Lysyl Oxidase Is a Tumor Suppressor Gene Inactivated by Methylation and Loss of Heterozygosity in Human Gastric Cancers

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ABSTRACT

Lysyl oxidase (LOX) and HRAS-like suppressor (HRASLS) are silenced in human gastric cancers and are reported to have growth-suppressive activities in ras-transformed mouse/rat fibroblasts. Here, we analyzed whether or not LOX and HRASLS are tumor suppressor genes in human gastric cancers. Loss of heterozygosity and promoter methylation of LOX were detected in 33% (9 of 27) and 27% (26 of 96) of gastric cancers, respectively. Biallelic methylation and loss of heterozygosity with promoter methylation were also demonstrated in gastric cancers. Silencing of LOX was also observed in colon, lung, and ovarian cancer cell lines. For mutations, only one possible somatic mutation was found by analysis of 96 gastric cancer samples and 58 gastric and other cancer cell lines. When LOX was introduced into a gastric cancer cell line, MKN28, in which LOX and HRASLS were silenced, it reduced the number of anchorage-dependent colonies to 57 to 61%, and the number of anchorage-independent colonies to 11 to 23%. Sizes of tumors formed in nude mice were reduced to 19 to 26%. Growth suppression in soft agar assay was also observed in another gastric cancer cell line, KATOIII. On the other hand, neither loss of heterozygosity nor a somatic mutation was detected in HRASLS, and its introduction into MKN28 did not suppress the growth in vitro or in vivo. These data showed that LOX is a tumor suppressor gene inactivated by methylation and loss of heterozygosity in gastric cancers, and possibly also in other cancers.

INTRODUCTION

Epigenetic alterations are deeply involved in cancer development and progression (1–3). Especially, methylation of promoter CpG islands is known to inhibit transcriptional initiation and cause permanent silencing of downstream genes. It is now known that some important tumor suppressor genes are inactivated, not only by mutations and deletions but also by promoter methylation. It is therefore expected that, if methylation-associated gene silencings are identified by genome-scanning analysis, some of the silenced genes might turn out to be tumor suppressor genes. For this, we previously developed a genome-scanning method to search for aberrantly methylated DNA fragments, methylation-sensitive representation difference analysis (MS-RDA; refs. 4, 5). In this method, unmethylated CpG-rich regions of the genome are enriched from two genomes, and the enriched libraries (amplicons) are compared by RDA (6).

In our previous study, MS-RDA was applied to human gastric cancers, the second most common cancer in the world (7), and the silencing of nine genes was identified (8). Five of the nine genes, lysyl oxidase (LOX), HRAS-like suppressor (HRASLS), thrombomodulin, HAND1, and filamin C, were shown to be frequently methylated in primary gastric cancers. Moreover, two of them, LOX and HRASLS, had been reported to suppress the growth of mouse/rat fibroblasts transformed by ras oncogene (9–12). LOX was down-regulated in ras-transformed NIH3T3 cells but was reexpressed in their revertants (9), and knock-down of LOX by its antisense cDNA caused transformation of rat fibroblasts (10). Contrary to this, a recent report showed that LOX expression increased in the in vitro invasion capacity of human breast cancer cell lines (13). HRASLS was isolated as a human homologue of mouse A-CI (12), which was reported to suppress the growth of ras-transformed mouse fibroblasts (11).

In human gastric cancer cells, it is still uncertain whether or not LOX and HRASLS have growth-suppressive activities. Moreover, inactivation mechanisms of these two genes have not been clarified enough. Here, we analyzed their inactivation mechanisms and tumor suppressor functions in gastric cancers.

MATERIALS AND METHODS

Tissue Samples and Cell Lines. Ninety-six primary gastric cancer samples were obtained from gastric cancer patients undergoing gastrectomy, with informed consents, and were frozen at −80°C until DNA/RNA extraction. Noncancerous samples were obtained by scraping off noncancerous gastric mucosa for 28 cases, and by excising noncancerous gastric wall for the other 68 cases. The former samples were used for genetic analysis (mutation and loss of heterozygosity (LOH)), epigenetic analysis (promoter methylation) and expression analysis, and the latter samples were used only for genetic analysis. Fifty-eight cancer cell lines (11 gastric, 14 lung, 13 colon, 13 ovarian, and 7 pancreatic cancer cell lines) and cultured normal epithelial cells of the lung, colon, ovary, and pancreas were obtained from American Type Culture Collection (Manassas, VA), Japanese Collection of Research Bioresources (Tokyo, Japan), RIKEN Cell Bank (Tsukuba, Japan), and Tohoku University Cell Resource Center for Biomedical Research (Sendai, Japan), or were donated by Dr. K. Yanagihara at National Cancer Center Research Institute (HSC39, HSC44, HSC57; ref. 8), Dr. W. Yasui at Hiroshima University (TMK1; ref. 14), Dr. S-W. Tsao at University of Hong Kong (HOSE6-3; ref. 15) and Dr. M-S. Tsao at University of Toronto (HPDE4; ref. 16).

