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Functional characterization of inhibitor of differentiation 1 (ID1) gene promoter regulatory elements

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ABSTRACT: Inhibitor of differentiation 1 (ID1) protein is highly expressed in undifferentiated cells and associated with tumor progression and metastasis. Restricted regulation of the gene expression is necessary for cell maintenance. We performed the characterization of the response elements involved in the regulation of ID1 promoter activity using deletions and mutations. In silico analysis was used to predict regulatory binding sites. Mutants of the 5′ upstream region of the ID1 promoter were constructed with nucleotide deletion or mutation, and the promoter activity was systematically analyzed. In silico analysis revealed several transcription binding sites presented in upstream region of the ID1 gene. Cluster of four Sp1 binding sites at position -1296 to -1078 contributed to the maximal expression activity of ID1 gene. The deletion and mutation of these Sp1 binding sites resulted in reduction of the expression level. Moreover, it is suggested that mutation of the Sp1 binding site at position -1078 could permit repressor proteins to suppress the gene expression. This study yields a new insight to a better understanding of the ID1 gene regulation, which pave the way to obtain the normal expression level of ID1 for cancer therapy development.

KEYWORDS: ID1 promoter, Sp1 binding site, regulatory elements, repressor

INTRODUCTION

Inhibitor of differentiation 1 (ID1) protein is a key regulatory protein which plays essential roles in the cell cycle and cellular processes and functions by inhibiting target proteins. The function of ID1 involved in cell-cycle regulation, as well as neural and stem cell developmental processes, bone formation and angiogenesis [1]. In cell cycle process, ID1 antagonized the transcription activation of differentiation-associated genes involved in arrest of cell-cycle and inhibited cell senescence. An aberrant expression of ID1 was associated with the progression and poor tumor prognosis [2]. Overexpression of ID1 protein was reported in many cancer types, including breast [3], bladder [4], lung [5], and cervix [6]. The ID1 gene locus is on chromosome 20 at 20q11.21, is 1,239 bp long, including 2 exons. The 5′-upstream region of ID1 gene contains several transcription factor binding sites [7]. An earlier report showed that there were 2 regions important in the regulation of the Id-1 promoter [8]. These regions are major target sites of many cell regulators. One of them is positioned at around 1.2 kb upstream of the transcription initiation site. This region contains Egr-1, BMP, Src, and YY1 binding sites [7, 9]. Egr-1 binding site is on -1130 bp upstream and BMP-responsive element is at -1200 bp upstream. Src responsive elements are found between -1361 to -1200 bp. Src is a positive modulator of the BMP2/Smad1/ID1 signaling pathway in neural stem cells. Inhibition of Src reduces the ID1 expression and is associated with reduced invasiveness of lung cancer cells [10, 11]. The second region is around 200 bp upstream of the ID1 transcription start position. Sp1 and NF-1 binding sites were reported on ID1 proximal promoter (-272 to -145 upstream) and played a role in constitutive expression of ID1 [8].

Transcription factor Sp1, member of Sp/Kruppele like family, is an important gene regulator. The binding site of Sp1 is a GC rich element on the regulatory region. Several studies revealed that more than 12,000 Sp1-binding sites are found throughout the entire human genome [12]. Sp1 transcription factor plays crucial roles both as activator and suppressor for a number of oncogenes and tumor suppressors, including genes involved in essential cellular functions such as proliferation, differentiation, DNA damage response, apoptosis, senescence [13]. In adult acute myeloid leukemia (AML), Sp1 indirectly up-regulated the secreted protein, acidic, cysteine-rich (SPARC) gene. SPARC mRNA expression was reduced in response to the inhibition of Sp1 function [14]. The direct interaction between the transcription factor NFATc2 and the N-terminal domain of Sp1 resulted in increased NFATc2 activity in pancreatic cancer cells [15]. In lung cancer cells, Sp1, together with c-Jun, regulated the cytosolic phospholipases A2 (cPLA2) expression [16]. Sp1 was also implicated in other cancer types and in tumorigenesis by mediating the expression of many oncogenes. The main objective of this study was to characterize the responsive elements present in the additional 5′ upstream sequence of ID1 promoter and to identify regulatory regions, through the deletion and mutation of binding sites that affect the ID1 gene regulation.

