HOP/OB1/NECC1 Promoter DNA Is Frequently Hypermethylated and Involved in Tumorigenic Ability in Esophageal Squamous Cell Carcinoma

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Abstract

Promoter DNA hypermethylation with gene silencing is a common feature of human cancer, and cancer-prone methylation is believed to be a landmark of tumor suppressor genes (TSG). Identification of novel methylated genes would not only aid in the development of tumor markers but also elucidate the biological behavior of human cancers. We identified several epigenetically silenced candidate TSGs by pharmacologic unmasking of esophageal squamous cell carcinoma (ESCC) cell lines by demethylating agents (5-aza-2′-deoxycitidine and trichostatin A) combined with ESCC expression profiles using expression microarray. HOP/OB1/NECC1 was identified as an epigenetically silenced candidate TSG and further examined for (*a*) expression status, (*b*) methylation status, and (*c*) functional involvement in cancer cell lines. (*a*) The HOP gene encodes two putative promoters (promoters A and B) associated with two open reading frames (HOPα and HOPβ, respectively), and HOPα and HOPβ were both down-regulated in ESCC independently. (*b*) Promoter B harbors dense CpG islands, in which we found dense methylation in a cancer-prone manner (55% in tumor tissues by TaqMan methylation-specific PCR), whereas promoter A does not harbor CpG islands. HOPβ silencing was associated with DNA methylation of promoter B in nine ESCC cell lines tested, and reactivated by optimal conditions of demethylating agents, whereas HOPα silencing was not reactivated by such treatments. Forced expression of HOP suppressed tumorigenesis in soft agar in four different squamous cell carcinoma cell lines. More convincingly, RNA interference knockdown of HOP in TE2 cells showed drastic restoration of the oncogenic phenotype. In conclusion, HOP is a putative TSG that harbors tumor inhibitory activity, and we for the first time showed that the final shutdown process of HOP expression is linked to promoter DNA hypermethylation under the double control of the discrete promoter regions in cancer. (Mol Cancer Res 2008;6(1):31–41)

Introduction

Gene silencing by DNA methylation synergistic with its associated histone deacetylation is recognized at the promoter region of tumor suppressor genes (TSG), resulting in shutdown of the gene expression in human cancer (1, 2). As a result, the oncogenic signal is thought to be more efficiently transduced to the downstream molecules involved in aspects of cancer progression such as invasion, angiogenesis, and metastasis. This hypothesis is supported by studies that showed that pharmacologic treatment of cell lines with demethylating-associated agents such as 5-aza-2′-deoxycitidine (5-aza-dC) and/or trichostatin A results in marked inhibition of tumorigenesis or angiogenesis, suggesting an important role for epigenetic silencing of TSGs in cancer progression (3, 4). We previously described a novel method of pharmacologic unmasking followed by microarray, which enabled us to identify unknown methylated TSG candidates in esophageal squamous cell carcinoma (ESCC; ref. 5). Subsequently, we improved the method of gene screening to detect more frequent and cancer-prone methylation, resulting in the identification of genes that have potent tumor-suppressive activity or display a clinically aggressive phenotype when methylated (6-8). For example, NMDAR2B is hypermethylated in more than 90% of primary ESCCs, and its expression induced drastic apoptosis in ESCC cells (8).

In the current study, we further advanced our search for novel methylated TSGs showing a critical role in tumorigenesis through a combination of analyzing our previous database with expression profiles of primary ESCC tissues (5), and for the first time identified the promoter hypermethylation of homeobox only protein (HOP)/OB1/NECC1 in human cancer and its potential role as a TSG.

Received 5/10/07; revised 8/9/07; accepted 8/15/07.
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doi:10.1158/1541-7786.MCR-07-0213

Results

Identification of Putative Candidate Methylated Genes by Pharmacologic Unmasking Microarray in Combination with Primary ESCC Expression Profiles

Three ESCC cell lines (KYSE30, KYSE410, and KYSE520; ref. 5) were treated with the methyltransferase inhibitor 5-aza-dC (1 and 5 μmol/L) in the absence or presence of the deacetylase inhibitor trichostatin A. Gene expression was then analyzed by microarray, and results were combined with the results of RNA expression profiles of primary ESCC tissues for the identification of novel methylated genes. Through the new detection method we developed (5, 8), we found methylated genes with 90% accuracy in a specific group of genes (we called this group the first priority group) from microarray data alone. In the present study, a combination of both the first and second priority groups combined with primary cancer expression profiles expected to yield additional relevant genes silenced in primary ESCC. Microarray analysis in primary ESCC versus noncancerous mucosal tissues revealed 2,228 up-regulated and 2,380 down-regulated genes in these cancers (data not shown). The former genes included cancer testis antigens (e.g., the MAGE family), metastatic genes (e.g., proteases such as the matrix metalloproteinase family and urokinase-type plasminogen activator systems), and extracellular matrix–related proteins (e.g., CD44, osteopontin, and SPARC; refs. 9, 10). The latter group included apolipoprotein D, transglutaminase-2, trypsinogen-4, and MAL (5, 11, 12). Combined analysis of both the higher priority group (1,035 genes; ref. 8) and down-regulated genes in primary ESCC tissues (2,380 genes) unexpectedly yielded only 50 targets among 12,599 genes.

Identification of HOP/OB1/NECC Promoter DNA Methylation in ESCC Cell Lines as Higher Priority Candidates

Promoter DNA hypermethylation associated with gene silencing in primary cancer results in completely or nearly absent expression in primary cancers compared with the corresponding normal tissues (5, 11). We thus reasoned that a contrast-variation value (CVV; described in Materials and Methods) could help assess the likelihood of cancer-prone methylation in primary cancers. For example, known methylated genes in primary ESCC cancers like transglutaminase-2 and trypsinogen-4 showed remarkable contrast between the tumor tissues and the corresponding normal tissues or even between the individual tumor tissues, resulting in higher CVV values of 25.7 and 19.2, respectively, than weakly methylated or unmethylated genes such as metallothionein 1G (CVV, 13.3), HEM45 (CVV, 4.7), MUC1 (CVV, 3.9), and IGFBP-4 (CVV, 2.4). Through such analysis, we selected a total of 34 candidates with CVV values of >5.0 in primary ESCC as the highest methylation priority group, and identified numerous novel methylated genes (MAL, HOP/OB1/NECC1, crystallin αB, KIAA0633/COBL, integrin α_7, Kip2/p57, FLJ10097 homology with p75 NTR-associated cell death executer, intestinal trefoil factor, Munc-13, and DKFZp564/FLJ90798) in ESCC cell lines (Table 1). However, as we observed in our previous studies (5-8, 11, 13), cell line data include unexpectedly numerous artifact methylation as compared with actual tumor tissues, and there has been remarkable discrepancy between cell lines and primary tumor tissues about promoter DNA methylation. For the top two genes (*MAL*, T-lymphocyte maturation-associated protein; and *HOP*, homeobox only protein), we therefore examined their promoter DNA methylation status in the 20 pairs of tumor/normal tissues and found DNA promoter hypermethylation of both genes in a cancer-prone manner, then focused on HOP methylation as our particular gene of interest. We had previously found MAL to be markedly down-regulated in ESCC using differential display and showed that it suppressed tumorigenesis by activating Fas in ESCC (12). We also discovered that MAL is hypermethylated in a very specific CpG region that contains wild-type binding sites in 25% of the primary ESCC tissues but not in the control tissues (data not shown).

