Synergistic Induction of DOC-2/DAB2 Gene Expression in Transitional Cell Carcinoma in the Presence of GATA6 and Histone Deacetylase Inhibitor

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Abstract

The down-regulation of DOC-2/DAB2 gene, which encodes a unique phosphoprotein modulating signal pathways elicited by exogenous stimuli, is often associated with several cancer types; however, the underlying mechanism is still unknown. Dramatically different expression levels of DOC-2/DAB2 mRNA and protein are observed among several human transitional cell carcinoma (TCC) cell lines, suggesting that transcriptional regulation may play a role in these cells. In this study, we have shown that the histone acetylation status associated with the 5' upstream regulatory sequence of DOC-2/DAB2 gene is one of the key determinants for its gene expression. In addition, GATA6 but not other GATA family members, such as GATA2 and GATA4, can specifically induce DOC-2/DAB2 promoter activity, although GATA transcription factors share a very similar DNA-binding sequence. We also show that increased histone acetylation and the presence of GATA6 have a synergistic effect on DOC-2/DAB2 promoter activity, which results in the elevation of DOC-2/DAB2 protein expression. Thus, we conclude that transcriptional regulation of DOC-2/DAB2 gene in human TCC is determined by histone acetylation and a specific transcription factor (i.e., GATA6), which underlie the reduced DOC-2/DAB2 protein expression in TCC cells. (Cancer Res 2005; 65(14): 6089-96)

Introduction

DOC-2/DAB2, originally isolated as a potential tumor suppressor gene from human ovarian carcinoma (1), is a phosphoprotein involved in modulating multiple signaling pathways (2–5) and protein trafficking (6). Decreased expression of DOC-2/DAB2 has been observed in several cancers (7–10), including prostate, mammary, colon, and choriocarcinoma. However, the underlying mechanism leading to the down-regulation of DOC-2/DAB2 gene expression is largely unknown. Moreover, data from Fulop et al. (10) and our previous study (7) showed that an increased expression of DOC-2/DAB2 can suppress the growth of choriocarcinoma and prostate cancer cells. These data indicate the tumor suppressive role of DOC-2/DAB2 in cancer development.

To understand the regulation of DOC-2/DAB2 gene, we recently showed that the increased promoter activity of DOC-2/DAB2 parallels the phorbol ester–induced megakaryocyte differentiation (11). Smedberg et al. (12) also show that the steady-state levels of DOC-2/DAB2 mRNA are elevated in retinoic acid–induced visceral endoderm differentiation of F9 mouse embryonic carcinoma cells. On the other hand, the GATA6-deficient embryos do not survive after E7.5 (13) and have a similar phenotype to that of DOC-2/DAB2–/– embryos. Morrissey et al. (14) further showed that DOC-2/DAB2 is differentially expressed in wild-type and GATA6-knockout mouse fetus because the promoter activity of DOC-2/DAB2 gene is modulated by the presence of GATA6 transcription factor. Six GATA transcription factors have been identified in vertebrates (15), each of which contains a highly conserved DNA-binding domain containing two zinc finger motifs (Cys-X2-Cys-X17-Cys-X2-Cys). They play a key role in regulating the nuclear events that modulate cell differentiation during development (15). Structurally, GATA4, GATA5, and GATA6 are more related and are expressed in an overlapping pattern in various mesoderm- and endoderm-derived tissues (16). During early development, GATA4 and GATA6 have a very similar gene expression pattern (17, 18). The coexpression of GATA4 and GATA6 can be detected as early as the egg cylinder stage in the visceral and parietal endoderm (19–22). Noticeably, in the urogenital ridge that develops to form the bladder, only GATA6 gene is detected (19), suggesting that GATA6 may play an important role in bladder physiology. Thus, these data indicate that each GATA factor has its own selective effect on modulating gene expression.

In this study, we observed a heterogeneous expression of DOC-2/DAB2 levels among several human transitional cell carcinoma (TCC) cell lines. We investigated the regulatory mechanism of altered DOC-2/DAB2 gene expression in these cells and documented the differential effect of GATA factor on the promoter activity of DOC-2/DAB2. This effect is mainly determined by the chromatin structure around DOC-2/DAB2 promoter. The outcome of this study provides the underlying mechanism leading to the down-regulation of DOC-2/DAB2 gene in human TCC cells.

