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Advances in Brief

Frequent Silencing of *Low Density Lipoprotein Receptor-Related Protein 1B (LRP1B)* Expression by Genetic and Epigenetic Mechanisms in Esophageal Squamous Cell Carcinoma

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Abstract

***Low-density lipoprotein receptor-related protein 1B* (*LRP1B*) is frequently deleted in tumors of various types, but its status and expression in esophageal squamous cell carcinomas (ESCs) have never been reported. In the course of a program to screen ESC cell lines for copy-number aberrations using array-based comparative genomic hybridization, we identified a homozygous deletion of *LRP1B*. Genomic PCR experiments revealed homozygous deletions of *LRP1B* in additional ESC cell lines (total, 6 of 43; 14.0%) and in primary esophageal tumors (30 of 70; 42.9%). Moreover, expression of *LRP1B* mRNA was frequently silenced in ESC lines without homozygous deletions (14 of 37; 37.8%). Using bisulfite-PCR analysis and sequencing, we found that *LRP1B*-nonexpressing cells without homozygous deletions were highly methylated at a CpG island of *LRP1B*, a sequence possessing promoter activity. Treatment with 5-aza-2′-deoxycytidine restored expression of *LRP1B* in those ESC lines. Histone acetylation status correlated directly with expression of *LRP1B* and inversely with the methylation status of the CpG island. Methylation of *LRP1B* was also detected in primary esophageal tumors. Restoration of LRP1B expression in ESC cells reduced colony formation. These results suggest that loss of LRP1B function in esophageal carcinogenesis most often occurs either by homozygous deletion or by transcriptional silencing through hypermethylation of its CpG island.**

Introduction

Esophageal squamous cell carcinoma (ESC) is one of the most common cancers worldwide, but the prognosis is extremely poor because of difficulties in early diagnosis and poor efficiency of treatment (1). Accumulated evidence suggests that multiple genetic alterations occurring sequentially in a cell lineage underlie the carcinogenetic process in solid tumors, including ESC. However, the molecular events leading to ESC are largely unknown. Unraveling the molecular mechanisms in this process could provide pivotal biomarkers for early detection of ESC and targets for development of more effective agents for treating this generally fatal disease.

We have been analyzing ESCs by comparative genomic hybridization (CGH) to identify chromosomal abnormalities that are likely to signal the presence of previously unidentified tumor-associated genes (2, 3) and have been able to successfully identify several genetic targets for amplification events (3–7). However, because a minimum of 5–10 Mb of DNA must be out of balance for low copy-number changes, particularly losses, to be visible by conventional CGH (8, 9), it is extremely difficult to isolate loci of tumor suppressor genes by this method. In addition, CGH does not provide information about sites of homozygous loss that could flag such loci. Because tumor suppressor genes, such as *SMAD4*, *RB1*, *PTEN*, and *p16INK4A*, were originally pinpointed by mapping regions of biallelic loss in cancer cells (10–13), the mapping of homozygous deletions in ESCs by high-throughput methods with high resolution and sensitivity should provide valuable clues for exploring tumor-suppressor genes associated with esophageal carcinogenesis, although genetic and epigenetic mechanisms other than homozygous loss could contribute to functional losses as well.

A recently developed CGH technique, CGH-array analysis, allows high-throughput and quantitative analysis of copy-number changes at high resolution throughout the genome, providing many advantages over conventional methods. CGH analysis using arrayed bacterial artificial chromosome (BAC)/P1-artificial chromosome (PAC) clones has successfully mapped high-level amplifications, low copy-number gains and losses, and complete genetic losses (14), allowing precise and rapid identification of tumor-suppressor genes as well as oncogenes in cancer genomes.

In the study reported here we identified homozygous loss of *low density lipoprotein receptor-related protein 1B* (*LRP1B*), which has been described in other tumors (15–18), by CGH-array analysis using our custom-made BAC/PAC-based array against a panel of ESC cell lines. We observed homozygous deletion of *LRP1B* frequently in both cell lines and primary tumors of ESC, but expression of this gene was often silenced, even in cells without homozygous loss. Because epigenetic mechanisms are important ways of transcriptionally silencing tumor-suppressor genes, we examined the role of DNA methylation in the expression of *LRP1B* and tested the effect of restored expression of LRP1B on growth of ESC cells.

Materials and Methods

Cell Lines and Primary Tumors. A total of 43 ESC cancer cell lines were used, of which 31 belonged to the KYSE series established from surgically resected tumors (19) and 12 were TE-series lines provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. All ESC cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Normal esophageal epithelial cell lines, NEK2 and

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HEEC1, were maintained in keratinocyte serum-free medium containing epidermal growth factor and bovine pituitary extract (Invitrogen, Carlsbad, CA; Refs. 20, 21).

Primary tumor samples were obtained during surgery from 104 patients who were treated at the National Cancer Institute Hospital in Tokyo or the Kyoto University Hospital, with written consent from each patient in the formal style and after approval by the local ethics committees. Samples from 70 of these patients were embedded in paraffin for laser-capture microdissection (LCM) after 24 h of methanol fixation, as described elsewhere (22); tissues from the other 34 patients were frozen immediately in liquid nitrogen and stored at −80°C until required. Genomic DNA and total RNA were isolated from each cell line or frozen primary tumor according to procedures described elsewhere (3).

CGH Array Analysis. To prepare our custom-made array (MCG Cancer Array-800), we selected 800 BAC/PAC clones carrying genes or sequence-tagged site markers of potential importance in cancer genesis or progression from the databases archived by the National Center for Biotechnology Information[8] or the University of California Santa Cruz Center for Biomolecular Science and Engineering,[9] and on the basis of results from a similarity-search program[10]. Each *Dpn*I/*Rsa*I/*Hae*III-restricted BAC/PAC DNA was amplified by two rounds of ligation-mediated PCR with a primer containing a 5′-amine group, printed in duplicate by inkjet-type spotter (GENESHOT; NGK Insulators, Nagoya, Japan), and covalently attached to an Oligo-DNA Microarray (Matsunami Glass, Osaka, Japan).

