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Frequent methylation-associated silencing of a candidate tumor-suppressor, *CRABP1*, in esophageal squamous-cell carcinoma

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Epigenetic alterations and the resulting inactivation of tumor suppressor genes often contribute to the development of various cancers. To identify novel candidates that may be silenced by aberrant methylation in esophageal squamous-cell carcinoma (ESCC), we analysed ESCC cell lines by a recently developed method known as bacterial artificial chromosome array-based methylated CpG island amplification (BAMCA), and selected candidates through BAMCA-assisted strategy. In the course of this program, we identified frequent CpG methylation-dependent silencing of the gene encoding cellular retinoic acid binding protein 1 (CRABP1) in our panel of ESCC cell lines. Expression of *CRABP1* mRNA was restored in gene-silenced ESCC cells after treatment with 5-aza 2′-deoxycytidine. The DNA methylation status of the *CRABP1* CpG island with clear promoter activity correlated inversely with expression of this gene. CpG methylation of *CRABP1* was frequently observed in primary ESCC tissues as well. Restoration of CRABP1 expression in ESCC cells lacking the protein reduced cell growth by inducing arrest at G_0–G_1, whereas knockdown of the gene in cells expressing CRABP1 promoted cell growth. Among 113 primary ESCC tumors, the absence of immunoreactive CRABP1 was significantly associated with de-differentiation of cancer cells and with distant lymph-node metastases in the patients. These results indicate that CRABP1 appears to have a tumor-suppressor function in esophageal epithelium, and its epigenetic silencing may play a pivotal role during esophageal carcinogenesis. Its expression status in biopsies or resected tumors might serve as an index for identifying ESCC patients for whom combined therapeutic modalities would be recommended.

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Introduction

Esophageal carcinoma is the sixth most frequent cause of deaths from cancer on a worldwide basis (Parkin *et al*., 2005), and esophageal squamous-cell carcinoma (ESCC) accounts for ~90% of esophageal carcinomas diagnosed in Asian countries. Although surgical techniques and perioperative management have progressed, the prognosis for patients with ESCC remains poor; this is largely because few ESCCs are diagnosed at an early stage and, even in apparently localized cases, early lymphogenous and hematogenous micrometastases may occur owing to the underlying anatomy (Roder *et al*., 1994). 'Combined-modality approaches', such as primary surgery with adjuvant chemotherapy or chemoradiotherapy, primary definitive chemoradiotherapy, or preoperative chemoradiotherapy followed by surgery, are known to be effective for eliminating micrometastases and facilitating complete tumor resection, the requisite for long-term survival. To identify correctly patients who should be treated with chemoradiotherapy, indices to enhance precision in clinical staging, especially for predicting latent distant mircometastases, are of critical importance. However, to the best of our knowledge no such indices are available at present.

Various genetic events that contribute to ESCC have been investigated in the context of mutations or disruptions in DNA sequence that either activate oncogenes or lead to loss of function of tumor-suppressor genes. However, the genetic changes that have been identified in ESCC cannot fully account for

the pathogenesis of this disease. Substantial evidence suggests that in addition to mutational inactivation or deletion of tumor-suppressor genes, epigenetic gene silencing plays an important role in development and progression of various human cancers (Jones and Baylin, 2002; Herman and Baylin, 2003; Laird, 2003; Egger *et al*., 2004). Since hypermethylation of promoter regions is a powerful and ubiquitous epigenetic mechanism of gene silencing, knowledge of methylation patterns across the cancer genome should help in identifying additional tumor-suppressor genes (Suzuki *et al*., 2002; Yamashita *et al*., 2002).

Alterations in DNA methylation patterns occur frequently in ESCC cells, involving many genes including *CRIP1*, *VHL*, *RARB*, *FHIT*, *RASSF1*, *PGP9.5*, *LRP1B* and *NMDAR2B* (Yamashita *et al*., 2002; Kuroki *et al*., 2003; Sonoda *et al*., 2004; Mandelker *et al*., 2005; Kim *et al*., 2006); however, of that group none has proven to be a sufficiently reliable marker for predicting micrometastasis in clinical settings. Numerous additional genes with potential tumor suppressor functions are likely to be targets for methylation-dependent silencing, and they might serve as indices for guiding therapeutic decisions for ESCC patients.

Recently, we developed a method of screening for aberrantly methylated sequences in cancer genomes, called 'bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (MCA)' (BAMCA; Inazawa *et al*., 2004), which incorporates our in-house BAC-based array combined with MCA (Toyota *et al*., 1999). In an attempt to identify epigenetic targets with biological and clinical significance in ESCC, we used BAMCA to screen four ESCC cell lines for aberrantly methylated regions, and analysed selected genes of interest within identified regions in terms of expression in large panels of ESCC cells. Using this strategy we successfully identified the gene encoding *cellular retinoic acid binding protein 1 (CRABP1)* as a candidate tumor suppressor for ESCC.

Results

Methylation analysis of ESCC cell lines by BAMCA

To identify epigenetic targets in ESCC, we chose an approach using BAMCA for initial screening of ESCC cell lines for aberrantly hypermethylated sequences (Misawa *et al*., 2005). A summary of our strategy and partial results is provided in Figure 1a and Supplementary Table S1. In view of the results of BAMCA using genomic DNA from each of the selected cell lines (TE-4, TE-5, KYSE-150 and KYSE-510) and from NEK2, a line derived from normal esophageal epithelium, as test and control DNAs respectively, we selected 61 BAC clones containing 105 genes that showed higher Cy3/Cy5 ratios (>1.5 after normalization) in all four cell lines. Since selected BAC clones may contain aberrantly methylated sequences, which are not associated with gene expression, as a result of their size, we analysed these 105 genes on the human genome database for (a) the existence of CpG islands and at least two *Sma*I sites, which may be recognized as methylated sites in BAMCA, around putative transcription start sites; and (b) expression patterns in multiple tissues and tumors (http://www.lsbm.org/database/index.html and http://www.ncbi.nih.gov/index.html). After that examination we focused on 59 genes whose expression we judged might be downregulated through hypermethylation of CpG sites. We then narrowed the selection of candidates further by sequentially analysing (a) the expression status of each gene; (b) restoration of gene expression after treatment with 5-aza 2′-deoxycytidine (5-aza-dCyd) and (c) the methylation status of CpG islands around each candidate gene in ESCC cell lines. Initial screening of five genes of interest, including one transporter, one channel protein and three transcription factors, selected from those 59 candidates based on known or predicted function revealed *CRABP1*, located within RP11-10K12 (Figure 1b), as a putative target for epigenetic downregulation in ESCC. Three CpG islands (CpG-1, 2 and 3) exist respectively around exons 1 and 2 and in intron 2 of the *CRABP1* gene (Figure 1c), according to CpGPLOT program (http://www.ebi.ac.uk/emboss/cpgplot/).

To confirm that *Sma*I sites around *CRABP1* were methylated in each of the four ESCC cell lines used in BAMCA, we determined methylation status by bisulfite sequencing. Two of three *Sma*I sites were hypermethylated in all four lines but no *Sma*I sites were methylated in NEK2 (Figure 1c), suggesting that regions between two of the *Sma*I sites might have been targets for adapter-polymerase chain reactions (PCR) amplification. Two *Sma*I sites around CpG-3 (sites B and C) were hypermethylated in TE-4 and -5, whereas the *Sma*I sites lying more 5′ of this gene (sites A and B) were hypermethylated in KYSE-150 and -510. Those results prompted us to examine whether a DNA methylation mechanism was the main reason for the silencing of *CRABP1*.