MATERIALS AND METHODS

Promoter *in silico* analysis

Human ID1 promoter sequence was retrieved from GenBank (accession No. U57645.1). The promoter sequence was analyzed using the online software PROMO version3 (http://alggen.lsi.upc.es). Binding sites with more than 90% similarity to consensus sequences were selected for further *in vitro* study.

Cell culture

The human embryonic kidney cells HEK-293 cells (ATCC® CRL-1573™, Manassas, USA) were used as a model cell line. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS, Gibco™, Carlsbad, CA, USA) and maintained at 37 °C in 5% CO_2 incubator. When the proliferating cells reached about 80% confluence, they were used for the further steps.

Deleted promoter vector construction

The ID1 promoter-luciferase plasmid, ID1-1472, was a gift from Dr. Takeda [17]. This plasmid was used as a PCR template to construct deleted ID1 promoter. All forward and reverse primers are designed for this study (Table S1). The amplification reaction was performed with denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 2 min using *Taq* DNA Polymerase (New England Biolabs, Massachusetts, USA). The PCR products were digested with the restriction enzymes *Bgl*II and *Hind*III (Promega, Madison, Wisconsin, USA) before being cloned into the luciferase expression vector pGL3 basic (Promega). The plasmid with deleted ID1 promoters were named as ID1-985. All constructs were verified by sequencing analysis of DNA (Macrogen, Seoul, Korea).

Construction of sited specific mutation vectors

ID1-1472 construct was used as a template to introduce Sp1mutations using PCR directed mutagenesis with primers listed in Table S1 [18]. Four mutations of Sp1 were created, namely Sp1m1, Sp1m2, Sp1m3 and Sp1m4. Mutations in the other binding sites, YY1, USF2, and ATF3 were created using specific primers and the Sp1m4 construct as a template. The PCR products carrying mutations were digested and ligated into the pGL3 vector. All mutations were confirmed by DNA sequencing analysis. The promoter activity was detected by luciferase assay system.

Transfection and Luciferase activity assay

HEK-293 cells were seeded in 24-well plates at the density of 1×10^4 cells per well prior transfection. Transfection was performed using Lipofectamine 2000 reagent according to the manufacturer's recommendation (Invitrogen, Waltham, Massachusetts, USA). Plasmids containing wild type or mutant ID1 promoter were mixed with Lipofectamine 2000 reagent. pGL3-Basic was used as a negative control and beta-galactosidase expression vector (Promega) served as a transfection efficiency control. Cells were harvested 48 h after transfection. Luciferase assay system (Promega) was used to measure luciferase activity with the Glomax 20/20 Luminator (Promega) at 595 nm. Beta-galactosidase activity was measured using beta-galactosidase enzyme assay system (Promega) at 420 nm with a Spark M10 multimode microplate reader (TECAN Trading, AG, Switzerland). Each assay was performed in triplicate. Transfections were carried out for at least three independent experiments. The luciferase activities were normalized with beta-galactosidase activity and presented in percentage.

Statistical analysis

Student's *t*-test and one way ANOVA were performed to determine a significant difference between various constructs. Statistical significance was considered when the *p*-value was less than 0.05.

RESULTS

In silico investigation of ID1 promoter

The *in silico* analysis of ID1 promoter showed several putative transcription factor binding sites of at least 90% similarity with canonical elements (Fig. 1). There were at least 4 groups of transcription binding sites: Sp1 binding site (5′-(C/T)(G/A)(G/A)CCGCCC(G/T)-3′), c-jun binding site (5′-(TGA[G/C]TCA)-3′), YY1 (5′-NNNATGGNNN-3′), ER-alpha (5′-GGTCAnnnTGACC-3′), USF2 (5′-CACGTG-3′) and ATF3 (5′-TGACGTCA-3′). Six Sp1 binding sites within the ID1 promoter were located at -1296, -1216, -1153, -1078, -194 and -163 bp upstream of the start codon. Four putative binding sites, for c-jun were presented at -1345, -1199, -977, and -824 bp. Seven YY1 binding sites were located at -1442, -1084, -862, -450, -422, -196, and -187 bp. Three binding sites for ER-alpha were found at -1440, -1199, and -206 bp. USF2, ATF3 and Smad binding sites were identified at -1136, -1026 and -1000 bp, respectively. Some of these binding sites were likely to be involved with ID1 promoter regulation.