Promoter DNA Methylation Status of HOPα and HOPβ in ESCC Cell Lines

In this study, we focused on HOP/OB1/NECC1 because HOP has been already shown to suppress *in vivo* tumorigenesis in choriocarcinoma, suggesting that HOP could be a bonafide TSG. The most reliable landmark of TSGs has been stated to be the presence of abnormal injuries representing the net identity of cancer including genomic or epigenomic alterations (2, 14). HOP has two discrete promoter regions that encode the identical gene representing two distinct transcripts, α and β (Fig. 1A). Whereas transcription start sites for HOPα do not include putative CpG islands, the promoter for transcript β harbors a CpG-rich region encompassing its transcription start sites. Thus, we examined the promoter methylation status of promoter B by bisulfite sequencing in nine ESCC cell lines (Fig. 1B). Representative bisulfite-treated genome sequencing is shown in Fig. 1C, where guanine (G) bands (*arrows*) represent complementary cytosines (C), which were methylated and therefore protected by bisulfite treatment. Exclusive G bands were found in TE1, TE4, KYSE30, KYSE150, and KYSE200, whereas exclusive adenine (A) bands, which represent complementary uracils (U) converted from unmethylated C by bisulfite treatment, were found in KYSE410 and KYSE520. In both TE2 and TE5, we found both discrete alleles (G bands representing methylation and A bands representing unmethylation; Fig. 1B).

Expression Status of HOP/OB1/NECC1 in Primary ESCC Tissues Compared with Corresponding Normal Tissues and Reactivation of HOPβ Expression by Demethylating Agents in ESCC Cell Lines

After confirming actual down-regulation of total HOP expression (HOP core) in the tumor tissues compared with the corresponding normal mucosal tissues by reverse transcription-PCR (RT-PCR; Fig. 2A), we examined HOPβ expression in the nine ESCC cell lines, comparing the methylation status with the expression status (Fig. 2B). The cell lines were also treated with demethylating-associated agents under several conditions (1 or 5 μmol/L 5-aza-dC in the absence or presence of trichostatin A) to show that demethylation of promoter B results in gene reactivation. HOPβ transcript was silenced in cell lines that harbor promoter B hypermethylation (TE1, TE4, KYSE30, KYSE150, and KYSE200) but reactivated by the optimal conditions of demethylation in each cell line. On the

Table 1. Top Priority Genes for Methylation in Primary ESCC by CVV Score

	Accession	Name	N1 + N2	C1	C2	C3	CVV	Methylation*	Derived from
1	X76220	MAL	P	D	D	D	226.8	M	1st
2	U51712	HOP/OB1/NECC1	P	D	D	D	158.2	M	2nd
3	ALO38340	Crystallin αB	P	D	NC	I	130.3	M	1st
4	J02854	20-kDa myosin light chain (MLC-2)	P	D	D	D	100.6	U	1st
5	L76465	PGDH	P	D	D	D	68.9	U	2nd
6	AB014533	KIAA0633 (COBL)	P	D	D	D	45.9	M	1st
7	U10550	Gem	P	D	D	D	38.2	U	1st
8	AB011143	KIAA0571 (GRB2-associated binding protein 2)	P	D	D	D	36.9	U	2nd
9	J02611	Apolipoprotein D	P	D	D	D	37.1	(M)	1st
10	D83402	Prostacyclin synthase	P	D	D	D	35.6	—	2nd
11	AF032108	Integrin α_7	P	D	D	D	34.4	M	1st
12	AFO39397	Crytallin-μ	P	D	NC	D	26.1	U	2nd
13	M55153	Transglutaminase-2	P	D	D	D	25.7	(M)	2nd
14	U22398	Kip2/p57	P	D	NC	NC	22.6	M	1st
15	X71345	Trypsinogen IVb	P	D	D	D	19.2	(M)	2nd
16	U68385	MEIS3	P	I	NC	D	18.2	—	1st
17	U35139	Necdin	P	D	D	D	17.5	—	1st
18	AL035494	FLJ10097 homology with p75NTR-associated cell death executor	P	D	D	D	16.3	M	2nd
19	M96740	NSCL-2	P	D	I	NC	15.5	—	1st
20	AJ012737	Filam in	P	D	D	D	14.8	—	2nd
21	J00073	Actin, α, cardiac muscle; actin	P	D	NC	D	13.3	—	2nd
22	J03910	Metallothionein 1G	P	D	D	D	12.3	(M)	1st
23	NM_002026	Fibronectin	P	D	NC	NC	10.6	—	2nd
24	L08044	Intestinal trefoilfactor	P	D	D	D	9.7	M	1st
25	D50918	KIAA0128 (septin 6)	P	NC	D	D	9.6	—	2nd
26	AF020202	Munc-13	P	D	D	D	9.0	M	1st
27	AL049949	DKFZp564 (FLJ90798)	P	D	D	D	8.7	M	1st
28	U29091	Selenium binding protein	P	D	NC	D	8.4	(M)	2nd
29	AB018264	KIAA0711 (TSPY-like 4)	P	D	D	D	8.2	(M)	1st
30	U85707	MEIS1	P	D	NC	D	7.7	—	1st
31	X56667	Calretinin	P	D	I	D	6.4	—	2nd
32	J05582	Mucin-1	P	D	D	D	6.3	—	2nd
33	X51405	Carboxypeptide E	P	D	D	D	5.7	—	2nd
34	M84526	Adipsin	P	D	D	D	5.2	(M)	1st

Abbreviations: N, normal tissue; C, cancer tissue; P, present in expression; D, decrease; I, increase; NC, no change.
*Methylation in ESCC cell lines was evaluated by direct sequencing. M, methylation; U, unmethylation; —, not assessed in this study; (), was examined in previous studies (5, 8).

other hand, HOPβ transcript was found to be expressed in the cell lines that harbor unmethylated alleles (TE2, TE5, KYSE410, and KYSE520), although expression was low in TE2. Promoter A did not harbor dense CpG sites, and almost all ESCC cell lines showed suppressed expression of HOPα (Fig. 2C), but failed to reactivate gene expression by demethylating-associated agents. This finding suggests that HOPα is not regulated by promoter methylation. Interestingly, according to our microarray data, HOPα was completely silenced in tumor tissues, yet abundantly expressed in normal tissues, whereas HOPβ was also decreased in a subset of cancers with silenced HOPα (Fig. 2A). This finding suggests that HOPα is accompanied by cancer transformation, and the final shutdown process may involve the methylation of promoter B. We further observed that transcripts of both TE2 and TE4 harbored no HOP mutations (data not shown).