Analysis of CRABP1 *expression in ESCC cell lines*

Among the 43 ESCC cell lines in our panel, 34 (79%) showed either no expression or remarkable under-expression of the *CRABP1* gene by reverse transcriptase (RT)–PCR (Figure 1d). Among the four ESCC cell lines examined by BAMCA, KYSE-150 and -510 showed loss of *CRABP1* expression, whereas TE-4 and -5 retained expression of this gene.

In ESCC cell lines lacking endogenous *CRABP1* expression (e.g., KYSE-510 and TE-14), expression of *CRABP1* mRNA was restored by demethylation with 5-aza-dCyd, but not by inhibition of histone deacethylation with trichostatin A (TSA, Figure 1e). TSA exerted no synergistic effect on the restoration of *CRABP1* mRNA by 5-aza-dCyd.

Methylation of CRABP1 *CpG islands in ESCC cell lines*

Hypermethylated *Sma*I sites observed in KYSE-150 and -510 cells lacking expression of *CRABP1* lie more 5′ than the methylated sites in TE-4 and -5 cells, which

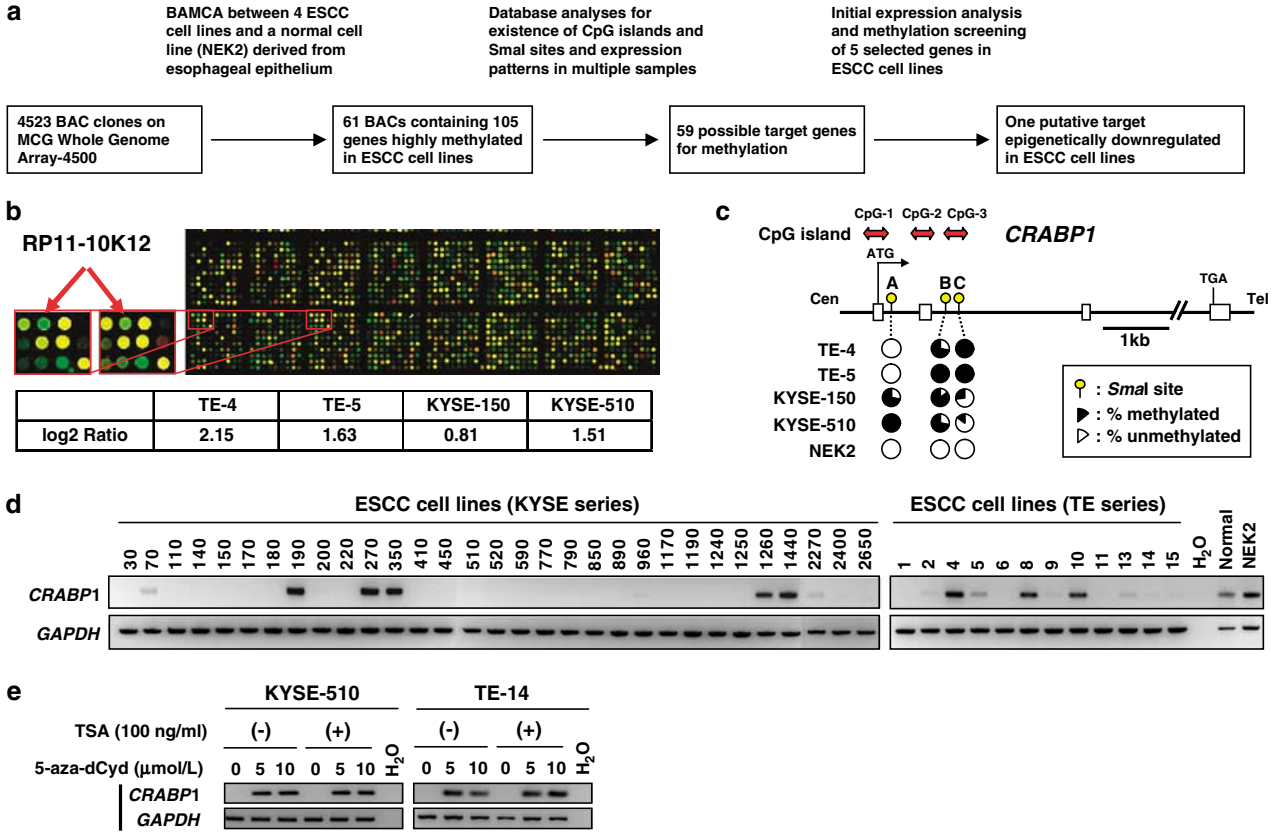

Figure 1 (**a**) Strategy for identifying putative epigenetic targets in ESCC cell lines. (**b**) Representative image of BAMCA applied to the TE-4 cell line. Green, yellow and red spots indicate BACs respectively containing highly methylated, similarly methylated and unmethylated fragments in TE-4 compared with NEK2; black spots, no detectable methylated sequences in either test or control samples. The RP11-10K12 BAC (arrows) including *CRABP1* was detected as a spot with a high Cy3/Cy5 ratio in all four cell lines tested by BAMCA. (**c**) Genomic structure of the *CRABP1* gene and methylation status of *Sma*I sites around its putative promoter sequence in the cell lines used for BAMCA. Three CpG islands (CpG-1, -2 and -3) exist respectively around exon 1, exon 2 and in intron 2 (GenBank accession no. NM_004378 for cDNA sequence and NT_010194 for genomic sequence); exons are indicated by clear boxes. Results of bisulfite sequencing of these five cell lines are shown below; open and filled fans in each circle represent ratios of unmethylated and methylated *Sma*I sites, respectively. (**d**) Representative results of RT–PCR analysis of *CRABP1* mRNA in ESCC cell lines, normal esophagus and NEK2 cell line. *GAPDH* was used as an internal control. (**e**) Representative results of RT–PCR analysis to restore *CRABP1* expression in *CRABP1*-nonexpressing ESCC cell lines after treatment with various concentrations of 5-aza-dCyd for 5 days and/or 100 ng/ml TSA for 12 h. *GAPDH* was used as an internal control.

retain its expression, indicating that methylation of the *Sma*I site located between CpG-1 and CpG-2 was inversely correlated with *CRABP1* expression. Therefore, we decided to compare the methylation status of the first two CpG islands, that is CpG-1 (a 355-bp genomic sequence covering exon 1; −142 IVS to +67) and CpG-2 (a 321-bp sequence covering exon 2; −117 IVS to +25), with the expression of *CRABP1* in ESCC cells.

First, we performed combined bisulfite restriction analysis (COBRA) to determine relationships between expression of *CRABP1* and methylation within CpG islands in ESCC cell lines and NEK2. Restricted fragments from methylated alleles in CpG-1 were detected primarily in ESCC lines without *CRABP1* expression, whereas unmethylated alleles were predominant in lines with its expression and in NEK2 (Figure 2a). On the other hand, fragments from methylated alleles in CpG-2 were detected in all ESCC lines regardless of *CRABP1* expression, implying that CpG-1 is a more critical site than CpG-2 for epigenetic events affecting *CRABP1* expression. Therefore, we focused on CpG-1 for further methylation analysis, using bisulfite-sequencing. Consistent with the results of COBRA, aberrant DNA methylation in the CpG-1 region was observed in cell lines lacking *CRABP1* expression, but not in cell lines expressing this gene (Figure 2b).

