Genetic or epigenetic silencing of low density lipoprotein receptor-related protein 1B expression in oral squamous cell carcinoma

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Previously, we have reported frequent silencing of the expression of *LRP1B* by genetic and epigenetic mechanisms in esophageal squamous cell carcinoma. As the same events might be involved in the development/progression of OSCC, we examined intragenic homozygous deletions, expression levels, and methylation status in the CpG island of this gene. Homozygous deletion was detected in only 1 of 18 (5.6%) OSCC lines, whereas the expression of *LRP1B* mRNA was silenced in 8 of 17 (47.1%) OSCC lines without homozygous deletion. An inverse correlation between mRNA expression and methylation status of the *LRP1B* CpG island was clearly observed in OSCC lines, and *LRP1B* mRNA expression was restored by treatment with 5-aza-dCyd. Frequent methylation of the *LRP1B* promoter was also observed in primary OSCC. Taken together, the results suggested that frequent inactivation of *LRP1B* mainly occurs by means of epigenetic mechanisms in OSCC, which might play an important role in oral tumorigenesis. (*Cancer Sci* 2006; 97: 1070–1074)

Oral cancer, predominantly OSCC, is the most common head and neck neoplasm, affecting >400 000 people worldwide every year.[1,2] Despite advances in surgical techniques, chemotherapy, and radiation, 50% of patients die of the disease or complications from it within 5 years.[3] Moreover, OSCC has a severe impact on quality of life through impairments of swallowing and speaking or esthetic disorders. Despite recent progress in the diagnosis and therapeutic methods for OSCC, the prognosis has not improved, reflecting the ineffectiveness of current treatment regimens.[4] An improved understanding of the molecular pathogenesis of OSCC is urgently needed to identify new targets and strategies for effective therapy.[5,6] However, the molecular mechanisms of the progression of OSCC are still unknown.

The carcinogenesis of OSCC is thought to be a multistep phenomenon in which a variety of genetic alterations can be segregated into early to late stages.[7] Recently, in addition, evidence has emerged that epigenetic mechanisms, such as altered DNA methylation patterns, play a significant role in the silencing of tumor suppressor genes and contribute to malignant transformation during carcinogenesis.[8] Although several genes, for example, *p14*, *p15*, *p16*, *RAR-beta*, *RASSF1A*, *E-cadherin*, *VHL*, *DAP-K*, *hMLH1*, and *MGMT*, have been reported to be silenced by aberrant DNA methylation,[9–16] some of them were infrequently methylated in OSCC compared with other tumors. In order to create the b.est possible panel of markers for the prediction of outcome, sensitivity to chemotherapy and radiation, and disease status OSCC, more candidates for tumor-suppressor genes targeted by promoter methylation will no doubt be tested in this disease.[16]

Recently, we have reported frequent inactivation of the *LRP1B* (2q22.1) through intragenic homozygous deletion or promoter hypermethylation in ESCC.[17] In the study reported here, homozygous deletion of *LRP1B* was observed in only 1 of 18 OSCC cell lines, whereas silencing of the expression of *LRP1B* mRNA through methylation of the promoter was observed in 8 of 18 OSCC cell lines, suggesting that *LRP1B* is mainly inactivated through an epigenetic mechanism in OSCC. Frequent hypermethylation of the *LRP1B* promoter was observed in primary tumors of OSCC as well, therefore an epigenetic mechanism, especially promoter hypermethylation, seems to be important for the inactivation of *LRP1B* in OSCC.

Materials and Methods

Cell lines and primary tumors. Eighteen OSCC cell lines were used in the present study. The HSC series was established in the First Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Tokyo Medical and Dental University (Tokyo, Japan). OM-1, OM-2, TSU, ZA, NA, Ca9-22, HOC-313, and HOC-815 were established in the Second Department of Oral and Maxillofacial Surgery at the same institution. KON, SKN-3, KOSC-2, and HO-1-N1 were obtained from the Japanese Cancer Resources Bank (Osaka, Japan).[10] All OSCC cells were maintained in Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin.