Promoter DNA Methylation of HOPβ in both ESCC Tissues and Corresponding Normal Tissues and Its Prognostic Relevance in ESCC Patients

We next examined HOP methylation status in primary ESCC tissues compared with corresponding normal esophageal tissues (representative cases shown in Fig. 3A). By the bisulfite-treated genome sequencing detection method, promoter B was frequently methylated (50%) in a cancer-prone manner (data not shown). Bisulfite-treated genome sequencing typically involves the sequencing of multiple clones generated from PCR products to conclude what the methylation pattern is; however, it is difficult to assess partial methylation. We thus changed our method of methylation analysis to a more quantitative one, using TaqMan methylation-specific PCR after standard curves were generated to allow for quantification of methylation. Promoter B methylation (defined as detection by TaqMan methylation-specific PCR) was found in 11 (55%) cases and was as always more hypermethylated in the tumor tissues than the corresponding normal tissues (Fig. 3B). We further examined the tumors of 50 ESCC patients who were well characterized by clinicopathologic data including patient prognosis (7) and found that a higher level of promoter B methylation [TaqMan methylation value (TaqMeth V) >60] significantly correlated with worse patient survival in primary ESCC (P = 0.02; Fig. 3D), suggesting that cell clones with promoter B methylation are clinically more aggressive.

Exogenous Expression of HOP Suppresses Colony Focus and Colony Formation in Soft Agar

HOP expression was also previously reported to be down-regulated in head and neck squamous cell carcinoma (15); thus, we aimed to reveal the functional involvement of HOP in squamous cell carcinomas including ESCC. We used two types

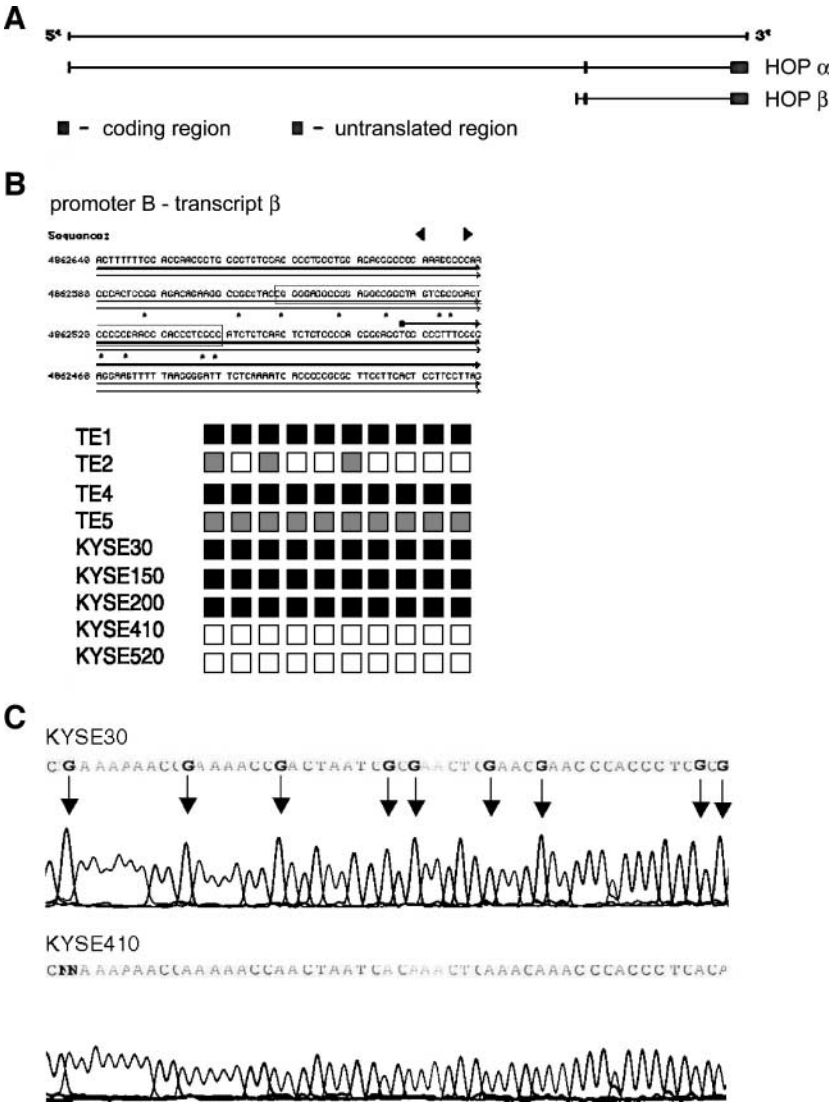

FIGURE 1. Methylation pattern of two different HOP variants in nine ESCC cell lines. **A.** HOP has two different promoter regions with distinct CpG islands and two known transcripts, α and β. **B.** Sequence of each promoter region (promoter A and promoter B) corresponding to transcripts α and β, respectively. Bold line, transcriptional region; initial larger box, presumed transcription start site. Asterisks, CpG sites. Methylation status of promoter B region of HOP is depicted for each cell line. Black box, complete (exclusive) methylation. Gray box, both methylated and unmethylated alleles included. White box, absence of methylation (exclusive unmethylation). **C.** Examples of exclusive methylation and unmethylation shown by sequencing of promoter B bisulfite-treated DNA. All guanines (*arrows*) present after sequencing represent methylated cytosines (protected) on the complementary strand after sequencing. The cell line is indicated on top left of each electrophenogram.

of assays to evaluate transformation ability: colony focus assay and soft-agar analysis. Because soft-agar analysis is the gold standard to examine such ability, we carried out colony focus assay as a preliminary test in six squamous cell carcinoma cell lines (TE1, KYSE200, KYSE410, KYSE013, 019, and 022; Fig. 4A). HOP transfection showed a dramatic reduction of colonies 2 weeks after G418 selection as compared with mock-transefected cells in all the squamous cell carcinoma cell lines tested. We then selected 120 clones (20 colonies from each cell line) after G418 selection, screened them for stable transfection by dot blot analysis, and finally expanded four stable clones with the highest HOP expression (KYSE200-HOP, TE1-HOP, 019-HOP, and 022-HOP; Fig. 4C). Protein production in each clone was confirmed by anti-Flag antibody M2 binding to the fusion 3× Flag-tagged HOP protein. The four stable transfectants showed a dramatic reduction of growth in soft agar as compared with the corresponding mock clones (Fig. 4B). Only TE1 still grew substantial colonies after HOP transfection, but the number relative to mock transfectant was markedly reduced.