Promoter activity of CpG-1

We examined regions within CpG-1 for promoter activity by means of a reporter assay, using five fragments constructed according to the result of the bisulfite-sequencing (Figure 2b). Fragments representing Regions 4 and 5, especially Region 5, revealed strong luciferase activity in comparison with Regions 1 and 2 in each cell line, regardless of the expression status of *CRABP1* (Figure 2c). These results indicated that a putative promoter is located in CpG-1, in particular

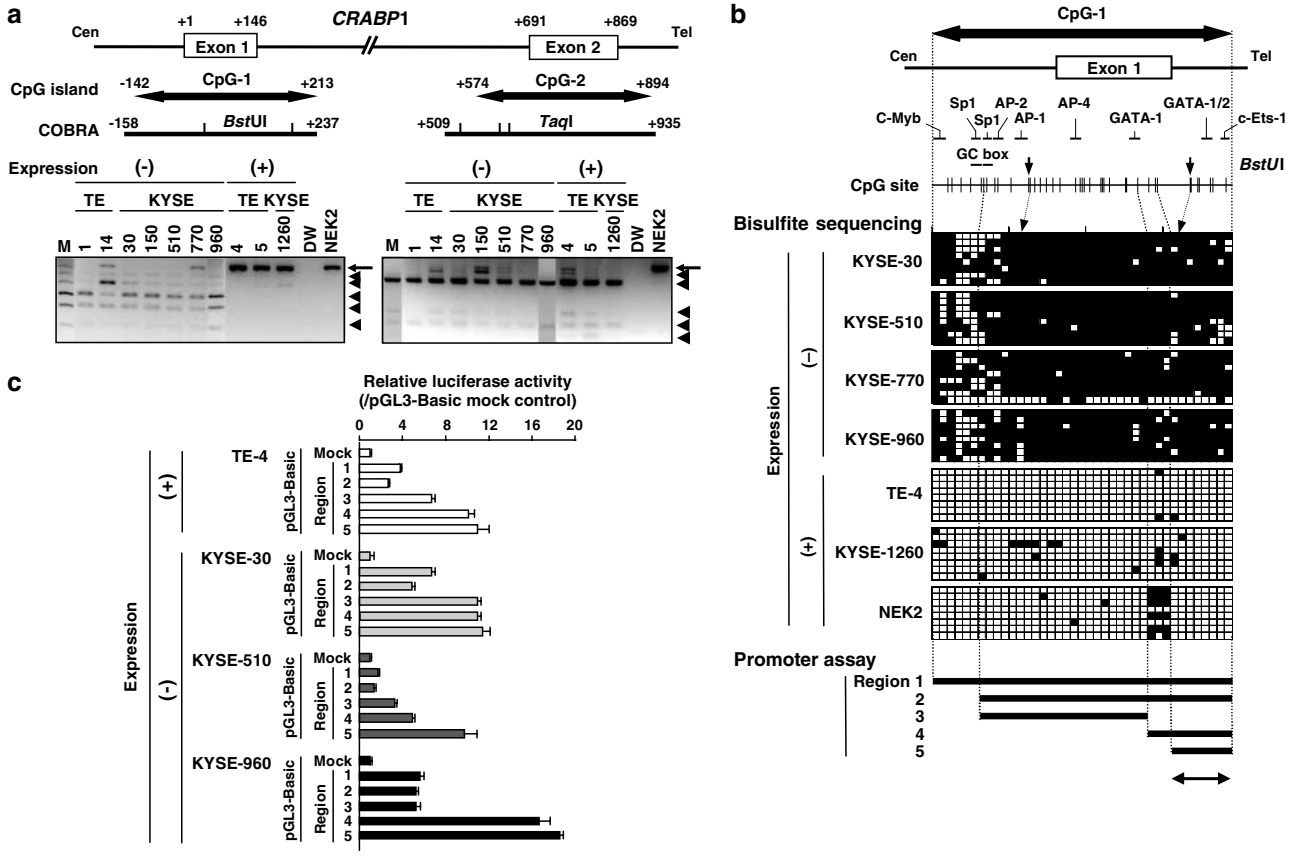

Figure 2 Methylation status of the CpG island of *CRABP1* in ESCC cell lines. (**a**) Representative results of COBRA involving CpG-1 and CpG-2 in ESCC cell lines with (+) or without (−) *CRABP1* expression, and in NEK2. A 395-bp PCR product including CpG-1 and a 427-bp PCR product including CpG-2 (horizontal bars with arrowheads) were restricted respectively by *Bst*UI and *Taq*I. Vertical tick marks, restriction sites. In the lower panel, arrows indicate unmethylated alleles; arrowheads, methylated alleles. Universal Methylated DNA (M) was used as a positive control. (**b**) (Top) Map of the CpG island around exon 1 (CpG-1) in *CRABP1*. CpG sites are represented by vertical tick marks. GC boxes and binding sites for representative transcription factors are shown by horizontal thin bars. (Middle) Results of bisulfite-sequencing performed in *CRABP1*-nonexpressing and expressing cell lines. Open and filled squares represent unmethylated and methylated CpG sites respectively, and each row represents a single clone. *Bst*UI restriction sites are indicated by arrows. (Bottom) Fragments examined in a promoter assay and the putative promoter region analysed in Figure 2c are indicated by horizontal bars and closed arrow, respectively. (**c**) Promoter activity of CpG-1. pGL3 basic empty vectors (mock) and reporter constructs, each containing one of five different sequences within CpG-1, were transfected into *CRABP1*-expressing cell line TE-4 and *CRABP1*-nonexpressing cell lines KYSE-30, -510 and -960. Luciferase activities were normalized *versus* an internal control. The data presented are the means ± s.d. of three separate experiments, each performed in triplicate.

Region 5 and that methylation of this promoter appears to be sufficient for repression of *CRABP1* transcription irrespective of the presence of transcription factors capable of inducing the gene (Figure 2b).

Analysis of CRABP1 *methylation and expression in primary ESCC tumors*

To determine the extent to which aberrant methylation of *CRABP1* might be involved in primary ESCCs, we examined the methylation status of this gene in 36 frozen sections of primary ESCCs, with corresponding non-cancerous esophageal mucosa in six cases of them, by COBRA of CpG-1 (Figure 3a). Clearly, methylated alleles were detected in 19 of the tumors (52.8%). Non-cancerous esophageal mucosa showed only unmethylated allele. To confirm the results of COBRA, we performed bisulfite sequencing of CpG-1 in individual alleles from representative primary ESCC tumors with corresponding non-cancerous tissues (Figure 3b). Hypermethylation, in a pattern similar to that observed in ESCC lines lacking *CRABP1* expression, was detected in methylation-positive ESCC tumors in COBRA, but not in methylation-negative tumors in COBRA and non-cancerous esophageal mucosa.

In six ESCC cases with tumor samples available for triple analyses, such as methylation analysis, real-time RT–PCR and immunohistochemistry, we compared expression status of CRABP1 with its methylation status (Figure 3c and d). Methylation-positive tumors tended to express lower *CRABP1* mRNA compared with methylation-negative tumors even in the small number of cases (Figure 3c). Two non-cancerous esophageal tissues in cases whose high quality RNA were available for mRNA analysis showed higher expression levels compared with corresponding tumors as well as other four ESCC tumors. In addition, methylation-negative tumors showed positive CRABP1