Single-Strand Conformation Polymorphism and Sequencing Analysis. Entire coding regions of LOX and HRASLS were analyzed by PCR-single-strand conformation polymorphism (SSCP; ref. 17). Approximately 50 ng of genomic DNA was amplified by PCR in the presence of [α-32P]dCTP, and when PCR products were longer than 300 bp, they were digested by restriction enzymes. The products were run in 5% acrylamide gels with or without glycerol as reported previously (18). PCR primers and conditions and restriction enzymes used are shown in Supplementary Table 1.4 When shifted bands were observed, the sample was cycle-sequenced with a BigDye Terminator kit (PE Biosystems, Foster City, CA) and an ABI automated DNA sequencer (PE Biosystems). Mutation of K-ras in gastric cancer cell lines was analyzed by direct sequencing with primers shown in Supplementary Table 2.

Loss of Heterozygosity Analysis. For each gene, LOH was analyzed by PCR-SSCP by using five microsatellite markers nearby (Supplementary Table 3). For LOX, LOH was also analyzed by using frequent polymorphisms found in sequence change analysis.

4 Supplementary Tables for this article can be found at Cancer Research Online (http://cancerres.aacrjournals.org).
Sodium Bisulfite Modification, Bisulfite Sequencing, and Methylation-specific PCR. One μg of DNA, digested with BamHI, underwent sodium bisulfite modification as described previously (19–21), and was suspended in 20 μL of 10 mmol/L Tris (pH 8)-1 mmol/L EDTA. For bisulfite sequencing, 1 μL of the solution was used for PCR with the primers common to methylated and unmethylated DNA sequences. PCR products were cloned into pGEM-T Easy vector (Promega, Madison, WI), and 10 clones were cycle-sequenced for each sample. For methylation-specific PCR (MSP), 1 μL of the solution was used for PCR with primers specific to methylated (M) or unmethylated (U) sequences with positive and negative controls (8). Primers were designed in the 5’ regions of the reported transcription initiation site of LOX (22) and HRASLS (Supplementary Tables 4 and 5).

Quantitative Reverse Transcription-PCR. cDNA was synthesized from 3 μg of total RNA treated with DNase I (Ambion, Austin, TX) with a Superscript II kit (Invitrogen, Carlsbad, CA). Real-time PCR was done with SYBR Green PCR Core Reagents (PE Biosystems) and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The quantity of mRNA was normalized to that of Proliferating cell nuclear antigen (PCNA), which is used for normalization of proliferation-dependent gene expressions (23). The primers and PCR conditions are shown in Supplementary Table 6.

5-Aza-2’-deoxycytidine Treatment. Cells were seeded at a density of 3 × 10^5 cells/10-cm dish on day 0 and were treated with freshly prepared 0.5 to 1 μmol/L 5-aza-2’-deoxycytidine (5-aza-dC; Sigma, St. Louis, MO) for 24 hours on days 1 and 3. After each treatment, the cells were placed in a fresh medium and were harvested on day 4.

Plasmid Construction and Transfection. Entire coding sequences of LOX [from nucleotide (nt.) –22 to nt. 1294] and HRASLS (from nt. –21 to nt. 577) were amplified by reverse transcription-PCR (RT-PCR) and were cloned into pGEM-T Easy vector. After confirmation of their sequences, the cDNAs were moved into pTRE2hyg vector (Clontech, Palo Alto, CA), cDNA in the pTRE2hyg vector was transfected into MKN28 or KATOIII with pRevTet-On vector (Clontech) with LipofectAMINE (Invitrogen), and cells resistant to hygromycin and neomycin were cloned.

As gastric cancer cell lines in which both LOX and HRASLS were silenced, MKN28 (established from intestinal-type gastric cancer) and KATOIII (established from diffuse-type gastric cancer) were selected. Transfected cells were cultured in RPMI 1640 (Invitrogen) with 10% Tet System-approved fetal bovine serum (Clontech), penicillin/streptomycin, hygromycin, and geneticin.