Primary OSCC tumor samples were obtained during surgery from 58 patients who were treated at the National Cancer Institute or Chulalongkorn University, Bangkok, Thailand, with prior written consent from each patient and approval by the local ethics committee. Tissues from the patients were immediately frozen in liquid nitrogen and stored at −80°C until required. Genomic DNA and/or total RNA were isolated from each cell line or frozen primary tumor according to procedures described elsewhere. As a control, we carried out the primary culture of oral gingival epithelial cells,[18] obtained from a non-smoking healthy adult male with approval from him and the local ethics committee for this study.

Drug treatment. Cells were treated with various concentrations of 5-aza-dCyd for 5 days and/or 100 ng/mL TSA for various periods. For the synergistic study, 5 or 10 mM 5-aza-dCyd was present in the cultures for 5 days, and/or 500 nM TSA was added for the last 12 h.

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Abbreviations: 5-aza-dCyd, 5-aza-2-deoxycytidine; CFS, common fragile sites; COBRA, combined bisulfite restriction analysis; ESCC, esophageal squamous cell carcinoma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LRP1B, low-density lipoprotein receptor-related protein 1B; OSCC, oral squamous cell carcinoma; PCR, polymerase chain reaction; RT-PCR, reverse transcription–polymerase chain reaction; TSA, trichostatin A.

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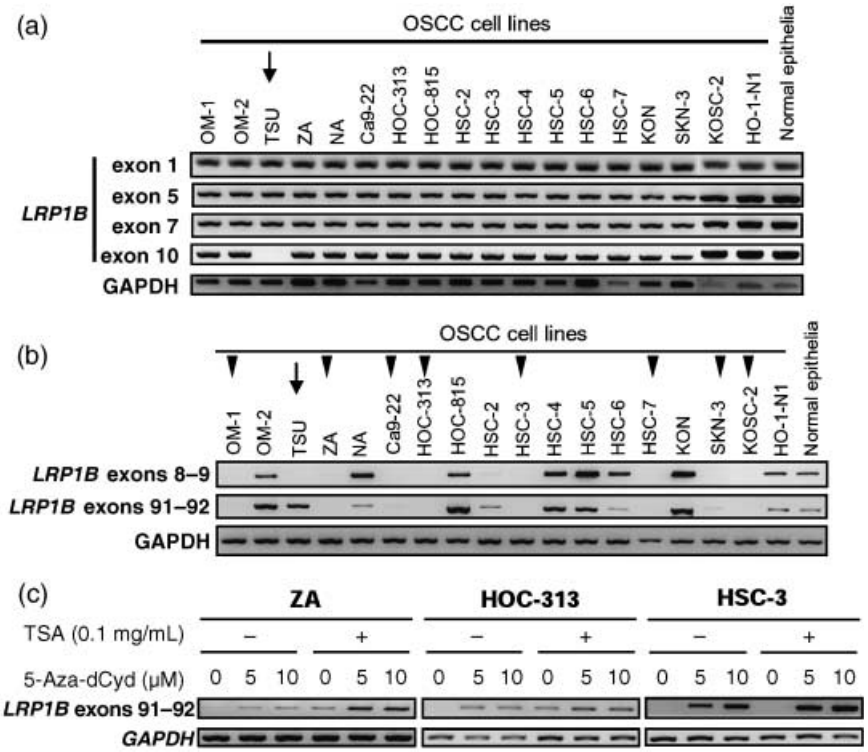

Fig. 1. Homozygous deletions and mRNA expression of the *LRP1B* gene in OSCC cell lines. (a) Representative result of a genomic PCR analysis for homozygous deletions of *LRP1B* in a panel of OSCC lines. A homozygous deletion in exon 10 of *LRP1B* was observed only in the TSU cell line (arrow). (b) Representative result of an RT-PCR analysis for *LRP1B* using primers designed for exons 8–9 or 91–92 in OSCC lines and primary culture of oral epithelial cells. Arrowheads indicate cell lines in which the RT-PCR products of *LRP1B* were undetectable or at lower levels than in primary cultures of oral gingival cells. Arrow indicates cell line having homozygous deletion of LRP1B (c) Restoration of the expression of exon 91–92 of *LRP1B* was observed after treatment with 5-Aza-dCyd (5 or 10 μM) and/or TSA (100 ng/mL) by RT-PCR analysis in three OSCC cell lines. Notably, *LRP1B* mRNA expression was synergistically restored with 5-Aza-dCyd and TSA in all lines tested. –, absence; +, presence.