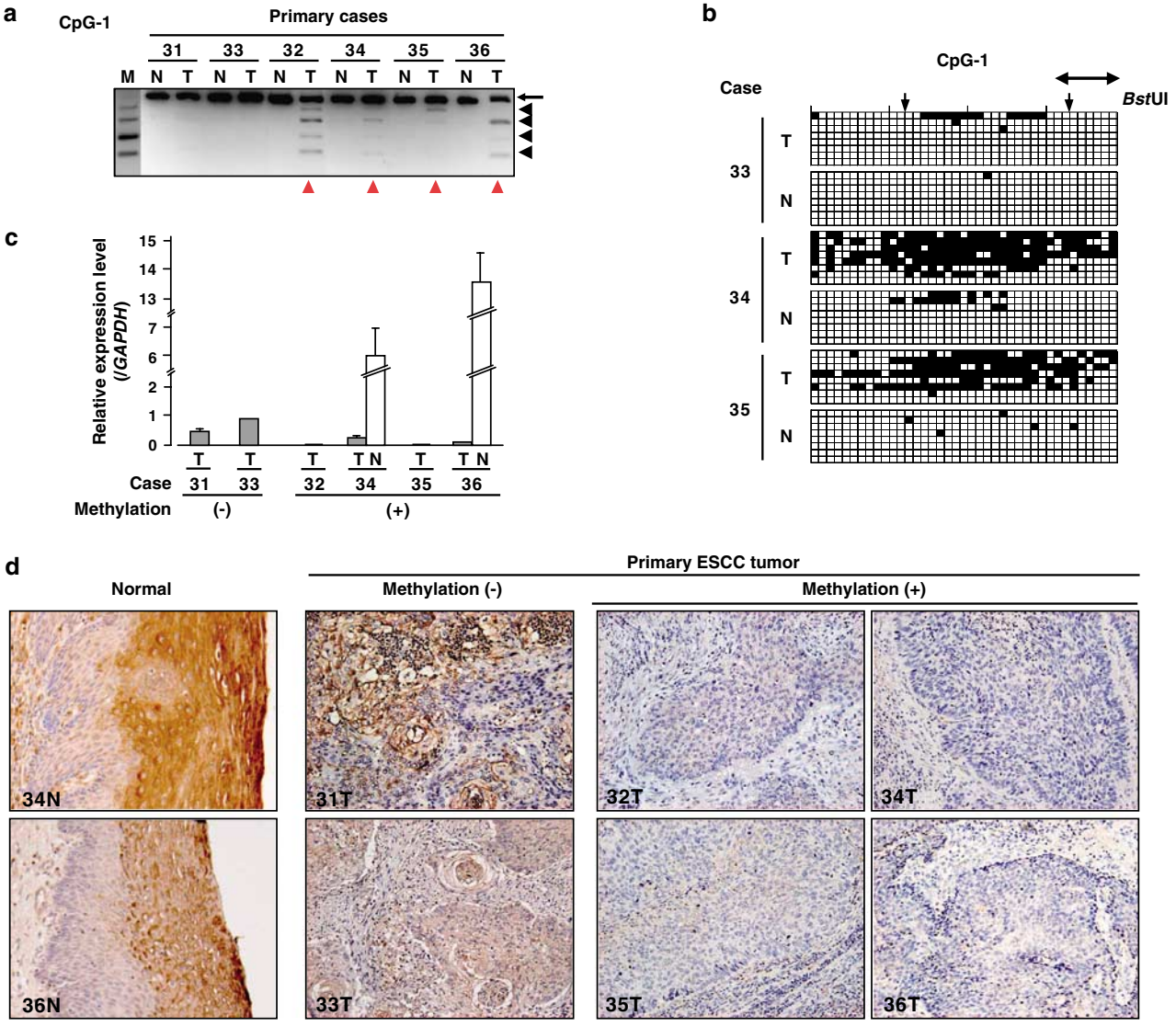

Figure 3 Methylation status of the CpG island and expression levels of *CRABP1* in primary tumors of ESCC. (**a**) Representative results of examination of CpG-1 by COBRA in surgically resected primary ESCC tumors (T) and corresponding non-cancerous esophageal mucosa (N). See legend for Figure 2a for interpretation. Red arrowheads indicate cases showed methylated pattern in tumors. (**b**) Results of bisulfite-sequencing of representative cases analysed in COBRA. See legend for Figures 2b and 3a for interpretation. Closed arrow indicates the putative promoter region (Figure 2c). (**c**) The level of CRABP1 mRNA expression determined by quantitative real-time RT–PCR. Among six cases, four methylation-positive ESCC tumors by COBRA showed lower *CRABP1* mRNA expression compared with two methylation-negative tumors. Non-cancerous esophageal tissues in cases 34 and 36 showed higher expression levels compared with corresponding tumors as well as other ESCC tumors. (**d**) Representative results of immunohistochemical staining of CRABP1 protein. Tumors in two unmethylated cases showed positive CRABP1 staining in $\geqslant 10\%$ of cancer cells, whereas four methylated cases showed positive CRABP1 staining in $<10\%$ of cancer cells. In neighboring non-neoplastic tissues, staining for CRABP1 started in the prickle-cell layer and increased in intensity toward the superficial layers, but was not observed in the basal layer. CRABP1 immunoreactivity was present mainly in the cytoplasm of normal esophageal epithelial cells, and also in cytoplasm of immunopositive malignant cells. Magnifications are $\times 200$.

immunostaining in $\geqslant 10\%$ of cancer cells, whereas methylation-positive showed positive CRABP1 staining in $<10\%$ of cancer cells (Figure 3d), suggesting that the methylation status in CpG-1 is likely to be inversely correlated with mRNA and protein expression of CRABP1. In neighboring non-neoplastic tissues, staining for CRABP1 started in the prickle-cell layer and increased in intensity toward the superficial layers, but was not observed in the basal layer. The results implied that hypermethylation of the CpG-1 might be a relatively common mechanism for silencing *CRABP1* during esophageal carcinogenesis.

Suppression of ESCC cell growth after restoration of CRABP1 expression

To investigate whether restoration of CRABP1 expression would suppress growth of ESCC cells lacking

endogenous *CRABP1*, we performed colony-formation assays using the full coding sequence of *CRABP1* cloned into a mammalian expression vector. Three weeks after transfection and subsequent selection of drug-resistant colonies, the numbers of large colonies produced by *CRABP1*-transfected KYSE-30 and -510 cells decreased markedly compared to cells containing empty vector (Figure 4a). Furthermore, transfected cell lines that stably expressed CRABP1, having been established from KYSE-30 and -510 cell lines, showed a lower growth rate compared to cell lines transfected with control vector alone (Figure 4b). On the basis of the fact that CRABP1 binds retinoic acid (RA) with a high affinity (Fiorella *et al*., 1993), we examined differences in responsiveness to RA between growth of transfectants stably expressing CRABP1 and empty-vector cell lines, using 0.01–100 μmol/l of all-*trans*-retinoic acid (ATRA). However, no significant difference was observed (data not shown).

Acceleration of ESCC cell growth after knockdown of CRABP1 expression

To confirm a growth-suppressive effect of CRABP1, we knocked down endogenously or exogenously expressed CRABP1 using *CRABP1*-specific small interfering RNA (*CRABP1*-siRNA; Figure 4c). Transient transfection of *CRABP1*-siRNA to TE-4 cells retaining expression of CRABP1 or to transfectants stably expressing CRABP1 effectively reduced expression of CRABP1 protein 48–96 h after transfection. Knockdown of CRABP1 accelerated cell growth in the TE-4 cell line, and restored the growth rate of stable transfectants, at least partially, compared with *luciferase*-specific small interfering RNA (siRNA) (*Luc*-siRNA) transfected counterparts.

Effect of CRABP1 expression on arrest of the cell cycle

To examine the mode of action of CRABP1 on the growth of ESCC cells, we analysed the cell cycle in *CRABP1*-stable transfectants and their control counterparts by fluorescence-activated cell sorting (FACS). Stable transfectants resulted in an accumulation of cells in G_0–G_1 and a decrease in S-phase cells compared with control clones (Figure 4d), suggesting that CRABP1 may arrest ESCC cells at the G_1–S checkpoint (G_0–G_1 arrest). No increase in the sub-G_1 phase was observed in *CRABP1*-stable transfectants compared with control clones, and no SA-β-gal-positive cells were seen in senescence assays (data not shown), suggesting that neither apoptosis nor senescence was a significant consequence of ectopic expression of CRABP1.