Materials and Methods

Cell culture and DNA transfection. T24, TCC, UMUC3, WH, SWBC1, 253J, and RT4 cells lines, as described previously (22), were routinely cultured in 5% fetal bovine serum T medium (Invitrogen Corporation, Carlsbad, CA). Cells were plated in a six-well plate with 70% to 80% confluence 1 day before transfection. The transfection was carried out with LipofectAMINE (Invitrogen) based on the manufacturer’s protocol. Two days after transfection, cell lysates were subjected to different assays. Three GATA expression vectors (GATA2 derived from human origin; GATA4 and GATA6 derived from mouse origin) were obtained from Dr. Zhi-Ping Liu (Department of Internal Medicine, UT Southwestern Medical Center at Dallas, Dallas, TX).

Western blot analysis. For preparing total cell lysate, cells were washed in cold PBS and lysed in SDS gel loading buffer [50 mmol/L Tris-HCl (pH 6.8),

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).
by proteinase K digestion, phenol extraction, and ethanol precipitation. To determine the amount of DOC-2/DAB2 DNA from both the total input and precipitate, the strand-specific nested PCR were used. For detecting the upstream regulatory sequence of DOC-2/DAB2 gene, we designed the primer set from the ΔB region (−215 to −30; Fig. 3A) and the first PCR amplifications were done first in a 50 μL reaction mixture containing 2 μL of DNA, 0.5 μmol/L downstream primer hDab1/17 (5′ ACATAC- ACATCCTGCAGTGGC3′), and 0.5 μmol/L upstream primer hDab380/73 (5′ GGCGGAGATATGTTGCTC3′) plus 5 units Taq DNA polymerase (Invitrogen). The reaction was carried out 40 cycles of Touch-Down amplification (95°C, 30 seconds; 60-49°C, 30 seconds; 72°C, 30 seconds). The second amplification was done by real-time quantitative PCR using the iCycler iQ Real-Time Detection System (Bio-Rad). A PCR reaction mixture (25 μL) contained 2× iQ SYBR Green Supermix (Bio-Rad), 0.5 μmol/L downstream primer hDab1/196 (5′ CTCGCGGAGCTCAGGAGAG3′), and 0.5 μmol/L upstream primer hDab298R (5′ GGTAACTCCTCCCTCAAGGT3′) plus 4 μL cDNA. The reaction was carried out at 40 cycles of amplification (95°C, 30 seconds; 57°C, 30 seconds; 72°C, 30 seconds). For the control, we chose the exon 10 from DOC-2/DAB2 gene (GenBank A009156) as a template to synthesize primer set as follows: first PCR (5′ GCCTTTTTG- GCCTTTTTGCACAATCTCAAA3′) and second PCR (5′ CTTCCACACGCGGACGCAAA3′) plus 5′ CTCAGGGG-GTCCAGGATGACGGAGC TA3′. Moreover, the same PCR reaction condition was applied. The relative level of acetyl H3 binding was calculated as follows: ΔCt (threshold cycle) of each sample = mean of Ct (precipitate) − mean of Ct (total input). The fold of induction of each sample = 2ΔCt(sample)/ΔCt(control).

Gel shift assay. The gel shift assay was done using LightShift Chemiluminescent EMSA kit (Pierce). Briefly, the complementary single-stranded DOC-2/DAB2 gene probes (−176 to+143, 5′ AGCGGAGCTTA-AGCGGATGAA3′) was first biotin labeled using terminal deoxynucleotidyl transferase from a biotin 5′-end DNA labeling kit (Pierce) and reannealed. The nuclear extract prepared from the TCC cell line was mixed with biotinylated probe (2×10−14 mol/L) in the presence of 50 ng/mL of poly(dI-dC), 5% glycerol, 37.5 mmol/L KCl, and 1 mmol/L EDTA with or without 200-fold excess unlabeled probe (competitor) at room temperature for 20 minutes. For the supershift, 2 μg GATA6 or GATA2 antibody was added into reaction mixture at room temperature for 20 minutes. The mixtures were loaded to 6% DNA retardation gel (Invitrogen) with 0.5× Tris-borate EDTA as the running buffer. After electrophoresis and transfer to nylon membrane, the DNA were cross-linked and the biotin-labeled DNA were detected by chemiluminescence using streptavidin–horseradish peroxidase conjugate (Pierce).
in human TCC cell lines, this system can provide a unique model to analyze the mechanism(s) leading to the altered expression of DOC-2/DAB2 gene. We hypothesized that the transcriptional regulation could play an important role in controlling DOC-2/DAB2 expression in these cell lines.