CGH array hybridizations were carried out as described by Snijders *et al.* (14) and Massion *et al.* (23) with modifications. *Dpn*II-restricted test and reference DNAs were labeled by random priming with Cy3- and Cy5-dCTP (Amersham Biosciences, Tokyo, Japan), respectively, precipitated together with ethanol in the presence of Cot-1 DNA, redissolved in a hybridization mixture [50% formamide, 10% dextran sulfate, 2× SSC, 4% SDS (pH 7)], and denatured at 75°C for 10 min. After incubation at 37°C for 30 min, the mixture was applied to array slides set up in custom-made hybridization chambers and incubated at 42°C on a rocking table for 48–72 h. After hybridization, the slides were washed once in a solution of 50% formamide–2× SSC (pH 7.0) for 15 min at 50°C, once in 2× SSC–0.1% SDS for 15 min at 50°C, and once in a 0.1 M sodium phosphate buffer containing 0.1% NP40 (pH 8) for 15 min at room temperature. The arrays were scanned with a GenePix 4000B (Axon Instruments, Foster City, CA; Fig. 1*A*), and acquired images were analyzed with GenePix Pro 4.1 imaging software (Axon Instruments). Fluorescence ratios were normalized so that the mean of the middle third of log2 ratios across the array was 0. Average ratios that deviated significantly (>2 SD) from 0 were considered abnormal.

LCM Samples and Adaptor-Ligation PCR of Genomic DNA. Methanol-fixed, paraffin-embedded tissues were prepared for LCM with a PixCell II LCM system (Arcturus Engineering, Mountain View, CA). Genomic DNA was isolated in lysis buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5% SDS]. The DNA was amplified by adaptor-ligation-mediated PCR after end-filling, as described by Tanabe *et al.* (24).

Screening of Homozygous Deletions by Genomic PCR. In view of previous reports (15–18) of homozygous deletions of *LRP1B* in other tumor types, we screened a panel of ESC DNAs for homozygous losses by PCR, using primers flanking exons 1, 5, 8, and 10 of *LRP1B* (GenBank accession no. NM_018557 for cDNA sequence and NT_005058 for genomic sequence). All primer sequences used in this study are available on request.

Reverse Transcription (RT)-PCR. Single-stranded cDNAs were generated from total RNAs using the SuperScript First-Strand Synthesis System (Invitrogen), and amplified with primers specific for exons 8–9 and exons 91–92 of the *LRP1B* gene. *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was amplified at the same time to estimate the efficiency of cDNA synthesis.

Promoter Constructs, *in Vitro* Methylation, and Promoter Reporter Assay. An 828-bp fragment (fragment 3 in Fig. 2*A*) of a CpG island in *LRP1B*, predicted by the CpGPLOT program,[11] and four other fragments (Fig. 2*A*) around *LRP1B* exon 1, including or not including this CpG island, were obtained by PCR. To examine the effect of methylation on CpG sites, we treated these fragments overnight with 3 units of *Sss*I (CpG) methylase (New England Biolabs, Beverly, MA) per μg of DNA in the presence (methylated) or absence (unmethylated) of 1 mM *S*-adenosylmethionine. Fragments were ligated into the pGL3-Basic vector (Promega, Madison, WI). Ligated products were purified on gels and used directly for transfection. Equal amounts of constructs containing either methylated or unmethylated fragments were introduced into cells with an internal control vector (pRL-hTK; Promega), by use of FuGENE 6 (Roche Diagnostics, Tokyo, Japan). A pGL3-Basic vector without insert served as a negative control. Firefly luciferase and *Renilla* luciferase activities were each measured 36 h after transfection by the Dual-Luciferase Reporter Assay System (Promega); relative luciferase activities were calculated and normalized *versus Renilla* luciferase activity.

Bisulfite-PCR Analysis and Bisulfite Sequencing. Genomic DNAs were treated with sodium bisulfite at 50°C overnight using an EZ DNA Methylation kit (Zymo Research, Orange, CA) and subjected to PCR using primer sets designed to amplify the CpG island of interest.

For bisulfite-PCR analysis, PCR products were digested with *Taq*I (New England Biolabs), which recognizes sequences unique to the methylated (bisulfite-unconverted) alleles but cannot recognize unmethylated (bisulfite-converted) alleles, and electrophoresed. After the gels were stained with ethidium bromide, the intensities of methylated alleles (as a percentage) were calculated by densitometry using MultiGauge 2.0 (Fuji Film, Tokyo, Japan). A methylation density cutoff point of 20% was considered significant. For bisulfite sequencing, PCR products were subcloned and sequenced.

Drug Treatment. Cells were treated with various concentrations of 5-aza 2′-deoxycytidine (5-aza-dCyd) for 5 days and/or 100 ng/ml trichostatin A (TSA) for various periods. For the synergistic study, 5 mM 5-aza-dCyd was present in the cultures for 5 days, and/or 500 nM TSA was added for the last 12 h.

Chromatin Immunoprecipitation (ChIP Assay). ChIP assays were carried out using ChIP Assay Kits with antibody to acetylated histone H4 (Upstate Biotechnology, Lake Placid, NY), after which PCR amplification was performed with approximately 1/100 of the immunoprecipitated DNA. PCR products were resolved on agarose gels, and the 5′ region of *GAPDH* was used as a control for normalization of each PCR product.