Next we examined expression of some molecules associated with the G_1-S checkpoint. KYSE-30 and -510 cells stably expressing CRABP1 showed increased levels of p27 protein compared with control clones (Figure 4e). In contrast, p21 protein was increased in KYSE-30 stable transfectants, but not in KYSE-510 transfectants. RT–PCR analysis revealed no increases in levels of *p21* or *p27* mRNAs in either transfectant line (Figure 4f), suggesting that the increased amounts of p27 or p21 had arisen as a result of post-transcriptional modification. To examine the effect of endogenous CRABP1 on expression of p21 and p27, we transfected *CRABP1*-siRNA or *Luc*-siRNA into TE-4 cells and subsequently determined protein levels 72 h after transfection (Figure 4g). The amount of p27 protein decreased with knockdown of CRABP1, but p21 was not affected.

Somatic mutation of CRABP1 *in ESCC*

To investigate possible involvement of mechanisms other than DNA methylation for inactivating *CRABP1*, we performed mutational analyses of the entire coding region using 43 cell lines and 30 primary tumors. Only one of the cell lines, KYSE-110, harbored a novel nucleotide change in the *CRABP1* gene, A1C in exon 1, resulting in loss of a start codon and consequent deletion of the first nine amino-acid residues. However, neither that change nor any other novel nucleotide change was present in the original tumor that had been implanted in nude mice to establish KYSE-110, suggesting that the nucleotide change in this cell line might have arisen during *in vitro* culture.

Association between CRABP1 expression and clinicopathologic factors in primary ESCCs

To verify downregulation of CRABP1 expression in primary ESCCs and to judge its clinicopathologic significance, we performed immunohistochemical analyses of CRABP1 protein in 113 primary tumor tissues. Among the 113 ESCCs, 39 (34.5%) showed positive immunoreactivity of CRABP1 in $\geqslant$10% of cancer cells (positive in Table 1), whereas 74 (65.5%) showed $<$10% or no immunoreactivity (negative in Table 1).

Negative CRABP1 expression was more frequent in poorly differentiated tumors than in well differentiated tumors (histopathological grading, $P=0.004$); in pT1 tumors compared with pT2/3 tumors (pT categories, $P=0.017$); in pM1/1a/1b (pM1; non-regional lymph-node metastasis) tumors compared with pM0 tumors (pM categories, $P=0.029$); and in stage I or IV tumors compared with stage II or III tumors (stage, $P=0.032$). Univariate analyses of overall survival by log–rank tests failed to demonstrate any significant association between CRABP1 expression patterns and overall survival of patients (data not shown).

Since metastasis to distant lymph nodes is an important criterion for selecting combined-modality therapy for patients with ESCC, we undertook a detailed analysis to determine whether CRABP1 immunostaining might be useful for predicting the existence of distant metastases. As shown in Table 2, most of the pM1 tumors (16/18, 88.9%) had negative CRABP1 immunoreactivity independent of the depth of tumor invasion (pT category). In pT2 or pT3 patients, whose micrometastases need to be correctly predicted for selection of therapeutic approaches, negative CRABP1 immunoreactivity was significantly correlated with non-regional lymph-node metastasis (pM1, $P=0.042$; Table 3).