Effects of Sp1 binding site mutants on the promoter activity

Luciferase activities of the ID1-1472 construct was used as 100% reference activity (Fig. 2). The activity of ID1-985 was decreased to 44.7% when compared to ID1-1472. The 492 bp deleted in ID1-985 contained numerous putative binding sites, including 4 Sp1-binding sites, 2 c-jun binding sites, 2 YY1 binding sites, 2 ER-alpha binding sites, as well as the USF2, ATF3, and Smad binding sites. Previous study revealed that Sp1 and NF1 binding sites located adjacent to

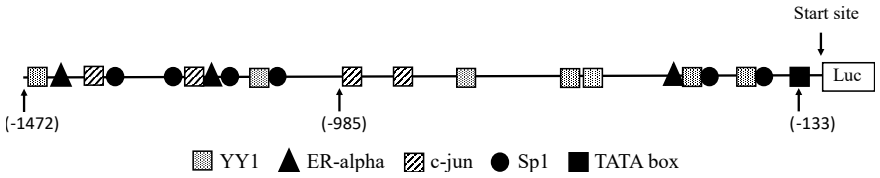

Fig. 1 The arrangement of transcription factor binding sites in ID1 promoter by in silico detection. The nucleotide positions of the deleted ID1 promoter constructs were indicated: -1472 and -985. The symbols indicate binding sites of transcription factors.

Table 1 Consensus sequences of Sp1-binding site located within -1472 to -985 bp of the 5′-upstream region of Id1 gene as shown in italics for mutation.

	Location	Wild type	Mutant
Sp1-binding site 1	-1296 to -1287	GGGGCGGAGA	GG*aaatt*AGA
Sp1-binding site 2	-1216 to -1207	GGGGCGGGGA	GG*atatt*GGA
Sp1-binding site 3	-1153 to -1144	CTCCCGCCCG	CTCC*ttt*CCG
Sp1-binding site 4	-1078 to -1069	CGACCGCCCG	CGACCG*ttt*G

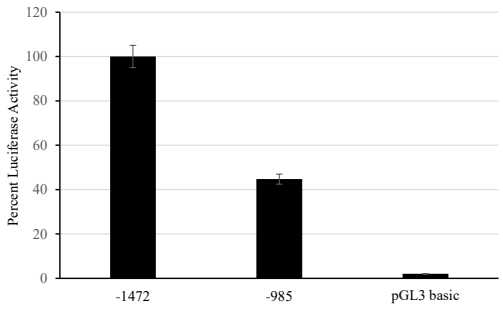

Fig. 2 Decrease of luciferase activity of ID1 promoter by deletion. The activity of ID1-985 was compared with that of ID1-1472. The latter activity was calculated to 100%.

ID1 start site, between -200 to -169, were involved in ID1 expression [8]. Therefore, we decided to further characterize the role of the 4 Sp1 binding sites located between -1472 to -985. Location of mutated Sp1 binding sites is presented in Table 1. All the mutants impaired ID1 promoter activity. When compared to the activity of ID1-1472, the activities of Sp1m1, Sp1m2 and Sp1m3 were reduced to 48.64%, 48.5% and 36.5%, respectively (Fig. 3). However, the reduction in the activity of Sp1m1, Sp1m2 and Sp1m3 showed no statistically significant difference from the activity of ID1-985. Only the Sp1m4 had less activity than that of ID1-985, or only 15.7%, with statistically significant difference.