RNA Interference Knockdown of HOP Restores Oncologic Properties Including Colony Formation in Soft Agar

Next, we explored the effect of HOP abrogation in ESCC cells. HOP mRNA expression was detected in TE2 and TE4 (Fig. 2C), whereas TE2 expressed more HOP protein than TE4 (Fig. 5A). We designed two sets of RNA interference constructs based on vector systems using pSuppressor. Both sets showed considerable reduction of endogenous expression of HOP protein after transient transfection in TE2 and TE4 (Fig. 5A). The Western blot shows a marginal difference between mock-transfected cells and RNA interference–treated cells. Because these experiments were done by transient transfection, expression reduction could be affected by transfection efficiency. To show endogenous HOP contribution to tumorigenesis, we selected stable clones that exhibited the most severe reduction of HOP at the mRNA level (TE2-HOP-i-1-1, TE-HOP-i-2-8, and TE2-HOP-i-2-43; Fig. 5B). These clones showed a remarkable change in phenotype. They tended to pile up onto other cells, and some aggregates could be taken off

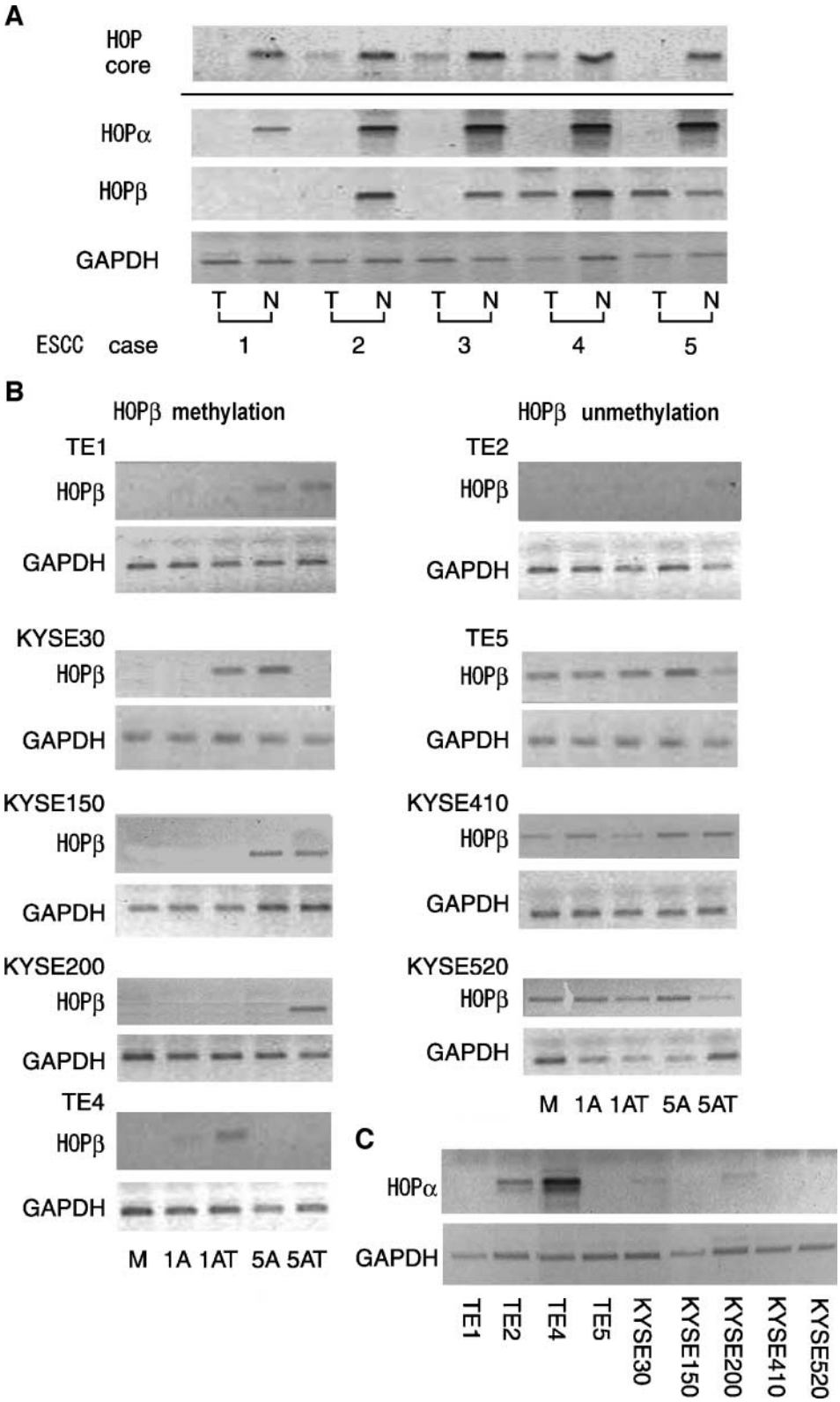

FIGURE 2. Expression analysis of HOP transcripts in primary ESCC and nine ESCC cell lines by RT-PCR. **A.** The common HOP core transcript was markedly down-regulated in primary tumor tissues (*T*) compared with the corresponding normal tissues (*N*). Note that α and β variants of the HOP gene showed independent patterns of expression. **B.** Reactivation of β variants of HOP by pharmacologic unmasking using 5-aza-dC (*A*) and trichostatin A (*T*) in cases with or without methylation. The number before treatments designates concentration of 5-aza-dC (*1,* 1 μmol/L; *5,* 5 μmol/L). Trichostatin A concentration was always constant (300 nmol/L); for example, 5AT: 5 μmol/L 5-aza-dC plus trichostatin A. **C.** HOPα expression in nine ESCC cell lines.