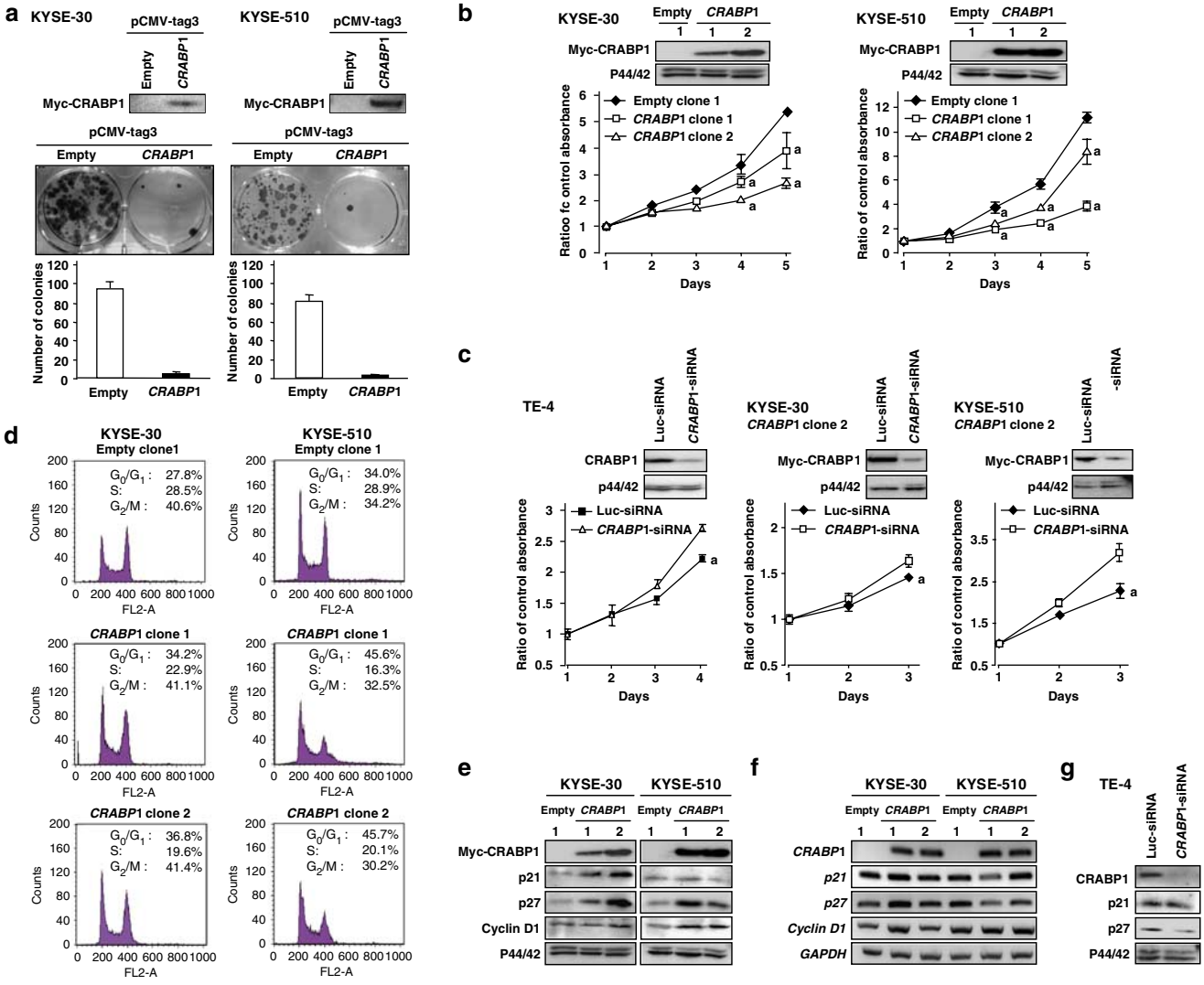

Figure 4 Effects of restoration or knockdown of CRABP1 expression on growth of ESCC cells. (**a**) Colony-formation assays using ESCC cell lines. Cells without *CRABP1* expression (KYSE-30 and -510) were transiently transfected with a Myc-tagged construct containing *CRABP1* (pCMV-Tag3-*CRABP1*), or empty vector (pCMV-Tag3-empty) and selected for 3 weeks with appropriate concentrations of G418. (Top) Western blotting prepared with 20 μg of protein extract and anti-Myc antibody, demonstrating that cells transiently transfected with pCMV-Tag3-*CRABP1* expressed Myc-tagged CRABP1. (Middle) Three weeks after transfection and subsequent selection of drug-resistant colonies, the colonies formed by *CRABAP1*-transfected cells were less numerous than those formed by empty vector-transfected cells. (Bottom) Quantitative analysis of colony formation. Colonies larger than 2 mm were counted, and results are presented as the means $\pm$ s.d. (histogram) of three separate experiments, each performed in triplicate. (**b**) Inhibitory effect of CRABP1 on the growth of KYSE-30 and -510 cells transfected with pCMV-Tag3-*CRABP1* or empty vector and selected with G418 to establish clones stably expressing CRABP1. (Top) Two clones transfected into KYSE-30 and -510 with pCMV-Tag3-*CRABP1* were subjected to Western blotting using anti-Myc-Tag antibody. (Bottom) Effect of stable CRABP1 expression on the growth of KYSE-30 and -510 cells. Cell viability was determined by WST assay at the indicated times in cell lines stably expressing CRABP1 and a cell line transfected with vector alone (empty clone). The data presented are the means $\pm$ s.d. of three separate experiments. Statistical analysis used the Mann–Whitney *U* test: a, empty clone versus CRABP1 clone; all, $P<0.05$. (**c**) Effect of CRABP1 knockdown by specific siRNA on the growth of TE-4 cells endogenously expressing CRABP1 and *CRABP1*-stable transfectants. Levels of CRABP1 protein were determined by Western blotting. See legend for Figure 4b for interpretation of the growth assay. (**d**) FACS analysis of *CRABP1*-stable transfectants (*CRABP1* clones) and control clones (empty clones) examined the mechanism behind the antiproliferative effect of CRABP1. *CRABP1*-stable transfectants established from KYSE-30 and -510 cells accumulated in G_0–G_1 phase and the population of S-phase cells decreased in comparison with control clones. (**e**) Expression of proteins associated with the G_1–S checkpoint. The level of each protein was determined on Western blotting using protein extract prepared from *CRABP1*-stable transfectants and their control counterparts (empty clones). (**f**) RT–PCR analysis of genes associated with the G_1–S checkpoint in *CRABP1*-stable transfectants and their control counterparts (empty clones). *GAPDH* served as an internal control. (**g**) *CRABP1*-siRNA or control *Luc*-siRNA was transfected into TE-4 cells, with the expression level of each protein subsequently determined by Western blot analysis. p27 protein decreased with knockdown of endogenous CRABP1 in this cell line, whereas the level of p21 protein was not changed.

Table 1 Association between clinicopathologic factors and CRABP1 expression

		CRABP1 immunoreactivity		
	N	*Positive (%)*	*Negative (%)*	P-*value**
Total	113	39 (34.5)	74 (65.5)	
Gender				
Male	93	31 (33.3)	62 (66.7)	0.569
Female	20	8 (40.0)	12 (60.0)	
Age (y)				
Mean	63.7			
>60	37	13 (35.1)	24 (64.9)	0.923
⩽60	76	26 (34.2)	50 (65.8)	
Location				
Cervical	5	1 (20.0)	4 (80.0)	0.833
Upper thoracic	9	4 (44.4)	5 (55.6)	
Mid-thoracic	56	19 (33.9)	37 (66.1)	
Lower thoracic	43	15 (34.9)	28 (65.1)	
Histopathological grading				
Well	38	20 (52.6)	18 (47.3)	**0.004**
Moderately	60	18 (30.0)	42 (70.0)	
Poorly	15	1 (6.7)	14 (93.3)	
pT categories				
pT1	19	2 (10.5)	17 (89.5)	**0.017**
pT2/3	94	37 (39.4)	57 (60.6)	
pN categories				
pN0	31	12 (38.7)	19 (61.3)	0.564
pN1	82	27 (32.9)	55 (67.1)	
pM categories				
pM0	95	37 (38.9)	58 (61.1)	**0.029**
pM1/1a/1b	18	2 (11.1)	16 (88.9)	
Stage				
I	9	1 (11.1)	8 (88.9)	**0.032**
II A/II B	36	16 (44.4)	20 (55.6)	
III	50	20 (40.0)	30 (60.0)	
IV/IV A/IV B	18	2 (11.1)	16 (88.9)	

Statistically significant values are in boldface type. **P*-values are from χ^2 or Fisher's exact test and were statistically significant when <0.05.

Table 2 Background data of each patient with distant metastasis (pM1, pM1a and pM1b)

CRABP1 immunoreactivity	*Age (years)*	*Gender*	*Differentiation*	*Location*	*pT*	*pN*	*pM*	*pStage*
Negative	75	M	Mod	Mt	1	0	1b	IVB
	54	M	Mod	Mt	1	0	1b	IVB
	62	M	Mod	Mt	1	1	1b	IVB
	76	M	Poor	Mt	1	1	1b	IVB
	60	M	Mod	Ce	2	1	1	IV
	71	M	Well	Lt	2	1	1b	IVB
	46	F	Mod	Lt	2	1	1b	IVB
	62	M	Poor	Mt	2	1	1b	IVB
	49	F	Mod	Ce	3	1	1	IVB
	45	M	Mod	Lt	3	1	1a	IVA
	76	M	Mod	Lt	3	1	1a	IVA
	84	M	Mod	Ut	3	1	1a	IVA
	61	M	Mod	Ut	3	1	1a	IVA
	62	M	Poor	Ut	3	1	1a	IVA
	65	M	Mod	Lt	3	1	1b	IVB
	75	M	Mod	Lt	3	1	1b	IVB
Positive	49	M	Mod	Mt	3	1	1b	IVB
	55	F	Mod	Mt	3	1	1b	IVB

Abbreviations: mod, moderately; poor, poorly; Ce, cervical; Ut, upper thoracic; Mt, mid-thoracic; Lt, lower thoracic.

Table 3 Association between pM categories of pT2/3 patients and CRABP1 expression

		CRABP1 immunoreactivity		
	N	*Positive (%)*	*Negative (%)*	P-*value**
M0	80	35 (43.8)	45 (56.3)	**0.042**
M1/1a/1b	14	2 (14.3)	12 (85.7)	

Statistically significant values are in boldface type. **P*-value is from Fisher's exact test and was statistically significant when <0.05.

Discussion

We searched for highly methylated DNA fragments in ESCC cell lines, and identified *CRABP1* as a target for the methylation of CpG islands observed in multiple cell lines and primary tumors of ESCC. Forced expression of CRABP1 in ESCC cells lacking its endogenous expression suppressed cell growth, whereas knockdown of this gene in cells expressing either endogenous or exogenous CRABP1 promoted cell growth. The level of immunoreactive CRABP1 protein in primary ESCC tumors appeared to be associated with the differentiation status of cancer cells and with distant lymph-node metastases, suggesting that loss of CRABP1 may play a pivotal role during esophageal tumorigenesis.

CRABP1 locates at 15q25.1, a chromosomal region that is rarely involved in loss of heterozygosity or shows copy-number losses in ESCCs (Shibagaki *et al*., 1994; Pimkhaokham *et al*., 2000). In addition, mutation of *CRABP1* was rare among cell lines and primary tumors we examined, suggesting that inactivation of this gene might occur mainly by biallelic methylation. Similar findings have been reported for several other genes, including *PGP9.5*, *NMDAR2B* and *CRIP1*, all of which are located in regions only occasionally deleted in ESCCs (Yamashita *et al*., 2002; Mandelker *et al*., 2005; Kim *et al*., 2006).

