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SOMATIC MUTATIONS OF THE LYSYL OXIDASE GENE ON CHROMOSOME 5Q23.1 IN COLORECTAL TUMORS

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Lysyl oxidase (LOX), a copper-dependent amine oxidase, has been implicated in tumor suppression and cell growth regulation. The chromosomal locus of LOX, 5q23, is affected by loss of heterozygosity (LOH) in colon cancer, suggesting that the LOX gene could be affected by LOH and consequently, loss or reduction of LOX function contribute to the tumorigenic process. Identification of microsatellite markers within the LOX locus has allowed us to map the LOX gene within the 5q23.1 region. Analysis of this locus and flanking loci in matched tumor and blood DNA samples from a panel of colorectal cancer patients, demonstrated that 38% (16/42) of informative samples were affected by LOH or allelic imbalance. Furthermore, 75% (6/8) of these tumor samples were shown to have significantly reduced LOX mRNA levels. Similar reduction in LOX levels were detected in a panel of matched normal colon and colon tumor samples. Tumor samples demonstrating LOH by RFLP, were subject to mutational analysis, including RT-PCR, exonic deletion detection by PCR, cDNA and genomic DNA sequencing, and were found to have a spectrum of alterations and mutations affecting the LOX gene. These results confirm that loss or reduction of LOX function during tumor development is a direct consequence of somatic mutations and is associated with colon tumor pathogenesis.

Key words: *chromosome 5; LOX; mutations; colon cancer*

Lysyl oxidase has been traditionally described as a copper-dependent extracellular amine oxidase responsible for the catalysis of the cross-linking of fibrillar collagens and elastin.[1] In addition to its role in the assembly of these extracellular matrix polymers, lysyl oxidase exhibits well documented growth regulatory activity and it has recently been implicated in intra-cellular functions.[2,3] Lysyl oxidase has been localized to the cytoskeleton[4] and to the nucleus, and an intra-nuclear lysyl oxidase activity has been demonstrated.[5]

Low levels of LOX mRNA were detected in several human tumor cell lines, in SV 40 transformed WI-38 cells, melanoma cells, fibrosarcoma, choriocarcinoma and rhabdomyosarcoma cells as a result of transcriptional downregulation and decrease in mRNA stability.[6,7] Similarly, reduced lysyl oxidase mRNA levels were observed in *ras*-transformed NIH 3T3 cells, rat fibroblasts and osteoblast cells.[8–10] The lower LOX activity of the transformed cells correlated with increased tumorigenicity in nude mice.[8]

Changes in lysyl oxidase gene expression have also been shown to be associated with invasive and metastatic prostate and breast cancer. Although significant lysyl oxidase gene expression was observed in normal prostatic epithelium and in normal prostatic stroma, the levels of lysyl oxidase mRNA were progressively reduced in malignant prostate epithelial cells and in primary and metastatic prostate tumors.[11] In breast tumors, abundant amount of lysyl oxidase protein was observed in benign breast lesions surrounding *in situ* ductal carcinomas and in the reactive fibrosis at the invasion front of infiltrating tumors. The amount of lysyl oxidase decreased markedly in late stromal reactions and there was no detectable lysyl oxidase in the loose scirrhous stroma of invading ductal carcinomas.[12,13]

Several regulatory mechanisms have been described that may explain the decrease of lysyl oxidase gene expression associated with tumorigenesis. Transcriptional downregulation in *ras*-transformed osteoblastic cells is probably mediated through a *ras*-regulatory element within the promoter of the lysyl oxidase gene.[10] LOX has also been shown to block the ras-MEK1 pathway downstream of Erk2.[3] TGF-β1 has been shown to increase LOX mRNA levels in various cultured cell types through a post-transcriptional regulatory mechanism that resulted in an inhibition of cell proliferation.[14] This response of LOX to TGF-β1 was lost in prostate cancer, resulting in a progressive and dramatic decrease of the amount of the LOX mRNA in prostate tumors.[11] The LOX gene was also shown to be the target of the antioncogenic interferon regulatory factor IRF-1 through an IRF response element within the LOX gene promoter in c-Ha-*ras* transformed embryonic fibroblasts.[15]

The lysyl oxidase gene has been localized to chromosome 5q23.[16,17,] Chromosome 5q interstitial and complete losses are recurring anomalies associated with a number of malignancies such as adenocarcinoma of the stomach,[18] lung carcinoma,[19,20] prostate cancer,[21] and male germ cell tumors.[22,23] Furthermore, a chromosome imbalance map based on over 300 cases of colon cancer showed approximately 15% loss of material of chromosome 5q14–5q31, with slightly higher percentage of loss for 5q21–31.[23]