Transient Transfection, Western Blotting, and Colony Formation Assay. A plasmid expressing a FLAG-tagged minireceptor construct of LRP1B (pBICEP-CMV-2-mLRP1B), which mimics the function of full-length LRP1B (25), was obtained by cloning the RT-PCR product into the pBICEP-CMV-2 eukaryotic expression vector (Sigma, St. Louis, MO) in frame along with the FLAG epitope. FLAG-mLRP1B or the empty vector (pBICEP-CMV-2-mock) control was transfected into cells for colony-formation assays, essentially as described elsewhere (7). Expression of mLRP1B protein in transiently transfected cells was confirmed 48 h after transfection with Western blot analysis using anti-FLAG antibody (Sigma) as described elsewhere (5). After 3 weeks of incubation with appropriate concentrations of G418 in 6-well plates, cells were fixed with 70% ethanol and stained with crystal violet.

Results and Discussion

Identification of Homozygous Deletions of LRP1B by CGH Array and Genomic PCR. High-throughput identification of homozygous deletions in cell genomes is a powerful way to identify candidate tumor-suppressor genes that are susceptible to biallelic inactivation in tumors of interest. To detect novel homozygous deletions in ESC, we began by applying CGH-array analysis to 43 ESC cell lines. Using the MCG Cancer Array-800, we identified complete loss of *LRP1B*, located at 2q22.1, (log2 ratio = −2.7) in one cell line (TE-6 cells; Fig. 1*A*), and a hemizygous pattern of loss (log2 ratio = −0.42 to −2.0) in 18 other lines (data not shown). The homozygously deleted region of *LRP1B* in TE-6 cells covered at least exons 3–33 (data not shown). Although homozygous deletions within the *LRP1B* gene have been reported in various other types of cancer (15–18), they have never been documented in ESC before. Almost all reported homozygous deletions of *LRP1B*, which consists of 92 exons spanning 1,900,274 bp,[8] have been intragenic (flanked by exons 2 and 10) and have resulted in frame-shifts or translation of truncated and

[8] Internet address: http://www.ncbi.nlm.nih.gov/.
[9] Internet address: http://genome.ucsc.edu/.
[10] Internet address: http://www.ncbi.nlm.nih.gov/BLAST/.
[11] Internet address: http://www.ebi.ac.uk/emboss/cpgplot/