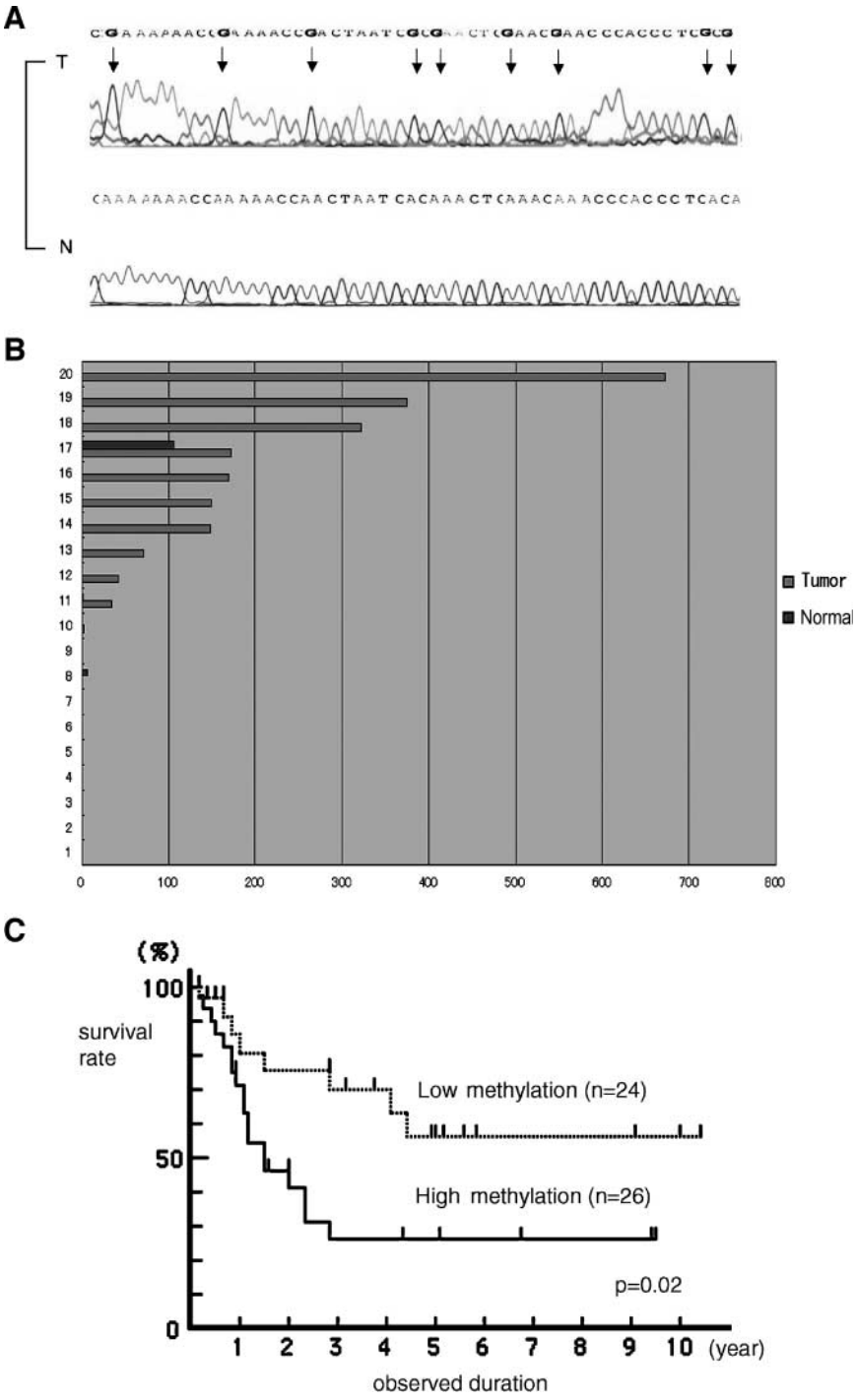

FIGURE 3. Methylation pattern of promoter B region of the HOP gene in primary ESCC tissues. **A.** Examples (methylated and unmethylated cases) of direct sequencing of promoter B after bisulfite treatment of DNA. All guanines (*arrows*) present after sequencing represent methylated cytosines (protected) on the complementary strand. T, tumor tissues; N, normal tissues. **B.** Quantitative evaluation of HOP methylation levels showing high levels in 10 of 20 (50%) cancers, and virtually lower levels in corresponding adjacent normal tissues (*X axis,* units; TaqMeth V). **C.** Kaplan-Meier curves in an expanded number of primary ESCCs comparing cases with higher TaqMeth V (≥25.0; n = 24) and those with lower TaqMeth V (<25.0) scores (n = 26). Cox-Mantel analysis comparing the two groups revealed a significant difference (P = 0.02); i.e., the group with higher methylation yielded a poorer prognosis than the group with lower methylation of HOP.

from the primary cell groups with successful growth in culture medium without attachment (Fig. 5C, *1* and *2*). These aggregates sometimes showed a signet-ring like appearance, characterized by round large cells in which the nucleus was distributed to the edge of the cytoplasm (Fig. 5C, *3*). In a confluent state, this phenotype regressed to some extent, but piled-up foci were still visible among the cells. Remarkably, three TE2-HOP knockdown clones showed a dramatic restoration of growth in soft agar (Fig. 5D). These findings suggest that loss of HOP expression plays an important role in ESCC cell growth and transformation.

Discussion

In this study, we applied a novel detection method to identify candidate methylated genes in combination with methods previously developed in our laboratory (5-8, 11, 13). We applied the combined expression profiles of primary tumor tissues to identify methylated genes, wherein the higher priority

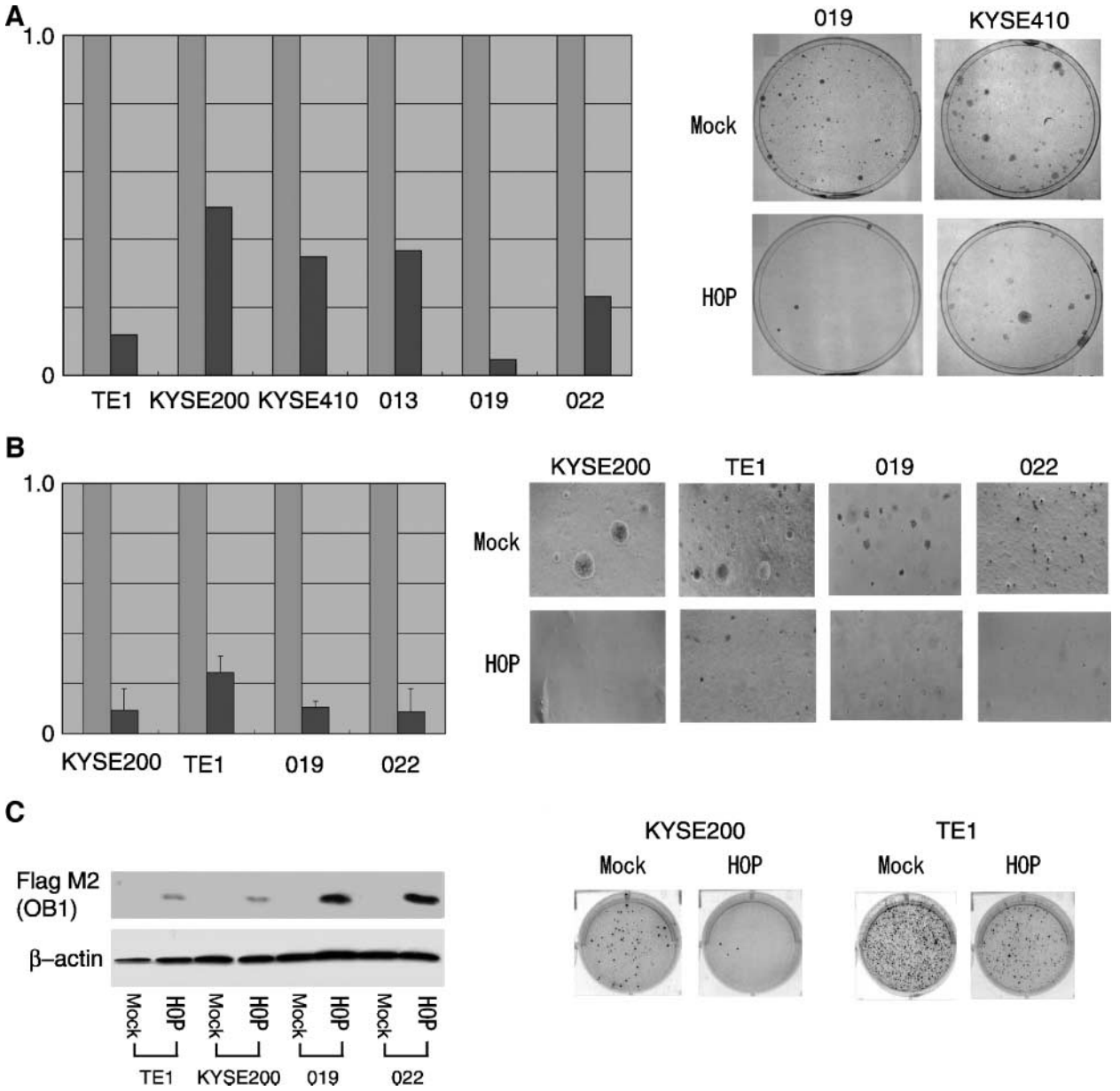

FIGURE 4. Functional relevance of HOP in human squamous cell carcinoma cells. **A.** Colony focus assay was done in six tumorigenic (in soft agar) cell lines (TE1, KYSE200, KYSE410, 013, 019, and 022). Colony numbers were markedly reduced 2 wk after transient transfection of HOP in all the cell lines tested. With control cells as 1.0, the colony forming ratio of the HOP transfectant is shown. **B.** Soft-agar assay showing that HOP stable transfectants were markedly suppressed in four different cell lines (TE1, KYSE200, 019, and 022). Top right, light microscopy of colony formation by KYSE200, TE1, 019, and 022 cells in soft agar. Bottom right, colony formation of KYSE200 and TE1 cells in soft agar. With control cells as 1.0, the colony forming ratio of the HOP transfectant is shown. **C.** Expression level of HOP fused to 3× Flag protein was determined by Western blot analysis with the Flag M2 antibody. β-Actin was used as an internal control.