Screening of homozygous deletions by genomic PCR. In view of previous reports of homozygous deletions of *LRP1B* in various types of tumors,[17,19–22] we screened a panel of OSCC cell lines for homozygous losses by genomic PCR using primers flanking exons 1, 5, 7 and 10 of *LRP1B* (GenBank accession number NM_018557 for cDNA sequence and NT_005058 for genomic sequence). All primer sequences used in this study are available on request.

RT-PCR. Single-stranded cDNAs were generated from total RNAs, and amplified with primers specific for exons 8–9 and 91–92 of the *LRP1B* gene (http://www.ncbi.nlm.nih.gov/).[17] GAPDH was amplified at the same time to estimate the efficiency of cDNA synthesis.

COBRA and bisulfite sequencing. To investigate the methylation of DNA, COBRA was carried out as described previously.[23] Genomic DNAs from frozen samples were treated with sodium bisulfite, and subjected to PCR using primer sets designed to amplify the CpG island of interest. For the COBRA, PCR products were digested with *Taq*I, which recognizes sequences unique to the methylated alleles but cannot recognize unmethylated alleles, and electrophoresed.[17] For bisulfite sequencing, PCR products were subcloned then sequenced.

Statistical analysis. The χ^2-test or Fisher's exact test were used to analyze differences in the frequencies of individual markers within subgroups of tumors. A two-sided *P* value less than 0.05 was required for significance.

Results and Discussion

Previously, we have identified frequent homozygous deletions of the *LRP1B* gene in cell lines and primary tumors of ESCC.[17] As at least 90% of oral cancers are classified as squamous cell carcinomas, we have hypothesized that the molecular events during the carcinogenesis of OSCC might be similar to those of ESCC, and screened for homozygous deletions of this gene in our panel of 18 OSCC cell lines. Genomic PCR using primer sets designed for different regions[17] showed the intragenic homozygous loss of *LRP1B* only in the TSU cell line (5.5%, Fig. 1a). As ESCC cell lines have shown more frequent intragenic homozygous losses (14%),[17] homozygous deletions might be a less frequent genetic mechanism for inactivating *LRP1B* in OSCC than ESCC. Further, unfortunately, we could not examine whether homozygous deletions occurred in primary OSCC tumors due to lack of primary tumor samples without contamination by non-cancerous tissues, such as those isolated with laser-capture microdissection.[17]

An intragenic homozygous deletion to inactivate the *LRP1B* gene is unlikely to be a frequent event in OSCC, so we next determined the expression level of the *LRP1B* gene in 18 OSCC lines by RT-PCR, using the primer sets for exons 8–9 and 91–92, as described previously.[17] TSU cells with homozygous deletions yielded RT-PCR products from exons 8–9 and 91–92, suggesting that intragenic homozygous deletion around exon 10 might not affect the expression of deleted mutants of *LRP1B* in this cell line. However, 8 of the 17 lines without the homozygous loss of *LRP1B* (47.1%) lacked the RT-PCR product (Fig. 1b), whereas primary cultures of gingival epithelial cells showed expression of *LRP1B*. As silencing of *LRP1B* expression was observed in OSCC cell lines to a similar extent to that in ESCC cell lines,[17] loss of *LRP1B* expression through mechanisms other than genomic deletion, including epigenetic events, might be important for inactivation of this gene in OSCC. Mouse *lrp1b* expression is mostly restricted to the brain,[24] but it was reported that human *LRP1B* expression is more widespread in various tissues,[25] suggesting that this putative tumor suppressor might play roles in several types of human cancers including OSCC.