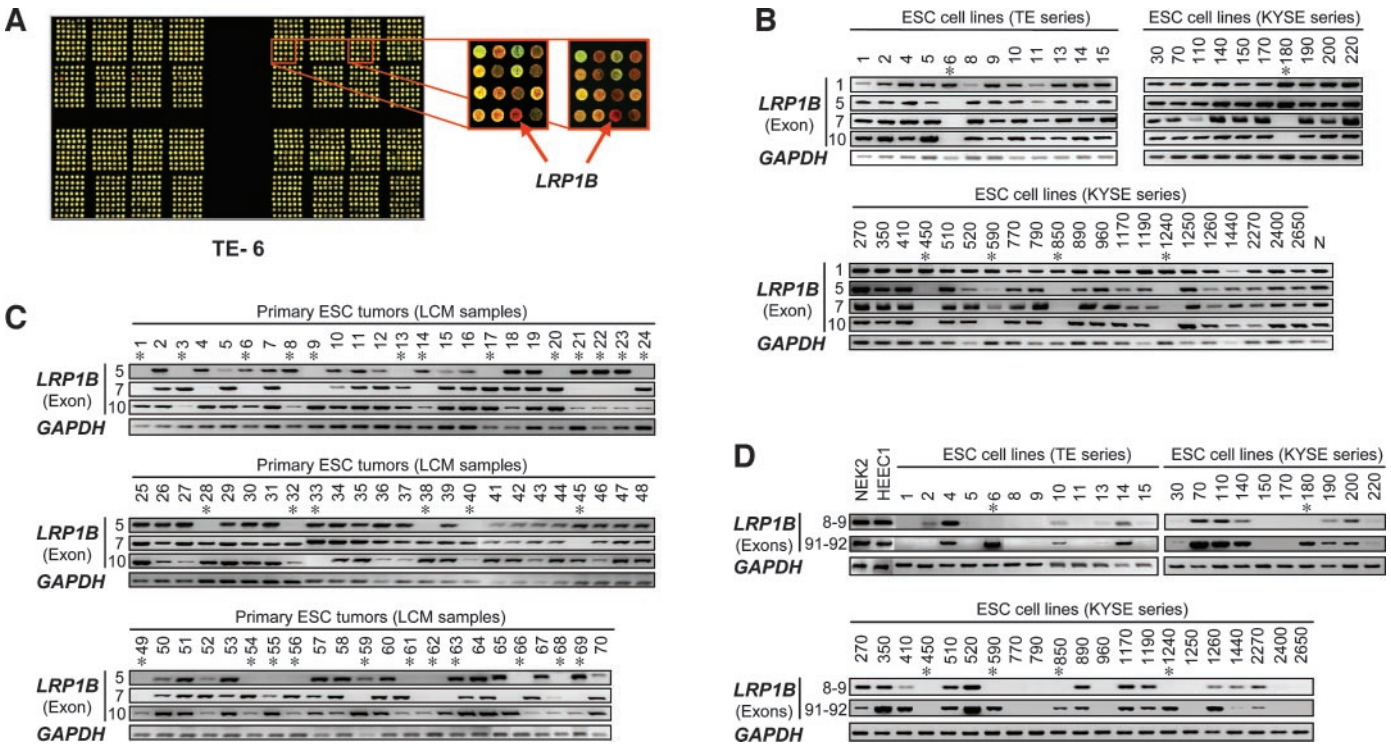

Fig. 1. Homozygous deletions and expression levels of *low-density lipoprotein receptor-related protein 1B* (*LRP1B*) in esophageal squamous cell carcinomas (ESCs). *A*, representative comparative genomic hybridization -array image of the TE-6 cell line. A decrease in copy number of *LRP1B* at 2q22.1 was detected as a clear *red* signal (ratio = −2.7). *B*, homozygous deletions of *LRP1B* exon 1, 5, 7, or 10 in ESC cell lines, detected by genomic PCR analyses. ∗, cell lines with homozygous deletions in any of the exons we analyzed. *N*, normal DNA; *GAPDH*, *glyceraldehyde-3-phosphate dehydrogenase*. *C*, homozygous deletion of *LRP1B* exon 5, 7, or 10 in laser-capture microdissection (*LCM*)-treated primary ESC tumors, also detected by genomic PCR analyses. ∗, tumors with homozygous deletion in any of the exons we analyzed. *D*, expression of *LRP1B* exons 8–9 and 91–92 in ESC cell lines and normal esophageal epithelial cell lines, NEK2 and HEEC1, detected by reverse transcription-PCR analysis. ∗, cell lines corresponding to those with homozygous deletions indicated in *B*. Note that 14 of the 38 cell lines without homozygous deletion of *LRP1B* (36.8%; TE-1, -5, -8, -9, and -11 and KYSE30, -150, -170, -770, -790, -960, -1250, -2400, and -2650) showed almost complete silencing of this gene.

inactive protein (15–18). On the basis of that information we chose to examine ESCs, using cell lines and LCM-treated primary tumors for homozygous losses of *LRP1B* by means of genomic PCR with primer sets designed for different regions. As shown in Fig. 1, *B* and *C*, we detected homozygous deletions of *LRP1B* in 6 of 43 ESC cell lines (14.0%); these deletions were even more frequent in LCM-treated primary ESC tumors (30 of 70; 42.9%), clearly suggesting that small intragenic homozygous deletions represent an important genetic mechanism for inactivating *LRP1B* in esophageal cells and that this event was not an artifact that arose during establishment of the cell lines. All five lines with homozygous loss of *LRP1B* only in genomic PCR showed hemizygous patterns of loss in CGH-array analysis, suggesting that deletions of *LRP1B* on chromosome 2 retained might be too small to by detected by our MCG Cancer Array-800 in those cell lines.

Loss of *LRP1B* Expression in ESC Cell Lines. We next determined expression levels of *LRP1B* by RT-PCR, using primer sets designed for two different regions, exons 8–9 within the frequently deleted region and exons 91–92 close to the 3′ end of the gene (Fig. 1*D*). Of the 43 cell lines examined, the 6 lines with homozygous deletions yielded RT-PCR products from exons 91–92 but not from exons 8–9. In addition, 14 of the 37 lines without homozygous loss of *LRP1B* (37.8%) lacked both RT-PCR products (Fig. 1, *B* and *D*). Normal esophageal epithelial cell lines (NEK2 and HEEC1) showed expression of *LRP1B*. These observations, which had never been reported, suggest that loss of *LRP1B* mRNA expression in some ESC cell lines might result from mechanisms other than genomic deletion, including epigenetic events.

Methylation of the *LRP1B* CpG Island. Hypermethylation in CpG-rich promoter or exonic regions is strongly associated with transcriptional silencing (26). CpG islands tend to be methylated in cancers more often than non-CpG regions, and hypermethylation at CpG islands in promoters appears to be a critical contributor to inactivation of tumor-suppressor genes (27). An 828-bp fragment that included parts of exon 1 and intron 1 (+718 to IVS +491) of *LRP1B* was identified by means of CpGPLOT[11] (Fig. 2*A*).

To test for promoter activity of this CpG island, we linked five fragments of *LRP1B* genomic sequence encompassing or adjacent to the island (Fig. 2*A*) to the luciferase reporter and transiently transfected them into HeLa and TE-4 cells. As shown in Fig. 2*B*, increased transcriptional activity was a feature of all constructs containing the CpG island, whereas constructs without it showed almost no transcriptional activity. Therefore, the CpG islands exert promoter activity without a transcriptional starting site, although it is possible that alternative transcriptional starting sites might exist within or 3′ to the CpG island. When fragments within reporter constructs were methylated by use of *Sss*I with *S*-adenosylmethionine, transcriptional activity was completely abolished (Fig. 2*B*). Complete methylation of the DNA fragment was confirmed by digestion with *Taq*I or *Hpa*II (data not shown). Similar results were obtained in other ESC cell lines regardless of *LRP1B* expression (data not shown). Therefore, complete methylation of the CpG island appears to be sufficient for repression of *LRP1B* expression, regardless of the presence of transcription factors capable of inducing this gene.

To explore the potential role of methylation of this CpG island in the transcriptional silencing of *LRP1B* in ESC, we first examined its methylation status in ESC cell lines by bisulfite-PCR analysis. As shown in Fig. 2*C*, cells lacking *LRP1B* expression but without homozygous deletions in the gene (KYSE170, -770, -790, and -960 and TE-1 and -8), were found to be aberrantly methylated, whereas no