Others have reported methylation of *CRABP1* in thyroid and colon cancers (Huang *et al*., 2003; Ogino *et al*., 2006), and reduced expression of *CRABP1* in thyroid and renal cancers (Huang *et al*., 2003; Hawthorn *et al*., 2004; Pfoertner *et al*., 2005). However, the link between *CRABP1* expression and DNA methylation in those types of cancer remains unknown; although methylation of *CRABP1* was noted in some thyroid-cancer cell lines and expression of the gene was reduced in some thyroid tumors, expression was not restored in those cells by treatment with 5-aza-dCyd (Huang *et al*., 2003). Therefore the significance of DNA methylation for inactivating CRABP1 as a tumor suppressor might differ among tissues. Of interest here is the fact that concentrations of retinoic-acid binding protein in squamous cell carcinomas and adenocarcinomas of the esophagus, as determined by binding of radiolabelled-retinoic acid and which may involve both CRABP1 and CRABP2, are significantly lower than they are in adjacent disease-free tissues, whereas most other neoplastic diseases show higher concentrations of total CRABP protein in tumors than in corresponding disease-free tissues (Dowlatshahi *et al*., 1984).

We observed the highest promoter activity within Regions 4 and 5 of CpG-1 of *CRABP1*, located from the end of exon 1 to intron 1. Although the structure and function of the promoter region of human *CRABP1* have not been reported before, our finding is different from the results of promoter analyses of murine *crabp1*. Two regions upstream of the transcription-start site in the mouse, a 5′ sequence from -7.8 kb to the first 40 nucleotides (Means *et al*., 2000), or a 5′ sequence from -3 to the $+114$ kb (Wei and Chang, 1996), showed promoter activity and regulation of transcription. However, our finding demonstrated that promoter activity was lower near the transcription-start site of human *CRABP1*. Although promoter activity near exon 1 in murine *crabp1* and its downstream sequence has never been evaluated, the discrepancy may depend on a difference in structure upstream of exon 1 between human *CRABP1* and murine *crabp1*. According to the genome database (http://genome.ucsc.edu/), nucleotide sequences of exon 1 and intron 1 are well conserved between the two species but those upstream of exon 1 are different, for example, five GC boxes lie upstream of murine exon 1 while only two GC boxes exists there in human *CRABP1*. When we combine the DNA-methylation pattern of CpG-1 with expression of *CRABP1* in cell lines and primary tumors of ESCC, the sequence around Region 5 in CpG-1 appears to be important as a promoter of human *CRABP1*. Expression of the murine *crabp1* is developmentally regulated through changes in the methylation pattern of its 5′-flanking region (Wei and Lee, 1994). Therefore, DNA methylation plays a role in silencing the *CRABP1* gene in both human and mouse, although the target sequences for this mechanism are likely to be different.

Of the two CRABP isoforms, CRABP1 is expressed in almost all tissues, where it binds RA at high affinity (Fiorella *et al*., 1993), and moderates cellular response to RA by facilitating catabolism and/or by sequestering RA, rendering it unavailable to nuclear receptors (Donovan *et al*., 1995). However, CRABP1 is not directly involved in the retinoid receptor-mediated RA-signaling pathway (Venepally *et al*., 1996), and has no effect on the RA-induced transcriptional activity of retinoic acid receptors (Dong *et al*., 1999). Our data are consistent with those findings. Indeed, homozygous mutant mice lacking functional crabp1 show normal development, growth and reproduction (de Bruijn *et al*., 1994; Gorry *et al*., 1994), although whether CRABP1 knockout accelerates tumorigenesis in mice, either with or without oncogenic stimuli, has not been determined. Therefore the functions, and possibly distinct roles, of

CRABP1 have not been fully defined. Our analyses using ESCC cells expressing CRABP1 ectopically, or CRABP1-knockdown ESCC cells, clearly demonstrated that this protein inhibits progression of the cell cycle at G_0–G_1 phase with a concomitant increase in p27, and of p21 to some degree. Although how CRABP1 induces expression of p27 protein and arrests the cell cycle at G_0–G_1 remains unknown, our results strongly suggest that CRABP1 may function as tumor suppressor in esophageal epithelium.

Immunohistochemical analysis of CRABP1 in primary ESCCs revealed significant correlation between expression of CRABP1 protein and histopathological grading, depth of invasion (pT), non-regional lymph-node metastasis (pM) and TNM stage. In neighboring non-neoplastic esophageal epithelia, CRABP1 protein was expressed in regions containing differentiated cells, but not in those containing actively dividing cells. Among ESCCs, poorly differentiated tumors showed negative CRABP1 expression more frequently than did well differentiated tumors. Those findings suggest that expression of CRABP1 may depend on the status of cellular differentiation, and that decreased expression of CRABP1 plays an important role during dedifferentiation of esophageal neoplasms. It was reported that lower expression pattern of p21 and p27 was observed in less differentiated or actively dividing cells of primary ESCC, although topological pattern of those proteins was not completely consistent with that of CRABP1 (Shirakawa *et al*., 2000; Shibata *et al*., 2001), at least partly supporting our findings observed in *CRABP1*-stable transfectants or *CRABP1*-knockdown cells. We note here that pT1 tumors showed negative CRABP1 expression more frequently than pT2/3 tumors did, a finding that may reflect, at least partly, the observed correlation between pT categories and histological grading: that is, pT1 tumors tended to be poorly differentiated (well differentiated cases, 21%; poorly differentiated cases, 32%), whereas pT2/3 tumors tended to be well differentiated (well differentiated cases, 36%; poorly differentiated cases, 13%). This correlation might also explain the higher frequency of negative CRABP1 immunoreactivity in stage I tumors compared with stage II/III tumors.

One of the most striking findings in the present study is that CRABP1 expression had a significant inverse correlation with pM categories, but not with metastasis to regional lymph nodes (pN). Several molecules correlated with lymph-node metastasis, including KAI1, EphA2 and CXCR4, have been identified (Miyazaki *et al*., 2000, 2003; Kaifi *et al*., 2005). However, molecules correlated particularly with distant lymph-node metastasis remain unknown, although metastasis to distant nodes is one of the important criteria for selecting suitable treatment options for patients with ESCC. ESCC metastasizes to a wide range of lymph nodes, occasionally beyond regional nodes even in the early stage of disease, but the extent of lymphadenectomy required to achieve complete clearance of all potentially involved nodes is controversial (Stein *et al*., 2005). Controversies also remain in regard to the primary treatment of resectable disease (Ohtsu, 2004). Therefore a molecular marker able to predict involvement of distant lymph nodes would be useful for choosing the most favorable options for treatment of individual ESCC patients. In our study, most of the ESCCs with pM1 showed negative CRABP1 immunoreactivity in a manner independent of the depth of tumor invasion. In pT2/3 patients, moreover, negative CRABP1 immunoreactivity significantly correlated with metastasis to non-regional lymph nodes. These results suggest that extended dissection of lymph nodes may be required for patients showing negative CRABP1 expression in the primary tumor or biopsy, and that combined treatment modalities may be more desirable in those cases. For CRABP1 to be useful as a clinical biomarker for prediction of latent, distant micrometastases in patients with ESCC, convenient and sensitive methods must be developed for detecting CRABP1 expression and/or methylation status in biopsy specimens or blood samples.

Materials and methods

Cell culture, drug treatment and primary tissue samples

A total of 43 ESCC cell lines were used, of which 31 belonged to the KYSE series established from surgically resected tumors (Shimada *et al*., 1992) and 12 were TE-series lines provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). All ESCC cells and normal esophageal epithelial cell line NEK2 were maintained as described elsewhere (Ito *et al*., 2003). To analyse restoration of genes of interest, cells were treated with or without various concentrations of 5-aza-dCyd for 5 days and/or 100 ng/ml TSA for the last 12 h.