To assess whether DNA demethylation could restore the expression of *LRP1B* mRNA, we treated OSCC cell lines lacking *LRP1B* expression with 5-aza-dCyd, a methyltransferase inhibitor, for 5 days. Induction of *LRP1B* mRNA expression occurred after treatment with 5-aza-dCyd in ZA, HOC-313 and HSC-3 cells (Fig. 1c). In addition, we observed an enhancement of *LRP1B* mRNA expression by 5-aza-dCyd given along with TSA, a histone

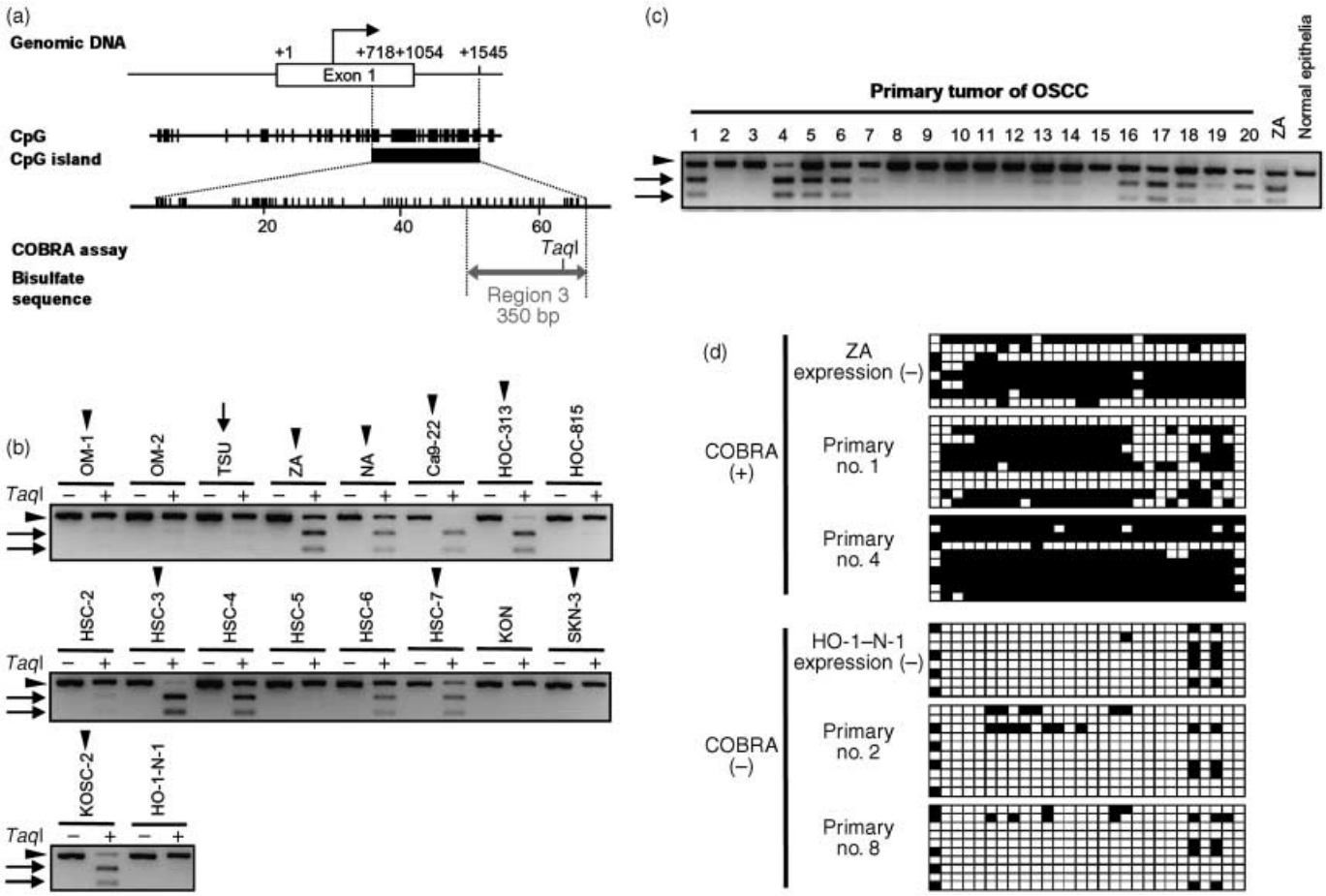

Fig. 2. Analysis of the methylation of the *LRP1B* gene in OSCC cell lines. (a) Schematic image of an 828-bp CpG island (horizontal thick bar, +718 to 1545) that includes parts of exon 1 and intron 1 of the *LRP1B* gene (GenBank accession number NM_018557). CpG sites are indicated by vertical bars on the axis. Exon 1 is indicated by an open box, and the transcription start site is marked by a right-angle arrow at +1. COBRA and bisulfite sequencing were carried out in region 3 (solid gray arrow) within the CpG island, which was identified as a hot spot for DNA methylation and a region with promoter activity in ESCC cells.[16] (b) Methylation status of the promoter region in our panel of OSCC cell lines detected by COBRA. Arrows indicate unmethylated alleles, whereas arrowheads indicate methylated alleles. (c) Representative results of COBRA of the hot spot within the *LRP1B* CpG island in primary OSCC tumors after digestion with a methylation-sensitive restriction enzyme (*Taq*I). Arrows indicate unmethylated alleles, whereas arrowheads indicate methylated alleles. The methylated alleles were examined by densitometry, and those with >20% methylation were considered hypermethylated.[16] (d) Representative results of bisulfite genomic sequencing of the hot spot within the *LRP1B* CpG island in four OSCC primary tumors and two cell lines as a control.

deacetylase inhibitor, in all cell lines we tested, although treatment with TSA alone had no effect on the expression only in HSC-3 cells, suggesting that histone deacetylation does play some role in the transcriptional silencing of *LRP1B* among methylated OSCC cells (Fig. 1c).