Differential effects of GATA transcription factors on DOC-2/DAB2 protein expression. Because GATA6 is known to be able to modulate the promoter of DOC-2/DAB2 gene activity in fetus and GATA6 is specifically associated with bladder development. We decided to examine whether each individual GATA transcription factor could modulate DOC-2/DAB2 protein expression in human TCC cell lines. We first determined the basal levels of three GATA family proteins in these TCC cell lines and found both GATA2 and GATA4 levels were very low or almost undetectable in these cells (Fig. 1B). In contrast, GATA6 levels varied among these cells. For example, the high GATA6 levels were detected in T24, TCC, and SWBC1; the intermediate GATA6 expression was detected in WH and 253J cell and very low GATA6 was seen in both UMUC3 and RT4 cells. This pattern of GATA6 expression is consistent with that of DOC-2/DAB2 expression in these cell lines. Thus, we further examined the potential impact of GATA6 on DOC-2/DAB2 gene expression. As shown in Fig. 2A, a dramatically increased level of DOC-2/DAB2 protein was detected in 253J cells transfected with GATA6 expression vector transiently. However, only a slight induction of DOC-2/DAB2 protein could be detected in 253J cells transfected with either GATA2 or GATA4 expression vector. After normalizing with actin levels, an ~16.9-fold increase in DOC-2/DAB2 was detected in 253J cells induced by GATA6. In contrast, only 2.4- and 6.7-fold increases in DOC-2/DAB2 were detected in 253J cells induced by GATA2 and GATA4, respectively. These data indicated a unique effect of GATA6 on DOC-2/DAB2 protein levels in TCC cells, which is not due to the differential expression of each GATA expression vector (Fig. 2B).

Modulation of DOC-2/DAB2 gene promoter activity by GATA6 in human transitional cell carcinoma cell lines. The induction of DOC-2/DAB2 protein by GATA6 transcription factor strongly indicated the key role of transcriptional regulation in the down-regulation of DOC-2/DAB2 gene among human TCC cell lines. Recent analyses of the human DOC2/DAB2 gene unveiled...
the presence of critical regulatory sequences located 5'-upstream from the first exon of DOC-2/DAB2 gene (Fig. 3A). Because GATA6 seems to be the most potent inducer for DOC-2/DAB2 gene, we first mapped the position of the cis-element for GATA6 on the DOC-2/DAB2 promoter. In general, the basal activity of luciferase activity in 253J cells was very low, and the presence of GATA6 could increase the luciferase activity over 20-fold (Fig. 3B).

Using a series of deletion mutants (Fig. 3A), we found that the luciferase activities of both DΔ and DΔB were similar; however, the luciferase activity of DC decreased gradually (Fig. 3B). We further showed the pattern of luciferase activity of DΔ in each cell line (Fig. 3C), consistent with the DOC-2/DAB2 expression pattern from each cell. Thus, we believe that this region ([C0]295 and [C0]120 bp upstream from the exon 1) contains the major GATA6 cis elements (Fig. 3A).

Synergistic effect of GATA6 and histone deacetylase inhibitor on DOC-2/DAB2 promoter activity in human bladder cell lines. The effect of each GATA transcription factor on the activity of DΔ was studied in UMUC3, WH, and 253J cells by transfecting each GATA expression vector. After transient transfection, the expression levels of each GATA in each cell line were determined and it seemed that GATA4 had a higher expression level than both GATA2 and GATA6 in three TCC cell lines tested (Fig. 2B and Supplemental Data). However, only GATA6 could elicit DOC-2/DAB2 gene promoter activity (Fig. 4A), indicating that other mechanism(s), such as epigenetic control, may play a role. One mechanism of epigenetic regulation is to change chromatin structure by modifying the histone protein via posttranslational modification (24, 25). The best-known histone modification is histone acetylation/deacetylation normally maintained by the equilibrium between histone acetyltransferase and histone deacetylase (HDAC) activities. To document the epigenetic effect on DOC-2/DAB2 promoter, DΔ was transfected into 253J, UMUC3, and WH cells in the presence of HDAC inhibitor (FK228). As shown in Fig. 4B, a significant induction of DΔ activity was detected only in WH cells but not in both UMUC3 or 253J, because the endogenous GATA6 levels were relatively higher in WH cells than in the two other cells (Fig. 1B). Noticeably, we observed a synergistic effect of GATA6 and FK228 on the DΔ activity in three TCC cell lines tested (Fig. 4C). In UMUC3 cells, combination of GATA6 and FK228 exhibited a significantly greater induction than either agent exhibited. However, in WH cells, FK228 treatment alone could elicit a significant induction of DΔ activity but not GATA6 alone. On the contrary, in 253J cells, GATA6 alone could elicit a significant induction of DΔ activity but not FK228 alone. These data suggested that the degree of histone acetylation associated with DOC-2/DAB2 promoter in 253J may be higher than in the other two cell lines.