Primary tumor samples obtained during surgery from 30 ESCC patients undergoing tumor resection at the Kyoto University Hospital (Kyoto, Japan) and from six ESCC patients undergoing tumor resection at the Tokyo Medical and Dental University Hospital (Tokyo, Japan) were frozen immediately in liquid nitrogen and stored at $-80^{\circ}C$ until required. In the later six cases, tumor and corresponding non-cancerous esophageal mucosa were obtained. An additional 113 ESCC tumor samples had been obtained from ESCC patients treated at the National Defense Medical College Hospital (Saitama, Japan) between February 1985 and September 1999, and embedded in paraffin after 24 h of formalin fixation. Relevant clinical and survival data were available for all 113 patients. Written consent was always obtained in the formal style and after approval by the local ethics committees. None of the patients in either group had received chemotherapy or radiation before surgery. Disease stage was defined in accordance with the TNM classification (Sobin and Wittekind, 2002). Patients with pT4 disease or distant organ metastases except for lymph nodes, and those who died within a month, were not included in this study. The median follow-up period for the surviving patients was 57 months (ranging from 2 to 123 months).

BAMCA

BAMCA were performed as described elsewhere (Misawa *et al*., 2005). Test DNA from each of ESCC cell lines was digested first with a methylation-sensitive *Sma*I and then

with methylation-insensitive *Xma*I. Adapters were ligated to *Xma*I-digested sticky ends, and PCR were performed with an adapter primer and Cy3-dCTP for labeling. Control DNA from NEK2 cells was treated in the same manner except that labeling was with Cy5-dCTP. Labeled test and control PCR products were co-hybridized to in-house BAC array (MCG whole genome array-4500; Inazawa *et al*., 2004).

RT–PCR
Single-stranded cDNAs generated from total RNAs were amplified with primers specific for each gene (Supplementary Table S2). Levels of mRNA expression in primary tumors were measured using a quantitative real-time fluorescence detection method (PRISM 7900HT, Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) was amplified at the same time to estimate the efficiency of cDNA synthesis. PCR amplification was performed in duplicate for each sample.

Methylation analysis
Genomic DNAs were treated with sodium bisulfite and subjected to PCR using primer sets designed to amplify regions of interest (Supplementary Table S2). For COBRA, PCR products were digested with *BstU*I or *Taq*I (Xiong and Laird, 1997). CpGenome Universal Methylated DNA (M, Chemicon International, Temecula, CA, USA) was used as a positive control. For bisulfite sequencing, the PCR products were subcloned and then sequenced.

Reporter assay
Fragments around or within a CpG island containing exon 1 of *CRABP1* were obtained by PCR, and ligated into the pGL3-basic reporter vector (Promega, Madison, WI, USA). Reporter assay was performed as described elsewhere (Sonoda *et al*., 2004) using each construct or a control pGL3-basic vector and an internal control pRL-hTK vector (Promega).

Western blotting
Anti-CRABP1 mouse monoclonal antibody was purchased from Sigma (St. Louis, MO, USA); anti-Myc-Tag and anti-p44/42 antibodies were from Cell Signaling Technology (Beverly, MA, USA); and anti-p21, anti-cyclinD1 and anti-p27Kip1 antibodies were from SantaCruz Biotechnology (Santa Cruz, CA, USA), Dako (Carpinteria, CA, USA) and BD Biosciences (Tokyo, Japan), respectively. Cells were lysed in Tris buffer (50 mmol/l, pH 7.5) containing 150 mmol/l NaCl, 1 mmol/l EDTA, 0.5% NP-40, 10% glycerol, 100 mmol/l NaF, 10 mmol/l sodium pyrophosphate, 2 mmol/l Na_2VO_3 and a protease inhibitor cocktail (Roche, Tokyo, Japan), and lysates were analysed as described elsewhere (Sonoda *et al*., 2004).

Colony-formation assays
A full-length *CRABP1* cDNA was obtained by RT–PCR and cloned into the pCMV-Tag3 vector (Stratagene, La Jolla, CA, USA) in-frame, along with the Myc-epitope. Plasmids expressing either a Myc-tagged CRABP1 (pCMV-Tag3-*CRABP1*) or empty vector (pCMV-Tag3-empty) were transfected into ESCC cells. Expression of CRABP1 protein in transfected cells was confirmed by Western blotting. Colony-formation assays were performed as described elsewhere (Misawa *et al*., 2005).

Establishment of clones stably expressing CRABP1 and cell-growth assay
Stable *CRABP1* transfectants and controls were obtained by transfecting pCMV-Tag3B-*CRABP1* or pCMV-Tag3B-empty into KYSE-30 and -510 cells, neither of which expresses *CRABP1*. For measurements of cell growth, 5×10^2 cells were seeded in 96-well plates. To assess the effect of ATRA on cell growth, ATRA (Sigma) or DMSO was added at various concentrations to fresh media every 48 h for 5 days. The numbers of viable cells were assessed by a colorimetric water-soluble tetrazolium salt (WST) assay as described elsewhere (Misawa *et al*., 2005).

Flow cytometry
For FACS analysis, cells were trypsinized, fixed in 70% ethanol overnight and sequentially treated with RNase A (40 U/ml, 30 min), and propidium iodide (20 μg/ml, 30 min) in phosphate buffered saline (PBS). Cells were analysed for DNA content using a FACSCaliber cytometer and Cell Quest software (BD Biosciences). Experiments were repeated twice and performed in triplicate each time.

Senescence assay
Senescent cells were identified by means of senescent cells staining kits (Sigma). We used late-passage cells from a primary culture of human dermal fibroblasts as a control.

Mutation analysis
We looked for mutations in *CRABP1* by means of direct sequencing, using primers designed for genomic sequences around each exon (Supplementary Table S2). Any base changes detected in tumor samples were confirmed by sequencing each product in both directions.

Transfection with synthetic siRNA
CRABP1-specific siRNA (*CRABP1*-siRNA) was purchased from Santa Cruz Biotechnology. A control siRNA for the luciferase gene (CGUACGCGGAAUACUUCGA, *Luc*-siRNA) was synthesized by Sigma. Each siRNA (50 nmol/l) was transfected into ESCC cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Immunohistochemistry and scoring method
Indirect immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections as described elsewhere (Imoto *et al*., 2001). Briefly, antigens were retrieved by microwave pretreatment in citrate buffer (pH 6.0) for 10 min at 95°C. After blocking in 2% normal swine serum, the slides were incubated with an anti-human CRABP1 antibody (Sigma, 1:100) overnight at 4°C, and then reacted with a dextran polymer reagent combined with secondary antibodies and peroxidase (Envision Plus; Dako). Antigen-antibody reactions were visualized with 0.2% diaminobenzidine tetrahydrochloride and hydrogen peroxide. The slides were counterstained with Mayer's hematoxylin.

A formalin-fixed ESCC cell line expressing CRABP1 (TE-4), in which $>50\%$ of cells showed cytoplasmic staining of CRABP1 protein, was used as a positive control. Tissue sections incubated with normal swine serum were included as negative controls. The percentage of the total cell population that expressed CRABP1 was evaluated for each case. Expression of CRABP1 was graded as either positive ($\geqslant 10\%$ of tumor-cell cytoplasm showing immunopositivity), or negative ($<10\%$ of tumor-cell cytoplasm showing immunopositivity, or no staining).

Statistical analyses
Differences between subgroups were tested by the nonparametric Mann–Whitney *U*-test. Correlations between CRABP1

expression in primary ESCCs and the clinicopathological variables pertaining to the corresponding patients were analysed by χ^2 or Fisher's exact tests. Survival data were analysed according to the method of Kaplan and Meier. The log–rank test was used to compare survival data with CRABP1 expression patterns. Differences were assessed with two-sided test, and considered significant at $P<0.05$.

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