In the previous study,[17] we demonstrated an inverse correlation between the expression level of *LRP1B* and DNA methylation status within a CpG island around exon 1 of this gene, and that hypermethylation around the region showing promoter activity within the CpG island seems to silence the expression of *LRP1B* mRNA. In the present study, therefore, we focused on this 'hot spot' to investigate the correlation between *LRP1B* expression status and methylation status of the CpG island with promoter activity in OSCC cells (Fig. 2a). For COBRA and the bisulfite sequencing analysis, we designed a primer set to amplify this hot spot (Fig. 2a). Of nine OSCC cell lines showing silenced or decreased expression of *LRP1B* compared with normal epithelia, seven showed clear hypermethylation in the hot spot in COBRA (Fig. 2b). Notably, five of these seven lines almost lacked the unmethylated allele. In contrast, only two of eight cell lines with the expression of *LRP1B* but without a homozygous deletion of this gene showed a methylated band, and those two cell lines still retained the unmethylated allele. These findings suggest that DNA hypermethylation within the hot spot of the *LRP1B* gene is strongly associated with a silencing of expression, although mechanisms other than homozygous deletion or DNA methylation might also contribute to the inactivation of *LRP1B*, as observed in several cell lines such as OM-1 and SKN-3 (Fig. 2b).

To analyze the methylation status of the hot spot within the CpG island of *LRP1B* in primary OSCCs, we applied COBRA to 58 OSCC primary tumors (Fig. 2c). Hypermethylation of the *LRP1B* CpG island was observed in 25 of 58 samples (43.1%), although the unmethylated allele (arrowhead in Fig. 2c) was also observed in almost all cases, probably due to the normal tissue components included in tumor samples. These findings indicate that the methylation of the *LRP1B* promoter region is not an artifact of the passage of OSCC cell lines *in vitro*, rather, it might be a relatively frequent cancer-related event during oral carcinogenesis. However, none of the clinicopathological characteristics, including stage of tumor, correlated with the methylation status of *LRP1B* (Table 1), although Liu *et al.*[26] have suggested that *LRP1B* inhibits metastasis. As we suggested