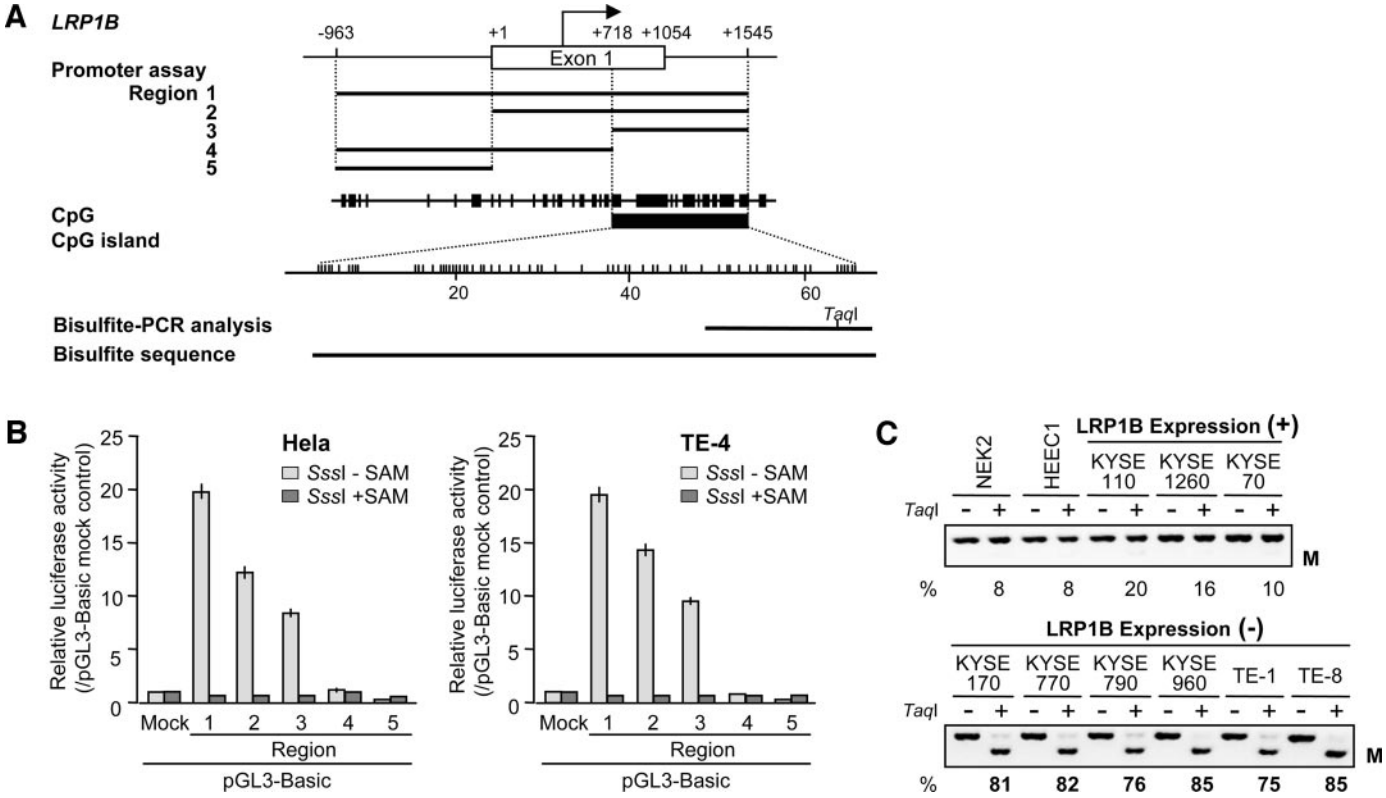

Fig. 2. Methylation status of the *low-density lipoprotein receptor-related protein 1B* (*LRP1B*) CpG island in esophageal squamous cell carcinoma (*ESC*) cell lines and primary tumors. *A*, schematic map of the 828-bp CpG island, which includes parts of exon 1 and intron 1 (+718 to IVS +491) of the *LRP1B* gene (GenBank accession no. NM_018557 for cDNA sequence and NT_005058 for genomic sequence). This island was identified by means of CpGPLOT (http://www.ebi.ac.uk/emboss/cpgplot/) and is indicated by a *thick horizontal bar*; CpG sites are indicated by *vertical bars* on the expanded *axis*. Exon 1 is indicated by an *open box*, and the transcription-start site is marked by a *right-angle arrow* at +1. The regions examined in a promoter assay (regions 1–5), bisulfite-PCR analysis, and bisulfite sequencing are indicated by *horizontal lines*. *B*, promoter activity of the *LRP1B* CpG island and its reduction by *in vitro* methylation. pGL3 basic vectors, each containing one of five different sequences around the CpG island (regions 1–5; see *panel A*) that were methylated *in vitro* by use of *Sss*I (CpG) methylase in the presence (methylated) or absence (unmethylated) of 1 mM *S*-adenosylmethionine (*SAM*), or pGL3 basic empty vector were transfected into HeLa and TE-4 cells. Luciferase activities were normalized *versus* an internal control. The data presented are the means ± SE (*bars*) of three separate experiments, each performed in triplicate. *C*, representative results of bisulfite-PCR analyses of the *LRP1B* CpG island in ESC cell lines after digestion with methylation-sensitive restriction enzyme (*Taq*I). *M*, methylated alleles examined by densitometry. Percentages of methylation are indicated *below* the gels, with values >20% shown in *bold*. *NEK2* and *HEEC1*, normal esophageal epithelial cell lines. *D*, bisulfite genomic sequencing of the *LRP1B* CpG island (a total 67 CpG sites between locations +718 and +1545 relative to the transcription start site) examined in *LRP1B*-expressing cell lines (KYSE110, -1260, and -70) and *LRP1B*-nonexpressing cell lines (KYSE170, -770, -790, and -960 and TE-1 and -8). Each *square* indicates a CpG site: □, unmethylated; ■, methylated. *E*, representative results of bisulfite-PCR analysis of the *LRP1B* CpG island in primary ESC tumors after digestion with a methylation-sensitive restriction enzyme (*Taq*I). *M*, methylated alleles. The methylated alleles were examined by densitometry, and percentages of methylation are indicated *below* the gels, with values >20% shown in *bold*. *F*, representative results of reverse transcription-PCR analysis to reveal *LRP1B* expression in ESC cell lines with and without treatment with 5-aza-deoxycytidine (*5-aza-dC*). These experiments used specific primers (exons 8–9) for *LRP1B* in *LRP1B*-nonexpressing cell lines without homozygous deletions (KYSE170, -770, -790, and -960 and TE-1, -5, -8, and -11) or with homozygous deletion (TE-6) after treatment with 5 mM 5-aza-dC for 5 days. Expression of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) mRNA demonstrated the efficiency of cDNA synthesis. *G*, representative documentation of *LRP1B* expression in *LRP1B*-nonexpressing ESC cells after treatment with 5-aza-dC and/or trichostatin A (*TSA*). Reverse transcription-PCR analysis was performed using specific primers (exons 8–9) for *LRP1B* in KYSE170 cells after treatment with 5-aza-dC for 5 days and/or TSA for 12 h. *H*, status of histone acetylation of *LRP1B* in ESC cells. The region around densely methylated CpG sites within the CpG island (shown in *D*) was amplified by PCR after chromatin immunoprecipitation (*ChIP*) assay using antibody to acetylated histone H4 in *LRP1B*-expressing cell lines (KYSE110, -1260, and -70) and in *LRP1B*-nonexpressing cell lines (KYSE170 and -960). Reactions were controlled in two ways: amplification of DNA before precipitation (*Input*) and amplification of the 5′ region of *GAPDH* after precipitation (*GAPDH*). Bands produced by the ChIP-PCR products of *LRP1B* and *GAPDH* were quantified by densitometry, and the ratios of the signals from *LRP1B* and *GAPDH* are shown in the *lower panel*. *I*, effect of 5-aza-dC or/and TSA treatment on the acetylation status of histone H4 at the *LRP1B* CpG island in ESC cells. KYSE170 cells were cultured with or without 5-aza-dC for 5 days or/and TSA for 12 h. ChIP assays were performed with antibody to acetylated histone H4 followed by PCR amplification, as in *H*. Bands produced by the ChIP-PCR products of *LRP1B* and *GAPDH* were quantified by densitometry, and the ratios of the signals from *LRP1B* and *GAPDH* are shown in the *lower panel*.