In addition to APC and MCC at chromosome 5q21, and IRF-1 at 5q31, other genes may be inactivated in a variety of malignancies through high frequency 5q21–31 somatic deletions,[15,19,21] and these deletions could possibly affect the lysyl oxidase gene at 5q23. To investigate, therefore, the possibility that the loss or reduced function of lysyl oxidase in the course of tumor development not only arises though regulatory mechanisms, but is a direct result of deletions and mutations affecting the LOX gene, we have tested the mutational status and expression level of the LOX gene in a cohort of patients with colorectal carcinomas.

MATERIAL AND METHODS

Patient population

Samples of normal tissue (whole blood) and tumor tissue were obtained from consenting patients with no obvious family history

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of colon cancer, who were undergoing surgery for colon cancer. After standard histological assessment, samples were removed from a representative area of the tumors for DNA and RNA isolation. The composition of the 66 patient panel was 54% female and 46% male, with an average age of 70.6 years (with a standard deviation of 11.5 years). All tumors were adenocarcinoma. Of these, 30% were removed from the proximal colon whereas the remainder were from the distal colon. The majority of the tumors were Stage B (73%), followed by Stage C (14%), Stage A (9%) and Stage D (4%).

PAC clone isolation and mapping

Two overlapping PAC clones, gs8423 and gs8424, both containing the entire LOX gene were obtained through Genome Systems Inc. (St. Louis, MO) using LOX gene-specific PCR primers. Purification of plasmid DNA was performed as recommended by Genome Systems, Inc. Plasmid DNA was digested with *Eco*RI and *Hind*III restriction enzymes. Restriction fragments were electrophoresed on 1% agarose gels, transferred onto nylon membrane and were hybridized to a ^{32}P-labeled $(dGT)_n$ probe to detect (CA) repeat sequences. *Pac*I and *Not*I restriction digests and Southern blot analysis of the PAC DNA was used to map these PAC clones using labeled LOX cDNA and LOX-specific oligonucleotide probes.

DNA and RNA isolation

DNA was isolated from blood using the method of Madisen *et al.*[24] DNA was isolated from tumor tissue using the QIAamp Tissue Kit (Qiagen Inc, Valencia, CA). Total RNA was isolated from tumor tissue samples using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX). Normal human smooth muscle cells were cultured to confluency before RNA extraction using the same method.

Southern blot analysis of tumor DNA samples

Aliquots of genomic DNA (5–10 μg) extracted from tumor samples were digested with *Pst*I restriction enzyme. DNA fragments were separated by electrophoresis through 1% agarose gels, blotted onto nitrocellulose filters and hybridized to a ^{32}P-labeled LOX cDNA probe.[18]

Microsatellite markers and genotyping

Three microsatellites at the LOX locus at 5q23, lms1, D5S467 and lms15, were used for loss of heterozygosity (LOH) analysis. Sequences for primers used to amplify these microsatellites are listed in Table I. Additional markers centromeric (D5S421, D5S471) and telomeric to the LOX locus (D5S490 and D5S642) and D5S346, a marker between the APC and MCC genes were also used in this LOH study (http://gdbwww.gdb.org/gdb/).

PCR reactions were performed in a final reaction volume of 25 μl with the following conditions: 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 20 ng genomic DNA, 1 μM each primer and 0.5 U AmpliTaq polymerase (Perkin-Elmer, Foster City, CA). The final nucleotide concentration for primers D5S346, D5S421, D5S471, lms1 and lms15 was 31 μM; for primers D5S467 and D5S642 124 μM and for primers D5S490 200 μM. In reactions with primers D5S421, D5S471, D5S490 and D5S642, 1% Triton X-100 and with primers D5S421, D5S467, lms15, D5S490 and D5S642, 10% final concentration of DMSO was included. All reactions were subjected to an initial denaturation step of 94°C for 3 min and a final extension of 72°C for 7 min. The thermocycle profile for markers D5S346, lms1, D5S467 and lms15 was 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec and for the remaining markers the annealing temperature was 55°C. PCR amplifications in all reactions were for 30 cycles, except for those PCR reactions containing DMSO, that required an additional 5 cycles. Labeled amplified products were mixed with an equal volume of formamide loading dye, denatured at 94°C for 5 min, then analyzed on 6% denaturing polyacrylamide gels. Autoradiography was performed with Fuji X-ray film overnight at −80°C.