We also examined whether the status of histone acetylation impacts the inductivity of each GATA factor on the activity of
DOC-2/DAB2 gene promoter. By transfecting each individual GATA vector into UMUC3, WH, and 253J, respectively (Fig. 4D-F), we found that FK228 does not change the specific effect of the GATA factors on DOC-2/DAB2 promoter; that is, only GATA6 can induce DOC-2/DAB2 promoter activity in these cells. These data lead us to conclude that the critical interaction between chromatin structure and specific transcription factors is a key determinant for DOC-2/DAB2 gene expression in human TCC cell lines.

The status of histone acetylation and GATA6 associated with the upstream regulatory sequence of DOC-2/DAB2 gene in transitional cell carcinoma cell lines. Increased histone acetylation associated with DNA can relax nucleosome structure, which initiates gene transcription by allowing transcription factors to bind to the promoter region of any given gene. To examine whether FK228 can enhance histone acetylation associated with the upstream regulatory region of DOC-2/DAB2 gene, we did chromatin immunoprecipitation assay to specifically measure the levels of acetyl H3 associated with the upstream regulatory sequence of DOC-2/DAB2 gene. As we expected, a higher acetyl H3 level associated with DOC-2/DAB2 gene was detected in 253J and TCC than that in UMUC3 and WH cells (Fig. 5A). In WH cells (Fig. 5B), FK228 or GATA6 cDNA transfection elicited a moderate increased acetyl H3 levels associated with only the upstream regulatory region but not the exon of DOC-2/DAB2 gene, suggesting changing acetyl H3 levels in the upstream regulatory region is critical for gene expression. Also, FK228 plus GATA6 cDNA transfection caused more significant increased acteyl H3 levels (Fig. 5B), suggesting that the cooperation between HDAC inhibitor and GATA6 can increase the accumulation of acetyl H3 in the DOC-2/DAB2 promoter region, resulting in its gene transcriptional activation.

To show the binding of GATA6 to the upstream regulatory sequence of DOC-2/DAB2 gene in the TCC cell line, a gel shift assay was done. As shown in Fig. 5C, the nuclear extract prepared from the TCC cell line contained the factor specifically binding to GATA cis-element (lane 1), which could be competed by the excess of unlabeled probe (lane 2). Moreover, GATA6 antibody (lane 4) but not GATA2 antibody (lane 3) was capable of retarding the mobility of this DNA-protein complex, indicating that the protein complex contains GATA6 transcription factor.

Synergistic effect of GATA6 and histone deacetylase inhibitor on DOC-2/DAB2 protein expression in human bladder cell lines. The effect of GATA6 and FK228 on DOC-2/DAB2 protein expression was also determined in UMUC3, WH, and 253J cells using Western blot analysis. In both UMUC3 and WH cells, FK228 alone caused 5- to 11-fold induction of DOC-2/DAB2 proteins, but GATA6 alone did not; a synergistic effect was observed in the combination of GATA6 and FK228 treatment (Fig. 6). In 253J cells, GATA6 alone caused 6.0-fold induction of DOC-2/DAB2 protein, but FK228 alone did not, whereas combination exhibited an additive effect (Fig. 6). The inductive pattern of DOC-2/DAB2 protein in these three cell lines is parallel with that of DOC-2/DAB2 promoter activity in each cell line, respectively (Fig. 4C), suggesting that transcriptional regulation of DOC-2/DAB2 should play a determinant role in overall protein levels in TCC cell lines.

Discussion

It seems that DOC-2/DAB2 plays a critical role in the development of visceral endoderm during mouse embryogenesis (19, 26). Moreover, loss of DOC-2/DAB2 expression has been associated with several cancer types because DOC-2/DAB2 is a potent growth inhibitor in these cancer cells. Mechanistically, DOC-2/DAB2 acts as a homeostatic factor in modulating many signal pathways elicited by phorbol ester or peptide growth factors (2–5, 27). Thus, studying the status of DOC-2/DAB2 in this cancer type is warranted.