group of genes from the reactivation profiles (5, 8) unexpectedly showed only a very limited correlation with gene expression in ESCC cell lines. This finding may be explained by artifact methylation in cell lines generated on establishment. Even when we identified methylation in cell lines, such methylation was only seen in a very limited number of genes in primary tumor tissues (8). However, the most important property of the genes we would like to identify is its clinical relevance to primary cancer. In primary tumor analysis, CVV could be a novel standard representing gene expression shutdown potential by methylation. Application of CVV in our algorithm was based on the notion that higher CVV does not necessarily mean that a gene was silenced by promoter DNA methylation, but that gene modification by methylation always displayed remarkable contrasts of gene expression in the presence and absence of promoter methylation. Through these methods, we identified numerous novel methylated genes as shown in Table 1.

We presented evidence that HOP is a true TSG by showing that HOP harbored cancer-prone methylation (Fig. 3C), and

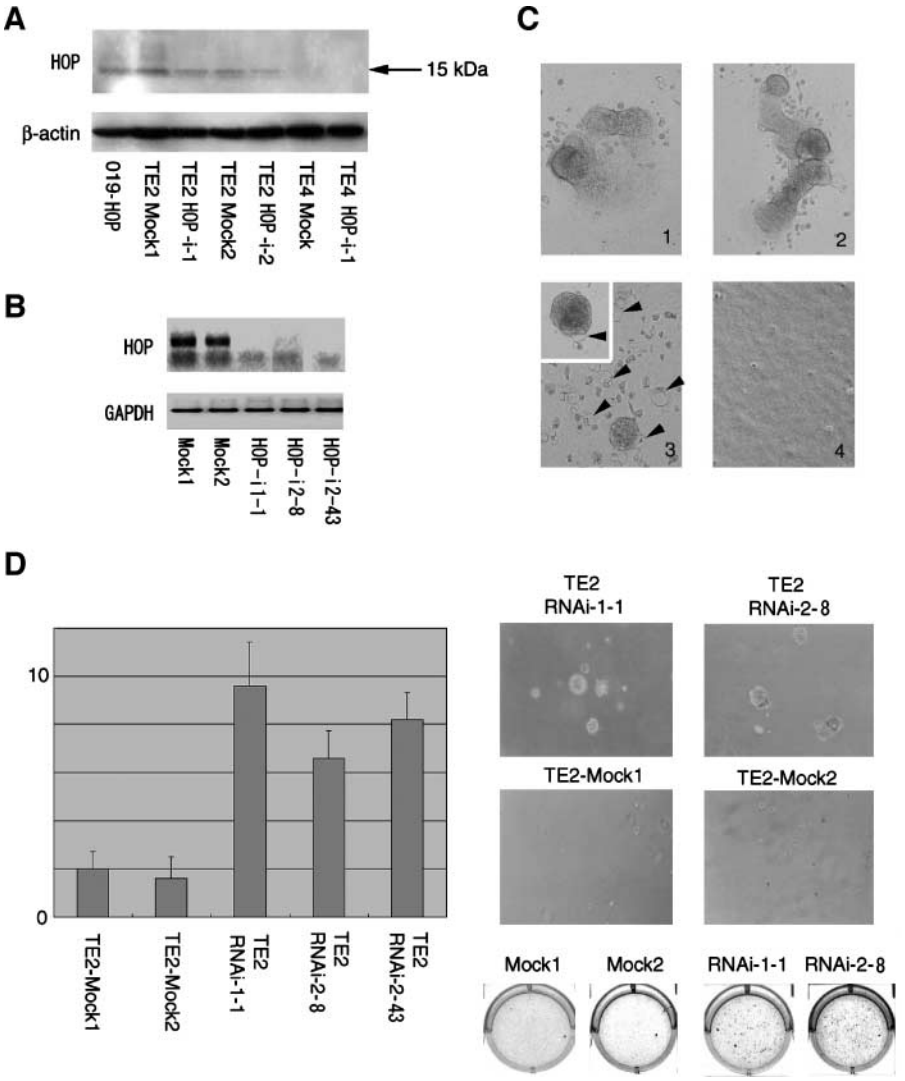

FIGURE 5. **A.** Validation of RNA interference of HOP using two kinds of interference sequences (*i-1* and *i-2*). Both sequences markedly down-regulated HOP at the protein level in TE2. Note that TE4 mock-transfected cells showed little HOP expression at the protein level. **B.** Establishment of stable clones after HOP knockdown (*i-1-1*, *i-2-8*, and *i-2-43*). Knockdown of endogenous HOP expression was evaluated by RT-PCR. **C.** After growing to confluence, clone i1-1 showed pile-up cell aggregates (*1* and *2*). Aggregate cells were sometimes dissociated from the primary portion and, intriguingly, a signet-ring like cell morphology was seen in the floating aggregates (*3*). Control cells displayed a sheet-like growth pattern with flattened distribution of cells (*4*). **D.** Soft-agar assays showed that HOP-suppressed cells recovered their tumorigenic ability in three clones as compared with mock cells (*i-1-1*, *i-2-8*, and *i-2-43*).

such a methylation pattern in tissues has been reported to be a landmark of many TSGs that actually have tumor-suppressive activity (16-19). HOP has been thus far reported to be down-regulated in several cancers such as choriocarcinoma (20) and head and neck squamous cell carcinoma (15); however, this is the first report to show that HOP is down-regulated in an epigenetic manner in human cancer even under the double control of the discrete promoter regions. We believe that methylation of TSGs is indicative of a suppressive vector during cancer promotion, and it would not necessarily reflect the absolute gene expression level. For example, minute methylation in the corresponding normal tissues might be equivalent to the potential of tumor-suppressive activity but would not reflect the absolute expression level in background tissues. Rather, final expression level would be regulated by constitutive signal transduction on the remaining unmethylated alleles in cancer cells.