Cell Growth and Anchorage-dependent and -independent Colony Formation Assay. To analyze growth curves, cells were seeded at a density of 2 × 10^3 cells/swell in a 96-well plate at 0 hour, and their growth was measured by absorbance at 450 nm with Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) at 12, 36, 60, 84, and 108 hours. To analyze anchorage-dependent growth, colony formation assay was performed. Cells were seeded at a density of 1 × 10^5 cells/10-cm dish and were cultured for 12 days with medium change on day 6. Colonies were fixed with 10% formalin and stained with 1% Giemsa solution on day 12. To analyze anchorage-independent growth, soft agar assay was performed. Using a 6-well plate, we overlaid 5 × 10^3 cells in 2 mL of top medium with 0.3% agarose, on 3 mL of bottom medium with 0.6% agarose (NuSieve 3:1 Agarose, Cambrex, Rockland, ME). On days 7 and 14, 0.5 mL of top medium was added, and on day 21 colonies were stained with iodonitrotetrazolium chloride (Sigma).

Tumor Formation Assay in Nude Mice. Five × 10^5 cells in 0.2 mL of phosphate-buffered saline were subcutaneously injected at two sites on the flanks of 5-week-old male BALB/cAcl-nu (nu/nu) mice (CLEA, Tokyo, Japan) on day 0. Tumor sizes were measured in three dimensions, and mice were sacrificed on day 42. All of the animal experiments were done in accordance with Guidelines for Animal Experiments in the National Cancer Center.

RESULTS

Mutations, Polymorphisms, and Loss of Heterozygosity of LOX and HRASLS. PCR-SSCP analysis was done with 96 primary gastric cancers, along with matched normal samples, and 58 cancer cell lines

Table 1  Sequence changes of LOX and HRASLS

<table>
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<tr>
<th>Exon</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>No. of primary samples</th>
<th>with the change</th>
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<td>Leu10Leu</td>
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<td>1</td>
<td>C440G*</td>
<td>Ala147Gly</td>
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<tr>
<td>Polymorphisms</td>
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<td>3</td>
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<td>Gly131Arg</td>
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</tbody>
</table>

NOTE: Entire coding regions of LOX and HRASLS were analyzed by PCR-SSCP.

* This nucleotide change was observed only in one ovarian cancer cell line, MCAS, for which noncancerous tissue was not available, and was considered as a somatic mutation or a rare polymorphism.
Promoter Methylation of LOX and HRASLS. Promoter methylation of LOX was detected by MSP in 26 (27%) of 96 primary gastric cancers. Among eight cancers that had the promoter methylation and were informative for a polymorphism at nt. 473, three cancers showed LOH (a representative result in Fig. 1A). This showed that one mode of LOX inactivation was a combination of promoter methylation and an allelic loss.

Then, the presence of biallelic methylation was analyzed in four pairs of primary cancers and corresponding normal samples and in two cancer cell lines with LOX methylation. The 5′ region and exonic region were separately analyzed by bisulfite sequencing because amplification of the two regions in one fragment was unsuccessful. KATOIII and two primary cancers (22C and A48C) were informative for a C/G polymorphism at nt. 225 and showed methylation of both alleles in the exonic region. In the 5′ region, all of the DNA molecules were methylated in KATOIII, and DNA molecules in proportions similar to the exonic region were methylated in 22C and A48C (Fig. 1B). This indicated that another mode of LOX inactivation was biallelic methylation of the promoter region. Although MKN28 did not have the polymorphism, it showed complete methylation.

As for HRASLS, MSP showed promoter methylation in 44 (46%) of 96 primary gastric cancers (data not shown).

Expression Loss of LOX and HRASLS. LOX expression was analyzed in 38 primary gastric cancers and eight noncancerous samples for which RNA was available (Fig. 2A). Twenty-eight cancers without methylated LOX tended to show its overexpression compared with normal samples. Among these cancers, six cancers with demonstrable LOH showed lower expression. Ten cancers with methylated LOX showed lower expression than the 28 cancers without methylation. One cancer with methylation and LOH, and another cancer with demonstrated biallelic methylation showed almost no expression.