hypermethylation was seen in *LRP1B*-expressing cells (KYSE110, -1260, and -70) or normal esophageal epithelial cell lines (NEK2 and HEEC1).

We assessed the methylation status of each CpG dinucleotide within the *LRP1B* CpG island in more detail by bisulfite sequencing. Results were consistent with those of bisulfite-PCR analysis: CpG sites on the CpG island, particularly sites 39–67, tended to be extensively methylated in *LRP1B*-nonexpressing cells without homozygous deletions (KYSE170, -770, -790, and -960 and TE-1 and -8), whereas almost all CpG sites were unmethylated in *LRP1B*-expressing cells (KYSE110, -1260, and -70; Fig. 2*D*). Taken together, these findings suggest that methylation of the *LRP1B* CpG island was tightly related to transcriptional silencing of *LRP1B* in ESC cells where homozygous loss was not a factor.

Methylation of the *LRP1B* CpG Island in Primary ESC Tumors. To determine whether aberrant methylation of *LRP1B* also takes place in primary ESCs, we carried out methylation analysis in a panel of primary tumors (Fig. 2*E*). Bisulfite-PCR showed that the *LRP1B* CpG island was clearly methylated in 14.7% (5 of 34) of those tumors. Because the DNAs had been isolated from snap-frozen tumors rather than LCM-treated samples because bisulfite treatment requires larger amounts of DNA, the lower frequency of methylation in primary tumors compared with cell lines could reflect unavoidable contamination of the specimens with noncancerous cells, leading to underestimation. Although corresponding normal esophageal tissues were not available to confirm this supposition, the results suggested that hypermethylation of the *LRP1B* CpG island may be a relatively common mechanism for inactivating LRP1B during esophageal car-