Asanoma et al. (20) showed that transfection of the HOP gene into choriocarcinoma cell lines showed remarkable alterations in cell morphology and suppression of *in vivo* tumorigenesis. In our study, we also showed that HOP suppresses soft colony formation in ESCC (Fig. 4). These data strongly suggest that HOP is a bonafide TSG. However, a more intriguing result is that HOP expression in ESCC cell lines is closely associated with tumorigenic potential (Table 2; Supplementary Fig. S1). Tumorigenic cell lines (TE1, KYSE30, KYSE150, and KYSE200) tested in this study were all silenced or reduced in expression for both variants of HOP, whereas nontumorigenic cell lines (TE2, TE5, and KYSE520) expressed at least one HOP variant. TE4 and KYSE410 were exceptions, but TE4 barely expressed HOP at the protein level despite abundant expression at the mRNA level, suggesting posttranslational regulation of HOPα. On the other hand, KYSE410 was expressed in mock-treated cells, but the optimal demethylating treatments reactivated HOPβ expression more, suggesting the existence of a more upstream TSG regulated in an epigenetic manner. Moreover, tumorigenesis was suppressed in TE1, KYSE200, and KYSE410 after 5-aza-dC treatment in concert with reactivation of HOPβ, and HOP expression pattern was consistent with tumorigenic phenotype in nine ESCC cell lines.

Table 2. Constitutive Tumorigenesis in Squamous Cell Carcinomas Affected by Major Oncogenic Pathway

	Tumorigenesis	5-Aza-dC	PI3K Inhibitor*	Erk Inhibitor †	Rapamycin
TE1	++	–	–	++	+
TE2	–	–	–	–	NA
TE4	+	–	–	+	–
TE5	–	–	–	–	NA
KYSE30	+	+	–	+	+
KYSE150	+	+	–	+	+
KYSE200	++	–	–	++	+
KYSE410	+	–	–	+	+
KYSE520	–	–	–	–	NA
011	–	–	–	–	NA
012	–	–	–	–	NA
013	+	–	–	+	NA
019	+	–	–	++	NA
022	+	–	–	+	NA
028	+	–	–	++	NA
Fadu	+	–	–	++	NA
SCC15	–	–	–	–	NA

Abbreviations: PI3K, phosphatidylinositol 3-kinase; Erk, extracellular signal–regulated kinase.
*LY294002 was used as a specific phosphatidylinositol 3-kinase inhibitor.
† PD 98059 was used as a specific extracellular signal–regulated kinase inhibitor.

Convincingly, clones with HOPβ silencing were more clinically aggressive (Fig. 3D), and HOP RNA interference knockdown in the constitutively HOP-expressing cell line TE2 resulted in recovered oncogenic properties accompanied by increased anchorage-independent growth (Fig. 5). These results further support the notion that HOP has an inhibitory role in ESCC promotion. Although the silencing mechanism of HOPα in actual tumors is unknown, it may be suppressed by promoter regulation by other oncogenic signals instead of either genomic or epigenetic mechanism.

The mechanism of HOP involvement in tumorigenesis is unknown. HOP was proposed to be an antagonist of serum responsive factor (SRF; refs. 21, 22), and SRF acts downstream of the phosphatidylinositol 3-kinase pathway and is required and sufficient for cell growth (23). Hence, we could postulate that HOP is critical for squamous cell carcinoma tumor formation through antagonism of SRF downstream of the phosphatidylinositol 3-kinase pathway. We examined SRF activity in nine ESCC cell lines using a reporter vector harboring 2× SRF and 2× mSRF (Supplementary Fig. S2A). Unexpectedly, SRF activity did not correlate with tumorigenic potential in ESCC cell lines tested. Moreover, neither the phosphatidylinositol 3-kinase inhibitor LY29004 nor HOP necessarily suppressed SRF activity in all the cells tested (Supplementary Fig. S2A and B), suggesting that the contribution of HOP to tumorigenesis is mainly mediated by mechanisms other than SRF antagonism. Recent reports suggested that HOP can inhibit SRF activity by recruiting histone deacetylase to the target gene promoter regions, but it could not bind DNA itself directly (24). Moreover, Asanoma et al. (20) also reported that a deletion mutant of HOP in which the DNA binding domain was removed did not affect its *in vivo* tumorigenic ability in choriocarcinoma, suggesting that HOP could affect various transcriptional factors other than SRF to inhibit tumorigenesis.

HOP functions directly downstream of Nkx2.5 (21, 22) and GATA transcription factor involved in heart development (25). Recently, GATA family members have been reported to be methylated in human cancers (26), suggesting that a pathway involved in heat development is also crucial for cancer development. When this gene was knocked out in mice, only a cardiac phenotype was reported to be obtained, and apparently this homeobox gene is involved in the development of the heart. Shin et al. (21) showed that mice homozygous for a HOP null allele segregated into two phenotypic classes characterized by an excess or deficiency of cardiac myocytes. They proposed that HOP modulates SRF activity during heart development and that its absence results in an imbalance between cardiomyocyte proliferation and differentiation, with consequent abnormalities in cardiac morphogenesis. Chen et al. (22) found that inactivation of HOP in mice by homologous recombination resulted in a partially penetrant embryonic lethal phenotype with severe developmental cardiac defects involved in the myocardium. Nevertheless, there has been no direct evidence of this gene's involvement in tumorigenesis using a mice model, presumably due to an insufficient follow-up term to detect phenotypic change, or alternatively, HOP silencing possibly is involved in tumor promotion and not tumor initiation. Undefined background conditions to initiate carcinogenic steps may be needed to elicit tumorigenesis instead of abrogation of HOP. To actually show this issue, we plan to carry out a double-knockout experiment using p53-knockout mice or use ESCC carcinogenic models we recently developed (27). Finally, conditional targeting for HOP in esophageal mucosal cells might be required to resolve this specific issue.

In conclusion, we added HOP to the list of cancer-prone methylation genes in primary ESCC and clearly showed that HOP has a tumor-suppressive activity, which could in turn reflect clinical aggressiveness. Through accumulation of such genes, we have applied panels of methylated genes for early cancer detection in human body fluids. We have recently shown that such detection is feasible in breast (28), bladder (29), and prostate cancers (30). As cancer-prone methylation is little known in ESCC, HOP could be a promising candidate for an ESCC tumor marker. In addition, as double control of the discrete promoters has been reported as crucial in the TSG RASSF1 (16), HOP is likely to be a crucial molecule involved in tumorigenesis in ESCC. Recently, RASSF1 methylation has been actually reported to be exclusive of RAS/B-Raf mutation in various cancers (31, 32), suggesting that methylation can affect the identical pathway abrogated by gene mutations. In accord with these findings, the GATA/HOP pathway may be on the identical pathway affecting tumorigenesis in ESCC. We believe that accumulated identification of such critical pathways by studying methylation patterns would lead to the discovery of novel therapeutic targets in ESCC.

