to confirm that there was no genomic DNA contamination of the no-antibody control. Quantitative PCR analysis of immunoprecipitated DNAs was performed by real-time PCR. The position