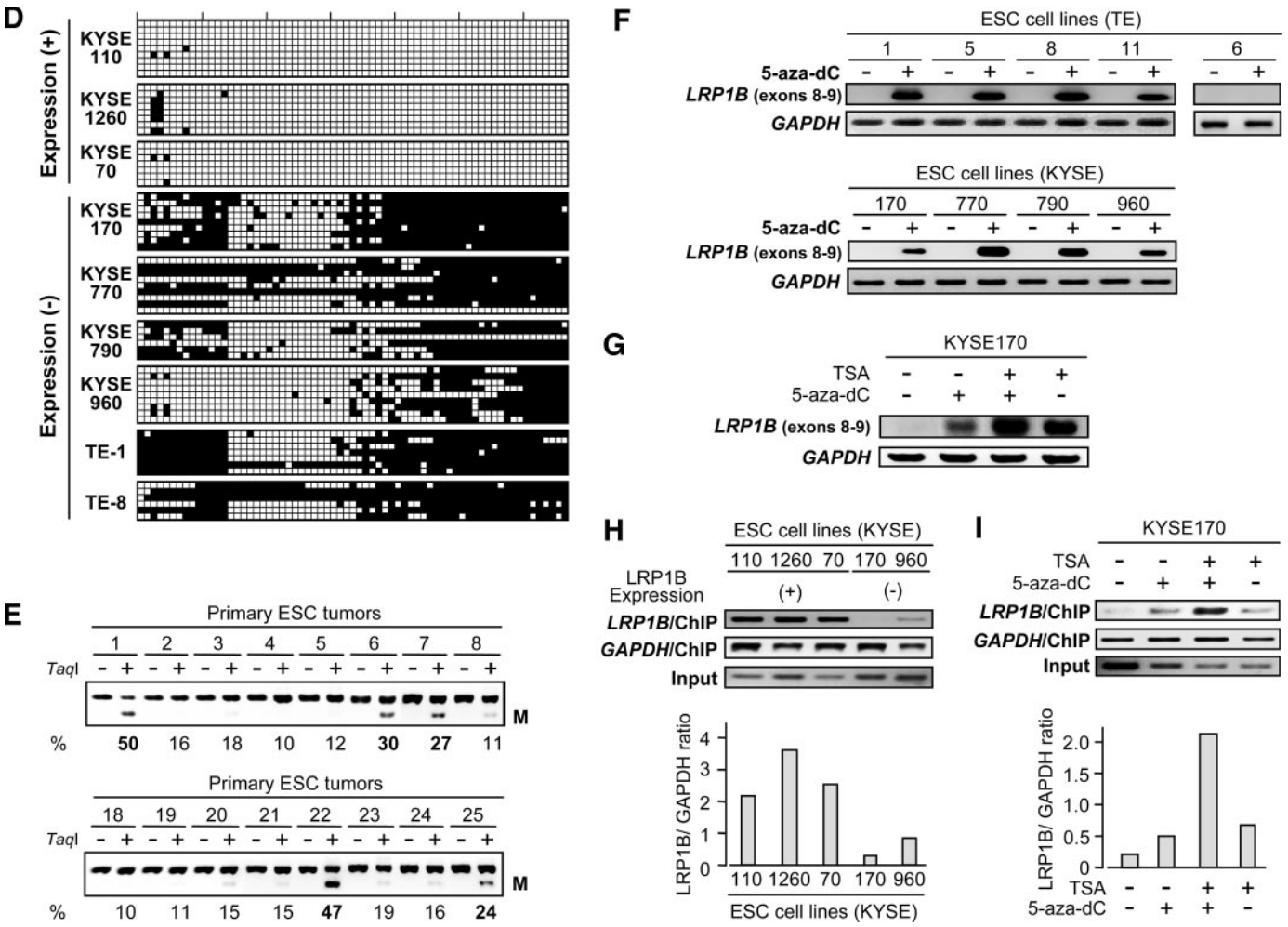

Fig. 2. *Continued.*

cinogenesis. These observations led us to conjecture that in addition to homozygous deletion, hypermethylation of the CpG island may be a frequent cause of inactivation of *LRP1B* in ESCs.

Effect of Demethylation by 5-Aza-dCyd on *LRP1B* Expression. To investigate whether demethylation could restore expression of *LRP1B* mRNA in ESC cells, we treated cells with 5-aza-dCyd, a methyltransferase inhibitor, for 5 days. Induction of *LRP1B* mRNA occurred after treatment with 5 mM of 5-aza-dCyd in cells lacking *LRP1B* expression but without its homozygous deletion (Fig. 2*F*). In contrast, 5-aza-dCyd treatment of TE-6 cells, which do harbor a homozygous deletion of *LRP1B*, failed to alter the level of *LRP1B* expression (Fig. 2*F*).

Relationship between CpG Methylation and Histone Acetylation. A growing body of data indicates that histone modification, including hypoacetylation of histones (26), is involved in the gene silencing caused by DNA methylation (28, 29). We therefore used the histone deacetylase inhibitor TSA, with or without 5-aza-dCyd, to examine the potential role of histone acetylation in the regulation of *LRP1B* expression. When KYSE170 cells, in which *LRP1B* is densely methylated, were treated with TSA, we detected an elevation in *LRP1B* expression greater than that induced by 5-aza-dCyd alone (Fig. 2*G*). This observation suggests that the expression of *LRP1B* induced by histone acetylation may not depend completely on methylation in ESC cells and that the positive methylation status observed in some of our ESC cell lines may be insufficient to inhibit histone acetylation-induced *LRP1B* expression. However, treating KYSE170 cells with both TSA and 5-aza-dCyd enhanced expression of *LRP1B* to a level greater than those seen with either drug alone (Fig. 2*G*), indicating some role for histone deacetylation in gene silencing of *LRP1B* among the methylated cell lines. To assess the degree of histone acetylation associated with *LRP1B* expression, we performed ChIP assays in five ESC cell lines (KYSE110, -1260, -70, -170, and -960), using antibody to acetylated histone H4. After amplification with primers specific for a portion of the *LRP1B* CpG island, we observed enrichment of hyperacetylated histone H4 in cells expressing *LRP1B* (KYSE110, -1260, and -70), whereas cells harboring a methylation-silenced *LRP1B* showed a remarkable decrease in hyperacetylated histone H4 (KYSE170 and -960; Fig. 2*H*). ChIP analysis using KYSE170 cells treated with 5-aza-dCyd, TSA, or 5-aza-dCyd plus TSA showed that 5-aza-dCyd or TSA alone increased histone acetylation at the *LRP1B* CpG island but that TSA plus 5-aza-dCyd synergistically enhanced histone acetylation (Fig. 2*I*). Thus, histone acetylation status correlated directly with expression of *LRP1B* mRNA and inversely with the methylation status of the *LRP1B* CpG island, although the correlations were incomplete. Those results confirmed that DNA methylation, in conjunction with histone deacetylation, is an important mechanism in ESC cells that do not express *LRP1B*.