TCC with an increasing tumor incidence has become the fourth most prevalent cancer among American men (28). Studies indicate that the amplification or overexpression of peptide growth factor or its receptor is associated with TCC progression. Therefore, we explored the status of DOC-2/DAB2 in this cancer and found that bladder cell lines had various levels of DOC-2/DAB2 expression, which provided an ideal system for studying DOC-2/DAB2 gene regulation (Fig. 1). To examine transcriptional regulation in each TCC cell line and to map the potential cis-element of DOC-2/DAB2 promoter inducible by GATA factor, we examined an approximate 300 bp DNA fragment containing the 5’ upstream regulatory region from the first exon of the DOC-2/DAB2 gene (Fig. 3A; ref. 11). Sequence analysis (16, 29) unveiled that at least 10 putative GATA binding sites were mapped in the ΔA, eight sites in the ΔB, and only two sites remained in ΔC (Fig. 3A). Consistently, the ΔA and ΔB exhibited higher activity induced by GATA6 than ΔC (Fig. 3B) and the binding of GATA6 to those consensus sequence was also observed (Fig. 5C), indicating that GATA6 is a potent transcription factor in modulating DOC-2/DAB2 gene expression in TCC cells. As shown in Fig. 3C, the basal promoter activity of ΔA in four TCC cell lines parallel with their steady-state levels of DOC-2/DAB2 mRNA levels (Fig. 1B). Thus, these data conclude that the region between −176 and −143 bp upstream from exon 1 of DOC-2/DAB2 gene contains critical cis-element(s) for the binding of GATA6.

The interaction between specific transcription factors and relaxed chromatin structure as a part of epigenetic regulatory mechanism is crucial for gene transcription. Overwhelming data warrant.

Figure 5. Analysis of the upstream regulatory region of DOC-2/DAB2 gene from human TCC cell lines for the status of acetyl H3 or GATA6 binding. A, basal acetyl H3 levels associated with the upstream regulatory region of DOC-2/DAB2 gene from each TCC cell line. The relative level of acetyl H3 was calculated by normalizing with UMUC3 (= 1). B, induction of acetyl H3 levels associated with the upstream regulatory region (white columns) or exon 10 (black columns) of DOC-2/DAB2 gene in the WH cell line treated with FK228 and/or GATA6 cDNA transfection. The relative level of acetyl H3 binding was calculated by normalizing with control from each cell line (= 1). C, binding of GATA6 to the upstream regulatory region of DOC-2/DAB2 gene in the TCC cell line.
have shown that histone deacetylation is often associated with loss of tumor suppressor gene expression in many cancer types (22), and it is now considered an alternative mechanism, in addition to gene mutation, to silence tumor suppressor genes. Apparently, DOC-2/DAB2 belongs to this category of tumor suppressor gene. The levels of DOC-2/DAB2 gene expression in human TCC cell lines are determined by the degree of histone acetylation and by the availability of GATA6 transcription factor. For example, in UMUC3 cells, the acetyl H3 levels in DOC-2/DAB2 promoter region are relatively low (Fig. 5A) and the endogenous GATA6 levels are also low (Fig. 1B). Both HDAC inhibitor and GATA6 vector transfection significantly increase the DOC-2/DAB2 gene reporter activity (Fig. 4C) and its protein expression (Fig. 6). It seems that FK228 could play an initial role in relaxing the nucleosome structure of DOC-2/DAB2 gene promoter region for further access of transcription factors. In WH cells, the acetyl H3 levels in the DOC-2/DAB2 promoter region remain very low (Fig. 5A); FK228 alone is sufficient to induce DOC-2/DAB2 promoter activity by increasing acetyl H3 levels (Fig. 5B). In contrast, in 253J cells, the histone H3 in the DOC-2/DAB2 promoter region is acetylated (Fig. 5A), so increased GATA6 levels is sufficient to induce DOC-2/DAB2 gene reporter activity and its protein expression (Fig. 4C and 6). In summary, in terms of DOC-2/DAB2 gene regulation, these TCC cell lines can be divided into four groups: high GATA6 and acetyl H3 (TCC); high GATA6 and low acetyl H3 (WH); low GATA6 and acetyl H3 (UMUC3); low GATA6 and high acetyl H3 (253 J). Although the underlying mechanism leading to high GATA6 and/or acetyl H3 levels in TCC cell lines is still unclear, it is likely that the enzymes responsible for epigenetic regulation, such as histone modification or DNA methylation, could play a role. Nevertheless, the outcome of this study has outlined a key underlying mechanism of the down-regulation of DOC-2/DAB2 often observed in several cancer types.

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