Loss of an allele or allele deletion was determined if 1 of the 2 alleles present in blood was absent or at least diminished by approximately 50% in tumor DNA quantification using an image acquisition and analysis system (Ambis Inc., San Diego, CA). Allelic imbalance was determined if 1 of the 2 alleles from tumor tissue was greater in intensity than the matching blood allele, or if any 2 alleles from tumor DNA differed in the ratio of intensity, when compared to the alleles in blood DNA. Although allelic imbalance can result from either loss of an allele, amplification or heterogeneity, we have grouped LOH and allelic imbalance in our results as they both indicate a contribution of this gene to tumor progression. New alleles were identified if additional alleles not present in blood DNA were noted in tumor DNA.

Matched Tumor/Normal Expression Array Analysis

A Matched Tumor/Expression Array (Clontech, Palo Alto, CA) was utilized according to the user manual. A ^{32}P-labeled human LOX cDNA clone (HLO-2)[18] was prepared by random primer labeling, and used for hybridization. The specific activity of the cDNA probe was 5×10^9 cpm/μg. The array was washed and exposed to a PhosphorImager plate. Signal intensities were quantified using the PhosphorImager:SI and the ImageQuant 5.0 software program (Molecular Dynamics, Sunnyvale, CA).

Mutational analysis of the LOX gene

For genomic sequencing of the LOX gene, exons and flanking intronic sequences were PCR amplified. The sequences of the intron derived primers for exons 1 through 5 are listed in Table I. PCR reactions were performed as described previously, except with 200 μM dNTP. For intronic primers flanking exons 2 and 3, 10% DMSO was used in the PCR reactions. Initial denaturation at 94°C for 3 min was followed by cycles of denaturation at 94°C for 30 sec; annealing for exon 1 primers was carried out at 60°C, for exon 2 and exon 4 primers at 48°C, exon 3 primers at 50°C, exon 5 at 46°C for 30 sec; followed by extension at 72°C for 30 sec. Final extensions were at 72°C for 7 min. Exon 1 was amplified for 40 cycles and all of the other exons for 35 cycles. Direct sequencing of the PCR products was performed using the ABI Prism BigDyeTerminator Cycle Sequencing Ready Reaction kit and an ABI 310 DNA sequencer (Perkin-Elmer). Sequence results were analyzed with the ABI Sequencing Analysis Software 3.0.

LOX allele deletion detection by a Pst*I RFLP*

DNA samples, heterozygous for a *Pst*I polymorphism in exon 1, were PCR amplified with primers flanking the polymorphic site.[25] PCR reactions were performed as described previously except with 5% DMSO and 31 μM dNTPs. The thermocycle profile consisted

TABLE I – SEQUENCES OF PCR PRIMERS USED TO AMPLIFY LOX GENE-ASSOCIATED MICROSATELLITE MARKERS AND FIVE EXONS OF THE LOX GENE

Primer	Sequence	Primer	Sequence
Ims1a	5′-GCT CAT TAA TGA GAG AAA C-3′	Exon 2a	5′-CCG GGT TGT TTC ACT CGT-3′
Ims1m	5′-ACA CCA GCA ATC TCA ACA-3′	Exon 2m	5′-CCC CTG AAG GTA GAC CG-3′
D5S467a	5′-CTA ACC AGA GGC TGC AAG-3′	Exon 3a	5′-ACT CTT GGA ACT GAT AG-3′
D5S467m	5′-TGA TCT TAG TGT GCC TTA GT-3′	Exon 3m	5′-CTG AGA AAT GAA AAG CAA-3′
Ims15a	5′-TTG CAG GAC TTC TCA GCC-3′	Exon 4a	5′-GCT TTC TCT GTA TGT AAC-3′
Ims15m	5′-CAG CCT CCA ATC TGA TTG-3′	Exon 4m	5′-ACC CGA TTC TCT CTG AGG-3′
Exon 1a	5′-TCA TCT GGA GTC ACC GCT GG-3′	Exon 5a	5′-GAC AGC TCA CTC TGA AA-3′
Exon 1m	5′-AGC TGG GGA CCA GGT GCA C-3′	Exon 5m	5′-TAA ATC AAG CAG GGA AGG G-3′

of an initial denaturation step of 94°C for 3 min followed by 35 cycles at 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec and a final extension step of 72°C for 7 min. The PCR products were digested with *Pst*I restriction enzyme and the resulting fragments were separated by electrophoresis through 10% polyacrylamide gels and stained with EtBr.