Suppression of Cell Growth after Restoration of LRP1B Expression. To gain further insight into the potential role of LRP1B in esophageal carcinogenesis, we investigated whether restoration of *LRP1B* expression would suppress growth of the ESC cells in which

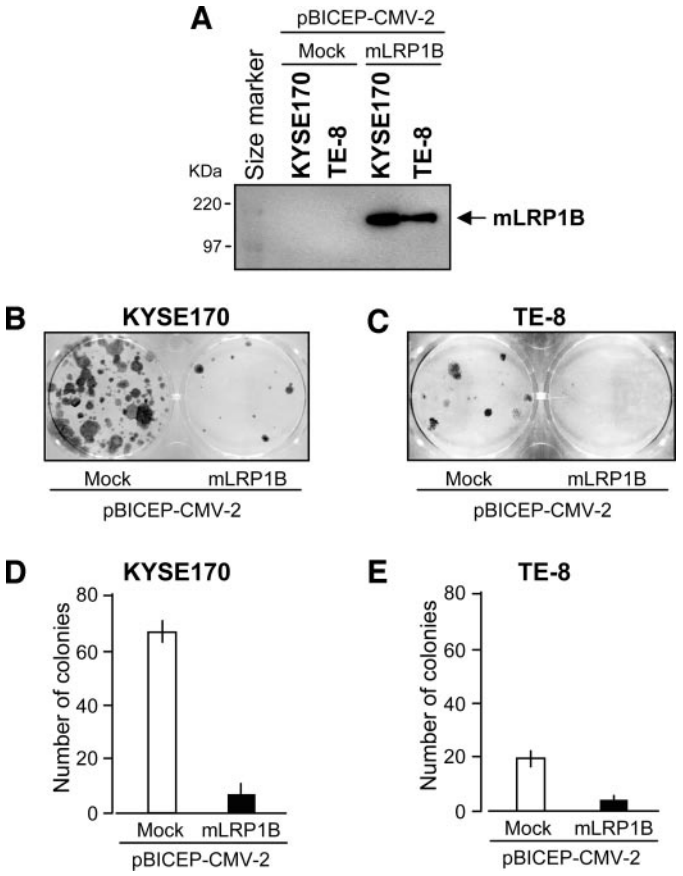

Fig. 3. Effect of restoration of low-density lipoprotein receptor-related protein 1B (*LRP1B*) expression on growth of esophageal squamous cell carcinoma cells. *A–C,* FLAG-tagged construct containing the mini-receptor of LRP1B (pBICEP-CMV-2-mLRP1B) or empty vector (pBICEP-CMV-2-mock) as a control was transfected into KYSE170 and TE-8 cells, which lack expression of the *LRP1B* gene because the CpG island is methylated. Western blot analysis using 10 μg of protein extracts and anti-FLAG antibody demonstrated that cells that were transiently transfected with pBICEP-CMV-2-mLRP1B expressed FLAG-tagged mLRP1B protein (*arrow* in *A*). Three weeks after transfection and subsequent selection of drug-resistant colonies in 6-well plates, the colonies formed by mLRP1B-transfected KYSE170 (*B*) and TE-8 (*C*) cells were less numerous than those formed by mock-transfected cells. *D* and *E,* quantitative analysis of colony formation in KYSE170 (*C*) and TE-8 (*D*) cells. Colonies larger than 2 mm were counted, and results are presented as the means ± SE (*bars*) of three separate experiments, each performed in triplicate.

the gene had been silenced. We performed colony-formation assays using mLRP1B, which was able to mimic the function of full-length LRP1B (25), instead of the full coding sequence. As shown in Fig. 3, three weeks after transfection and subsequent selection of drug-resistant colonies, we found that the numbers of colonies produced by mLRP1B-transfected KYSE170 and TE-8 cells decreased remarkably compared with cells containing empty vector. We have never been able to obtain LRP1B-stable transfectants from cells that do not express this gene (data not shown).

With respect to the tumor-suppressor activity of LRP1B, Liu *et al.* (25) have suggested that this molecule may inhibit metastasis. However, our findings of frequent inactivation of *LRP1B* in ESC suggest that this event is likely to be involved in multiple phenotypes other than metastasis of this disease. Recently, another tumor-suppressor gene, *TSCL1*, which also is often inactivated by methylation in ESCs, was shown to be involved in multiple phenotypes related to malignancy of ESC, such as cell growth, motility, and invasion *in vitro* and formation of tumors *in vivo* (20). Loss of LRP1B may contribute to esophageal carcinogenesis in a similar way, by abrogating multiple functions.

Because *LRP1B* has such a large number of exons and because only one mutation of this gene has been reported to date, in a non-small cell lung cancer cell line (15), we chose not to analyze this gene for mutations in the present study. However, because we frequently did observe not only homozygous deletions but methylation of *LRP1B* in ESC, which has not been published previously, we believe that *LRP1B* mutations, even if they occur, may be a relatively rare cause of inactivation, although mutation analysis of *LRP1B* in ESC will be needed to confirm this hypothesis. In our CGH-array analysis of ESC cell lines, many, but not all, of the lines without *LRP1B* homozygous deletions exhibited hemizygous losses of this gene (13 of 37 lines; 35.1%; data not shown). Those findings suggest that most "two hit" (30) *LRP1B*-inactivating events in ESCs are likely to be (*a*) deletion of both alleles, (*b*) deletion of one allele and methylation of the other, or (*c*) methylation of both alleles. In any case, analysis of *LRP1B* expression will have to be undertaken in numerous primary ESC tumors to clarify the clinicopathological significance of *LRP1B* inactivation in this disease.

In summary, we have demonstrated that expression of *LRP1B* mRNA is frequently lost in ESCs as a consequence of either homozygous deletions or DNA methylation and that reexpression of this gene inhibits growth of ESC cells. These two types of events affecting the *LRP1B* gene may be useful as novel diagnostic markers for ESC because of their high frequencies, although it remains unclear whether precancerous lesions of this tumor contain either of those alterations. The apparent multiplicity of tumor-suppressing activities of LRP1B, however, suggests that this molecule might be a useful starting point for development of novel therapeutic strategies.

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