Materials and Methods

Microarray and RT-PCR Analysis

We carried out oligonucleotide microarray analysis on the GeneChip Human Genome 95 Ev (Affymetrix) containing 12,599 genes per manufacturer's instruction. Genes down-regulated in primary ESCC tissues against the corresponding normal-appearing mucosa were identified according to the manufacturer's algorithm. Expressions of tumor tissues and normal tissues were compared after correction considering the

total distribution of the gene expression per Affymetrix recommendation, in which the normal/tumor ratio was calculated and arranged. We believe that genes of functional importance showed remarkable contrast in gene expression during malignant conversion or even among individual tumors, and we designated CVV as the abbreviation for contrast-variation value, the maximum of either the corresponding normal/tumor expression ratio or T_{min}/T_{max} ratio, where both the tumor and the normal tissues were derived from those we examined (normal tissues, $n = 2$; tumor tissues, $n = 3$).

RT-PCR Analysis for Cell Lines after 5-Aza-dC and Trichostatin A Treatments

Cells were split to low density (1×10^6 per T-75 flask) 12 to 24 h before treatment. Cells were then treated for 4 days with 5 μmol/L 5-aza-dC (Sigma, Inc.) dissolved in 50% acetic acid or were mock treated with PBS including the same amount of acetic acid. As indicated, for some cell lines, trichostatin A was used to inhibit histone deacetylase as previously described (5). We isolated total RNA using Qiazol (Invitrogen, Inc.), reverse transcribed total RNA (8 μg) with Moloney murine leukemia virus (Invitrogen), and used one hundredth of the cDNA as a template for PCR. RT-PCR was done for 24 to 30 cycles at 95 °C for 1 min, 54 or 56 °C for 1 min, and 72 °C for 1 min or by touchdown PCR depending on the gene. Primer sequences for HOP are as follows: HOPcore F, 5′-CAGAGGACCAGGTGGAAATCC-3′; HOPα F, 5′-CAAACCCAGGGCTTGCGCTT-3′; HOPβ F, 5′-GGTCCCCCTTTCGGGAGGAA-3′; HOP R, 5′-GCGGAGGAGAGAAACAGAGAT-3′; GAPDH F, 5′-CGTCTTCACCACCATGGAGA-3′; and GAPDH R, 5′-CGGCCATCACGCCACACAGTTT-3′.

Sequencing and Real-time Quantitative PCR for Bisulfite-Treated DNA

DNA was prepared as previously described (5). The primers for sequencing were designed to recognize the DNA sequence alterations caused by bisulfite treatment as follows: HOP B F, 5′-TACAACCCTACCTAAAAAAAACCCC-3′; HOP B R, 5′-TTTTTTGGGGATAGATTTGATAGAT-3′. TaqMan methylation-specific PCR, the fluorescent probe, and primers were designed to hybridize to the amplified region of DNA (Q-HOP B F, 5′-CCAAAACGCCAACCCACTACG-3′; Q-HOP B R, 5′-GTGGGTTCGTTCGAGTTCGCG-3′; Q-HOP B probe, 5′-CGCCTACCGAAAAAACCGAAAACCGACTAATCGCG-3′). β-Actin was used as an internal control as previously described (7). For all reactions, 3 μL of bisulfite-treated DNA were added to a final volume of 20 μL. Serial dilutions of human leukocyte DNA (*in vitro* methylated) were used to construct a calibration curve, and all reactions were carried out in duplicate. The methylation ratio was defined as quantity of fluorescence intensity derived from HOP promoter amplification divided by the fluorescence intensity from β-actin amplification, multiplied by 100 [we designated this value as the TaqMan methylation value (TaqMeth V)].

Cell Lines and Tissue Samples

Five ESCC cell lines (KYSE30, KYSE150, KYSE200, KYSE410, and KYSE520) were kindly provided by Dr. Shimada of the Department of Surgery and Surgical Basic Science, Graduate School of Medicine, Kyoto University (Kyoto, Japan). Four ESCC cell lines (TE1, TE2, TE4, and TE5) were obtained from the Cell Response Center for Biomedical Research Institute of the Department of Aging and Cancer, Tohoku University (Sendai, Japan). Eight head and neck squamous cell carcinoma cell lines (011, 012, 013, 019, 022, 028, Fadu, and SCC15) were established at Johns Hopkins University, Department of Otolaryngology-Head and Neck Surgery (Baltimore, MD). All the squamous cell carcinoma cell lines were grown in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone). All primary ESCC cancers and corresponding normal appearing mucosal tissues were obtained from surgical specimens dissected at the Medical Institute of Bioregulation Hospital, Kyushu University, as previously described (7).

Development of Stable Clones with HOP Overexpression in ESCC Cell Lines

A full-length cDNA of HOP was isolated from normal-appearing esophageal mucosa using PCR with the primer sets 5′-CAGAAGCTTCCACCATGTCGGCGGAGACCGCGAGCGG-3′ and 5′-CAGGGATCCGTCTGTGACGGATCTGCACTCTG-3′. The restricted PCR products were ligated into p3xFLAG-CMV (Sigma) within the *Hin*dIII-*Bam*HI sites. The sequence of the cloned HOP cDNA was verified by sequence analysis and confirmed for absence of mutation in the open reading frame. Using this vector, we developed stable transfectants in four squamous cell carcinoma cell lines (TE1, KYSE200, 019, and 022) in which HOP was not constitutively expressed at the mRNA level. We carried out dot blot analysis for 20 clones resistant to G418 (600 μg/mL: Clontech, Inc.) in each cell line using antimouse Flag antibody M2 and selected clones with the most abundant expression of Flag-tagged HOP confirmed by Western blot analysis with M2 antibody. Cells were suspended in 200 μL of radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS] containing 1 mmol/L phenylmethylsulfonyl fluoride. The lysates were separated by standard Western blotting (33).

Anchorage-Independent Colony Formation Analysis in Soft Agar

Anchorage-independent cell growth was analyzed by plating 0.36% top agarose (Bacto Agar, Becton Dickinson, Inc.) containing 1×10^5 cells on a surface of 0.72% bottom agarose in six-well plates at 37°C. Cells were fed weekly by overlying fresh soft-agar solution, and colonies were photographed and counted under a light microscope after 3 weeks of incubation. Each experiment was done in triplicate wells and repeated thrice. Phosphatidylinositol 3-kinase inhibitor LY29004, extracellular signal–regulated kinase inhibitor PD 98059, rapamycin, and 5-aza-dC (all from Sigma) were used at final concentrations of 100, 10, 1, and 10 μmol/L, respectively.

RNA Interference of HOP

pSuppressor is a mammalian expression vector that suppresses endogenous RNA directing the synthesis of

siRNA-like transcripts with a polymerase III H1 RNA gene promoter. It produces a small RNA transcript lacking a polyadenosyl tail and has a well-defined start of transcription and a termination signal consisting of five thymidines in a row (T5; ref. 34). Cleavage of the transcript at the termination site occurs after the second uridine, yielding a transcript resembling the ends of synthetic siRNAs, which also contain two 3′ overhanging T or U nucleotides. We designed the HOP gene–specific inserts with a 19-nucleotide sequence (CTTCAACAAGGTCGACAAG) for the i-1 experiment and with a 18-nucleotide sequence (GGTCGACAAGCACCCGGA) for the i-2 experiment. The resulting transcript is predicted to fold back on itself to form a 19- or 18-bp stem-loop structure. Transfections were carried out using an optimized calcium phosphate method.

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Mol Cancer Res 2008;6:31-41.