Correlation between methylation and expression loss was clearly observed. (PCNA, proliferating cell nuclear antigen.)
LYSL oxidase in gastric cancers

Fig. 3. Analysis of in vitro tumor suppressor activity. A, expression of introduced LOX (left) and HRASLS (right), analyzed by quantitative RT-PCR. Whereas vector-only clones (M28V1 and M28V2) showed no expression of LOX or HRASLS, sense LOX clones (M28sLOX1 and M28sLOX2) and sense HRASLS clones (M28sHRASLS1 and M28sHRASLS2) had mRNA expression levels comparable with normal samples or unmethylated cancer cell lines. Expression of antisense mRNA was detected in antisense LOX clones (M28asLOX1 and M28asLOX2). B, colony formation assay. Bottom panel, average numbers of colonies obtained in three plates are summarized with SD. Sense LOX clones formed fewer and smaller colonies than did vector-only and antisense clones. The number of colonies that the sense HRASLS clones formed was similar to the number of the control clones. C, soft agar assay. Bottom panel, average numbers of colonies are summarized with SD. The number of colonies was markedly decreased in sense LOX clones. D, expression of introduced LOX (left) and number of colonies in soft agar assay (right) produced by using clones derived from KATOIII. Whereas vector-only clones (KIIIV1 and KIIIV2) showed no LOX expression, sense LOX clones (KIIIsLOX1 and KIIIsLOX2) had LOX mRNA expression comparable with normal samples or with unmethylated cancer cells. The sense clones showed decreased anchorage-independent colony formation. (PCNA, proliferating cell nuclear antigen.)

and two clones with sense HRASLS cDNA (M28sHRASLS1 and M28sHRASLS2) expressed the introduced genes at levels similar to those in normal samples or unmethylated cancer cell lines at their basal expression levels (Fig. 3A), and no induction by tetracycline was used for the following experiments. In two control clones transfected with an empty vector (M28V1 and M28V2), no expression of the endogenous LOX or HRASLS gene was observed.

Doubling times of M28sLOX1 and M28sLOX2 were 32.9 and 33.4 hours and were reduced to 89 to 90% of those of two control clones, M28V1 (30.1 hours) and M28V2 (29.3 hours). In anchorage-dependent colony formation assay, the numbers of colonies formed by the two LOX sense clones were decreased to 61 and 57%, respectively, of that of M28V1 (P < 0.01, t test; Fig. 3B). The sizes of colonies were also decreased. In soft agar assay, the numbers of colonies formed by the two LOX sense clones were remarkably decreased to 11 and 23% of that of M28V1, respectively (P < 0.001; Fig. 3C). On the other hand, the two HRASLS sense clones did not show growth-suppressive activity in any assay (Fig. 3B and C). These demonstrated tumor suppressor activity of LOX in gastric cancer cells in vitro.

To confirm the finding in MKN28 cells, LOX cDNA was introduced into KATOIII cells. Two LOX sense clones (KIIIsLOX1 and KIIIsLOX2) were obtained, and the LOX expression levels in them were comparable with those in normal samples or unmethylated cancer cell lines (Fig. 3D). Doubling times of KIIIsLOX1 and KIIIsLOX2 were 35.9 hours and 36.4 hours, respectively, and were almost the same as those of two control clones, KIIIV1 (35.2 hours) and KIIIV2 (35.7 hours). KATOIII was a floating cell line and had no tumorigenicity in nude mice, and we were able to perform only soft agar assay. The two LOX sense clones showed marked decreases in the numbers of colonies to 11% (P < 0.001) and 14% (P < 0.01) of that of a control clone KIIIV1 (Fig. 3D).

Analysis of In vivo Tumor Suppresser Activity of LOX and HRASLS. Tumorigenicity in nude mice was examined by subcutaneous injection of the clones obtained from MKN28 (Fig. 4). The sizes of tumors formed by the two LOX sense clones were 19 and 26%, respectively, of the tumors formed by M28V1 on day 42 (P < 0.01). In contrast to the two LOX sense clones, the sizes of tumors formed by the two HRASLS sense clones were similar to those of the vector-only clones.

DISCUSSION

Inactivation mechanisms of LOX, LOH with promoter methylation, and biallelic promoter methylation, and its growth-suppressive activity were here demonstrated in human gastric cancers. Although we made an extensive search for LOX mutations with 96 primary cancer samples and 58 cancer cell lines, only one possible mutation (Ala147Gly) was found in an ovarian cancer cell line. Somatic mutations of LOX were thus considered to be rare, as reported in colon cancers (1 of 66; ref. 24). Instead, promoter methylation and LOH were observed in 27 and 33%, respectively. LOX is located on chromosome 5q (25), and frequent LOH of this locus has been reported in gastric and other cancers (26, 27). Although target genes of LOH at 5q are considered to be APC and MCC at 5q21 or IRF-1 at 5q31 (28, 29), LOX could be an additional target gene. Notably, methylation-associated silencing was observed in lung, colon, and ovarian cancer cell lines as well, suggesting involvement of LOX in various human cancers.