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Table 1. Relation between clinicopathological data from 58 patients with primary OSCC tumors and hypermethylation of the *LRP1B* CpG island

	n	*LRP1B* hypermethylation†	*P*‡
Gender			
Male	26	9 (34.6%)	0.2393
Female	32	16 (50.0%)	
Age (years)§			
Median (range)	66.9 (28–90)		
>60	40	19 (47.5%)	0.4965
≤60	16	6 (37.5%)	
Smoking¶			
Smoker	20	6 (30.0%)	0.1415
Non-smoker	23	12 (52.2%)	
Alcohol††			
Drinker	15	4 (26.7%)	0.0924
Non-drinker	22	13 (59.1%)	
TNM classification			
T stage			
T1	3	1 (33.3%)	>0.9999
T2–4	55	24 (43.6%)	
N stage			
N0	38	16 (42.1%)	0.8324
N1–3	20	9 (45.0%)	
M stage			
M0	47	19 (40.4%)	0.3946
M1	11	6 (54.5%)	
Stage			
I, II	20	7 (35.0%)	0.3660
III, IV	38	18 (47.4%)	

†Methylation status was determined by methylation-specific PCR as described in Materials and Methods. ‡*P* values are from the χ^2-test or Fisher's exact test and were statistically significant when <0.05 (2-sided). §No information in two cases. ¶No information in 15 cases. ††No information in 21 cases. T, tumor; N, node; M, metastases.

in a previous study in ESCC,[17] frequent inactivation of *LRP1B* is likely to be involved in multiple phenotypes other than metastasis in various types of tumors.

To confirm the methylation status of each CpG dinucleotide within the *LRP1B* CpG island in more detail, we carried out bisulfite sequencing using some of the cell lines and primary tumors. As shown in Figure 2d, the samples showing hypermethylation in COBRA tended to be extensively methylated, whereas the samples showing hypomethylation in COBRA were unmethylated at almost all CpG sites within the hot spot of the *LRP1B* CpG island.

CFS are large regions of genomic instability present in all individuals. CFS are non-randomly distributed throughout the human genome, and so far more than 110 loci have been defined (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=search&term=). The four most active CFS regions are FRA3B (3p14.3), FRA16D (16q23.2), FRAXB (Xp22.31), and FRA6E (6q26).[27,28] Three of the four, FRA3B, FRA16D, and FRA6E, have been reported to be consistently deleted during the course of cancer development in various types of tumors. Very large genes, *FHIT*, *WWOX* and *Parkin* are located at those three CFS sites, respectively, and all of them span more than 1 Mb of genomic sequence.[28] These three genes are frequently involved in genomic alterations in cancers, including deletions or rearrangements, and the proteins encoded by them are frequently not expressed in these same cancers,[29] suggesting that the genes function as tumor suppressors. Furthermore, two large CFS genes, *GRID2* and *RORA*, have recently been proposed as candidate tumor suppressors, residing within the unstable and evolutionally conserved chromosomal regions 4q22 and 15q22.2 (FRA15A), respectively.[30,31] *LRP1B* is also a very large gene, containing 92 exons and spanning 1.9 Mb of genomic sequence within FRA2F (2q22.1, http://www.ncbi.nlm.nih.gov/).[17] Inactivation of this gene due to homozygous deletions or epigenetic events has been reported in various cancers[19–22] and by us in ESCC.[16] Thus, *LRP1B* is another very large CFS gene that is inactivated in multiple tumors.

In the present study, we have demonstrated that *LRP1B* spanning FRA2F is frequently inactivated in OSCC as a consequence of either homozygous deletions or DNA methylation, especially the methylation of CpG islands. The methylation of the *LRP1B* gene might be a novel diagnostic marker for OSCC because of its high frequency, although it remains unclear whether precancerous lesions of this tumor contain either of these alterations.

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