Identification of the potential binding sites of repressors on the ID1 promoter

The promoter activity of the Sp1m4 was much lower than that of ID1-985. It was implied that the mutation of Sp1m4 might permit some repressors to act on the ID1 promoter. Neighboring sequence of the Sp1

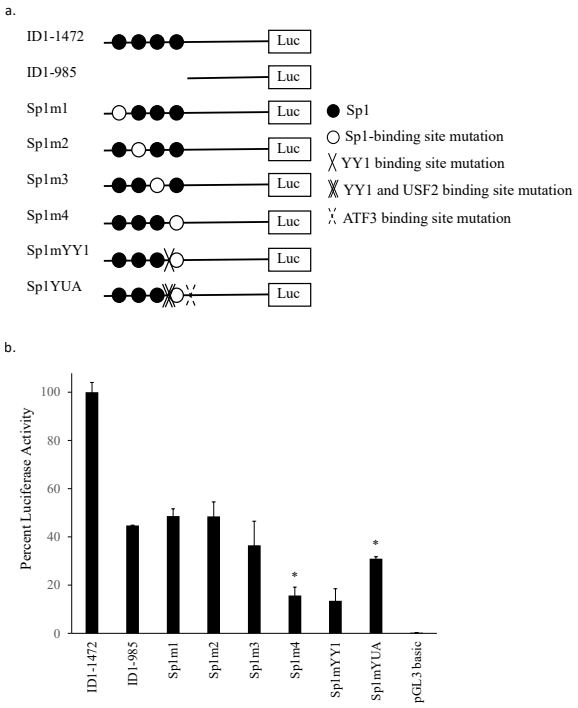

Fig. 3 The effect of Sp1-binding site mutation on ID1 expression level. (a) Diagram of the plasmid constructs with different mutations on Sp1 binding sites. The symbols indicate binding sites of transcription factors and mutation introduced on the transcription factor binding site. (b) Comparison of the expression level of created ID1 promotors. Activity of ID1-1472 was calculated to 100%. Activity of other constructs were calculated in percentage. The expression levels of all constructs were performed in three separate experiments to verify the results. Asterisk (*) indicates statistically difference at $p < 0.05$.

Fig. 4 Nucleotide sequence around the Sp1 binding site 4. The binding sites and nucleotide positions were shown. The canonical sequences of the predicted binding sites are in parentheses; USF2 (5′-CACGTG-3′) at -1136 bp, YY1 (5′-NNNATGGNNN-3′) at -1085 bp, SP1.4 (5′-(C/T)(G/A)(G/A)CCGCCC(G/T)-3′) at -1078 bp, and ATF3 (5′-TGACGTCA-3′) at -1026 bp.

binding site 4 was intensively examined for possible repressor binding sites. The most suspected one was the YY1 binding site which located nearby to the Sp1 binding site 4 (Fig. 4). The mutation of YY1 binding site was then introduced to Sp1m4 by PCR and named Sp1mYY1. However, the expression level of this construct showed no significant difference from Sp1m4. Thus, YY1 alone was not a key repressor in this case. We further investigated other specific binding sites within this region. Besides YY1 binding site, USF-2 binding site was located from -1131 to -1136 of upstream of Sp1 binding site 4 whilst ATF3 was presented from -1019 to -1026 of its downstream. To study the effects of these binding sites on the ID1 expression regulation, the mutations of USF2 and ATF3 were created in the Sp1mYY1 construct. The expression level of this construct was increased to 31%, higher than that of Sp1m4 and Sp1mYY1, but slightly lower than ID1-985 (Fig. 3). The activity of Sp1m4 with YY1, USF2 and ATF3 mutations, clone Sp1mYUA, was different from that of Sp1m4 or ID1-985 clones with statistical significance.

DISCUSSION

The up-regulation of ID1 was associated with the progression of many types of tumors including breast cancer, cervical cancer, prostate cancer and acute myeloid leukemia [19]. ID1 protein is involved with cell proliferation via PI3K/Akt/mTOR signaling in hemangioma-derived endothelial [20], and EGF [21]. However, the mechanism of ID1 regulation in those genes remained unclear. At the transcriptional level, several proteins are identified to involve with ID1 promoter regulation. Investigation of the ID1 promoter, in this study, showed that the -1472 to -985 bp segment contributed to the highest promoter activity compatible to the previous report [7]. Deletion of this region resulted in the sharply reduced ID1 promoter activity of 50%.