LOX has been known as an extracellular enzyme that catalyzes the oxidative deamination of peptidyl lysine to α-aminoacidic-β-semialdehyde, initiating covalent cross-linking of collagens and elastins (30). In addition, LOX has been reported to suppress growth of mouse/rat fibroblasts transformed by ras oncogene (9, 10). Because it was not known whether or not LOX has growth-suppressive activities in gastric cancer cells, we here introduced LOX cDNA into MKN28 gastric cancer cells, in which LOX was silenced. The introduction induced a slight decrease in cellular growth rate, but it showed remarkable reduction of anchorage-dependent and -independent colony formations and tumor formation in nude mice. By introduction of LOX into KATOIII gastric cancer cells, reduction of anchorage-independent colony formation was also observed. These data demonstrated that LOX has a tumor suppressive function in human gastric cancer.
cancer cells and indicated that loss of its expression could be involved in cancer development.

Tumor suppressor function and silencing of LOX in gastric cancers were in accordance with reports that described expression loss of LOX in colon and prostate cancers (24, 31). On the other hand, up-regulation of LOX was reported to correlate with higher staging in renal cell cancers (32), and, in particular, expression of LOX in human breast cancer cell lines increased their in vitro invasion capacity (13). One possible explanation for these contradictory reports on LOX function is that its function could be dependent on cell types, as pointed out by Kirschmann et al. (13). Another point to be considered is that tumor suppressor genes can be overexpressed to counteract rapid proliferation of cancer cells in which they are still functioning, as observed in the case of p16 (33). LOX was also overexpressed in gastric cancers without its silencing in this study.

The molecular mechanism of how LOX exerts its tumor suppressor activity is still unclear. Although LOX was initially identified as an extracellular enzyme, its fully catalytic form has been shown to be present in the nuclei of vascular smooth muscle cells and fibroblasts (34, 35). On the basis of its specific interaction with histones H1 and H2, LOX was suggested to exert its tumor suppressor activity through transcriptional regulation of specific genes (36). From the viewpoint of cellular signaling, LOX was originally shown to be down-regulated in ras-transformants and up-regulated in revertants (9), and down-regulation of LOX led to cellular transformation associated with ras activation (10). Recently, LOX was shown to inhibit nuclear factor κB, a crucial factor that mediates ras-induced transformation, by activating IkB (inhibitor of κB) through the inhibition of the IkB kinase complex and by down-regulation of phosphatidylinositol 3'-kinase and Akt kinases (37). Although K-ras mutations are known to be rare (0–4%) in human gastric cancers (38, 39), and we did not find K-ras mutations in MKN28 and KATOIII (data not shown), nuclear factor κB could be activated by an alternative pathway, such as activation of Her-2/neu (40, 41). In this case, the major target of tumor suppressor activity of LOX would be the blocking of NF-κB signaling. However, more investigations are necessary to clarify what activity of LOX in what signaling pathway is critical for its tumor suppressor activity.

In contrast to LOX, HRASLS did not undergo LOH with promoter methylation. This was in accordance with the fact that LOH of chromosome 3q, where HRASLS is located (12), has not been reported in gastric cancers. Human HRASLS is a homologue of mouse A-C1 (12), which inhibited the growth of ras-transformed mouse fibroblasts (11). However, the introduction of HRASLS into MKN28 cells did not suppress their growth either in vitro or in vivo. Although there remains a possibility that HRASLS still possesses tumor suppressor activity in different cell types, it did not have tumor suppressor activity, at least, in MKN28 gastric cancer cells.

One of the nine genes the silencing of which we identified by a methylation-based genome-scanning method, MS-RDA (8), turned out to be a tumor suppressor gene. This supports the methylation-based approach to tumor suppressor genes, which was first achieved in the case of SOCS-1 in human hepatocellular carcinomas (42). However, at the same time, we have to note that there are a significant number of genes in cancer cells of which the promoter CpG islands are methylated and of which the expressions are lost (43). Therefore, the role of a gene that is silenced in cancer cells needs to be cautiously analyzed and interpreted.

In the present study, we found that LOX is a tumor suppressor gene inactivated by methylation and LOH in human gastric cancers, and that genome scanning for aberrant methylations is useful to isolate candidate tumor suppressor genes.

REFERENCES

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