LOX cDNA analysis

First strand cDNA was synthesized using SuperScript Preamplification System (Gibco BRL, Gaithersburg, MD) and total RNA extracted from tumor tissue samples. PCR amplifications were undertaken using specific primers for the genes LOX, G3PDH and β-catenin. Primers 5′-CCT GGC TGT TAT GAT AC-3′ and 5′-GAG GCA TAC GCA TGA TG-3′ generated a 152-bp fragment of the LOX cDNA. These PCR reactions were performed as described previously, except with 1 μl of cDNA and 124 μM dNTPs. PCR reaction conditions were an initial denaturation at 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 7 min.

A 452 bp DNA fragment derived from the G3PDH cDNA was amplified using G3PDH primer pairs (Clontech, Palo Alto, CA). PCR reactions using these primers were performed as above, except with 200 μM dNTPs. The conditions for PCR reactions were identical to above except for an annealing temperature of 57°C.

The PCR amplification of β-catenin cDNA was performed with primers catf (5′-CAT AAC CTT TCC CAT CAT C-3′) and catr (5′-AAT CAA TCC AAC AGT AGC C-3′). The amplified 849 bp fragment represented the region between 874–1723 bp of the β-catenin cDNA. PCR conditions were as above, except for 0.8 μM of each primer, 1 mM dNTPs and 2.5 U AmpliTaq. Reaction conditions were identical to above except for an annealing temperature of 50°C. The amplified fragments were analyzed on 6% acrylamide or 2% agarose gels.

RESULTS

Lysyl oxidase gene maps to marker D5S467 at 5q23.1

The lysyl oxidase gene had been mapped earlier to 5q23 by FISH[16,17] but its precise position relative to D5S markers was unknown. To incorporate the lysyl oxidase gene into the chromosome 5q linkage map we have used a previously characterized YAC clone[26] and 2 newly isolated PAC clones (gs8423 and gs8424) that mapped within this YAC clone and contained the entire LOX gene. We have identified 6 microsatellites on the PAC clones surrounding the LOX gene, and 3 of these were further characterized to be polymorphic and were used for microsatellite studies. A comparison of these microsatellite sequences to the GenBank database indicated that 2 of these markers, lms1 and lms15, were new microsatellites. The third microsatellite was found to be identical to a previously described marker, D5S467.

Southern blot and sequence analysis of PAC clone gs8423 enabled us to establish the order of these markers relative to the LOX gene as follows: centromere–lms1–D5S467–LOX–lms15–telomere (Fig. 1). Microsatellite lms1 lies 20 kb, D5S467 5 kb centromeric to the LOX gene and lms15 is located 7 kb telomeric to the LOX gene. This result placed the lysyl oxidase gene telomeric to D5S467 at 5q23.1, between D5S471 and D5S818 markers (Fig. 1).

Loss of heterozygosity at 5q23 in colorectal tumor DNA samples

The LOX gene locus at 5q23.1 was subjected to LOH mapping in tumor and blood DNA samples from 66 patients with colon cancer using microsatellites lms1, D5S467 and lms15 spanning 40 kb at the LOX gene locus and additional flanking centromeric (D5S346, D5S421 and D5S471) and telomeric (D5S490 and D5S642) markers within the 5q21.3–23.2 region.

LOH and allelic imbalance at the three markers flanking the LOX gene in tumor DNA occurred at about the same frequency (all percentages are followed by absolute numbers in parentheses):