By using computer analysis, plenty of putative transcription factor binding sites gathered on this area including Sp1, c-jun, ER-alpha and YY1 (Fig. 1). Various patterns of Sp1 binding sites were reported as the key regulation of several gene promoters such as TGF-beta [22] and hTERT [23]. Sp1 binding site on -272 bp of the ID1 promoter was required for the constitutive expression of ID1 in both non-invasive and metastasis breast cancer [8] and trophoblast stem cells [17] by maintaining the undifferentiated stage of cells. In addition, the computer analysis of region between -1472 to -985 bp demonstrated 4 more Sp1 binding sites in this segment. Our *in vitro* findings were the first to report suggesting the importance of these 4 Sp1 binding sites for the ID1 promoter expression. The site-direct mutation of each Sp1 binding sites affected the ID1 promoter expression. Among these Sp1 binding sites, the Sp1 binding site 4 (-1078 bp) had the most dramatical effect on ID1 promoter expression. The decreasing of promoter activity due to Sp1 binding site 4 mutation was much more than the deletion of -1472 to -985 bp segment which contained those 4 Sp1 binding sites. It was suggested that the mutation of Sp1 binding site 4 induces the main suppressive regulation of ID1 promoter. This Sp1 binding site 4 mutation alone allowed the activation of some repressors on Id1 promoter such as YY1, USF2 and ATF3. This could be supported since the mutations of YY1, USF2 and ATF binding sites which are introduced in the Sp1mYUA clone together with the Sp1 binding site 4 mutation exhibited partial recovery of ID1 promoter expression activity. The effects of transcription factors on Sp1 function were reported previously. Addition of Sp1 affected the repressive function of YY1 due to competition between Sp1 and YY1 [24]. ATF3 interacted with Sp1 in process of nerve regeneration [25]. Interactions between USF2 and Sp1 involved in regulation of human deoxycytidine kinase promoter activity [26]. Thus failure of Sp1 binding to either of the mutated Sp1 binding sites 1 – 4 could interfere function of the other transcription factors resulting in reduction of the promoter activity.

In conclusion, our study reported new region in the ID1 promoter which involved in the control of ID1 expression. In computer analysis, many binding sites were identified in this region, including Sp1, YY1, USF2 and ATF3. The mutation of Sp1 binding site, especially Sp1 binding site 4, reduced the activity of ID1 promoter. Interrupting of the Sp1 binding might permit other cellular proteins to repress the ID1 gene expression. These findings may be useful information to improve cancer treatment in the future.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2023.079.

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Appendix A. Supplementary data

Table S1 Primer sequences used in this study.

Primer name	Primer sequences
ID1-1472 forward	5′ TTT AGA TCT CTC TTC ATA CAG TGC CCG CT 3′
ID1-985 forward	5′ TTT AGA TCT TTC AGA CGC TGA CAC AGA CC 3′
ID1 Reversed	5′ TTT AAG CTT TCT TGG CGA CTG GCT GAA ACA 3′
Sp1m1F	5′ GG GTA GCT GGA AAT TAG AGG TGG G 3′
SP1m1R	5′ CC CAC CTC TAA TTT CCA GCT ACC C 3′
Sp1m2F	5′ C AGG TGT GGA TAT TGG AGG TAA GGT G 3′
SP1m2R	5′ CAC CTT ACC TCC AAT ATC CAC ACC TG 3′
Sp1m3F	5′ CAG GCC TCC CTT TCG GGG TCT GC 3′
Sp1m3R	5′ GCA GAC CCC GAA AGG GAG GCC TG 3′
Sp1m4F	5′ CAT GGC GAC CGT TTG CGC GG 3′
Sp1m4R	5′ CCG CGC AAA CGG TCG CCA TG 3′
YY1mF	5′ GTC GTC TAC GCG GCG ACC G 3′
YY1mR	5′ CGG TCG CCG CGT AGA CGA C 3′
USF2mF	5′ GTC TGC AGG ACT CGG GCT G 3′
USF2mR	5′ CAG CCC GAG TCC TGC AGA C 3′
ATF3mF	5′ GAA TGG GTT ATT TCA CAG GC 3′
ATF3mR	5′ GCC TGT GAA ATA ACC CAT TC 3′

* The underlined type indicates the mutation.