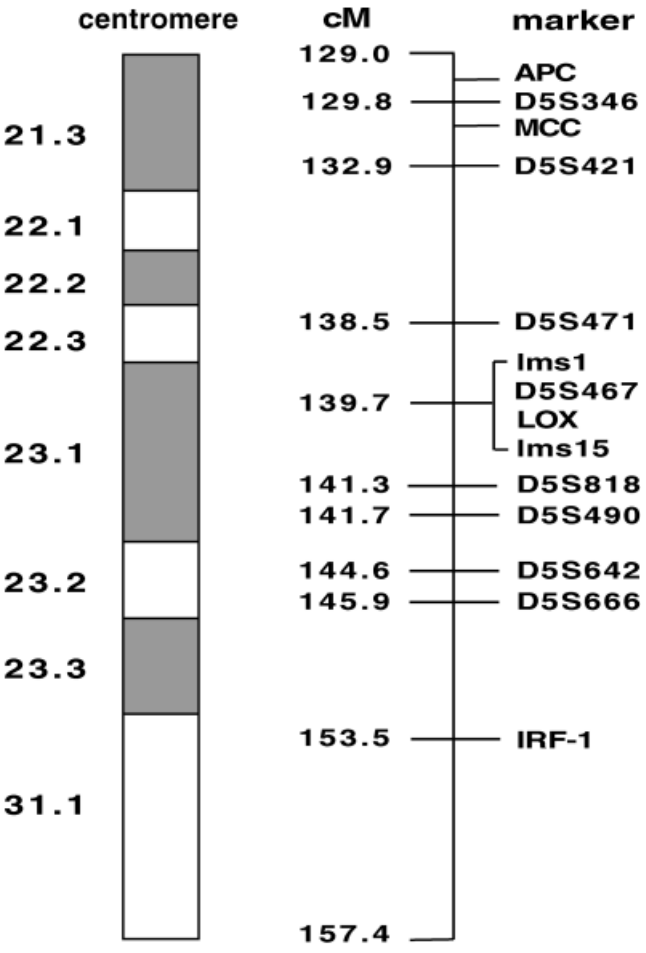

FIGURE 1 – Chromosomal localization of the LOX gene at 5q23.1. Map positions and physical distances in centimorgans (cM) are indicated. lms1 and lms15 are novel microsatellites. The positions of the LOX, APC, MCC and IRF-1 genes and the relative positions of microsatellite markers are also indicated.

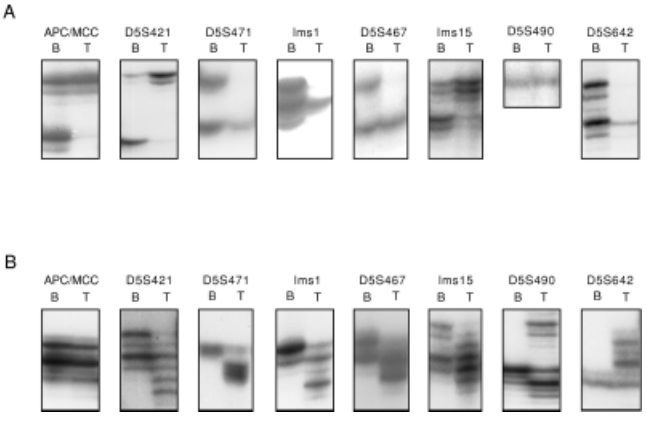

FIGURE 2 – Representative loss of heterozygosity (LOH) and microsatellite changes (MI) at the LOX gene and flanking loci in colon cancer. (*a*) All markers tested in colon tumor DNA relative to blood DNA samples demonstrated LOH in Tumor 19. Each panel contains an autoradiogram of the microsatellites PCR amplified from blood (B lanes) and tumor (T lanes) DNA. (*b*) Microsatellite instability was detected at all markers tested in Tumor 56.

32.2% (10/31) at the lms1, 35.7% (15/42) at D5S467 and 31.2% (15/48) at the lms15 locus. At all these 3 loci combined, 38.1% (16/42) of patients showed allelic changes of 1, 2 or all 3 microsatellite markers in colon tumor DNA samples, in cases when at least 2 of the markers were informative at the LOX gene locus. Representative alleles and LOH for the microsatellite markers tested are shown in Figure 2*a*.

The most centromeric marker tested in our study, D5S346, located between the APC and MCC genes at q21.3 revealed 37.1% (23/62) LOH and allelic imbalance in all informative patients. The D5S421 marker at q21.3 showed 37.7% (23/61) LOH and allelic imbalance; the third centromeric marker relative to the LOX gene (D5S471), 36.4% (20/55) LOH and allelic imbalance. The marker telomeric to the LOX gene, D5S490 at q23.1, had a lower value of 23.8% (10/42) and the most telomeric microsatellite, D5S642 at q23.2, demonstrated 38.8% (19/49) LOH and allelic imbalance. The results of these studies are summarized in Figure 3. Of the total number of patients, 9.1% (6/66) were noninformative for any of the 3 LOX microsatellites.

An additional 15.2% (10/66) of patients showed the presence of new alleles in their tumor DNA (MI) and 70.0% (7/10) of these had new alleles either at several or at all informative markers, suggesting possible DNA mismatch repair defects (RER+).[27] In general, most of the new alleles appeared to be shorter than the normal counterparts. Figure 2*b* illustrates examples of new alleles in Tumor 56 at all loci tested.

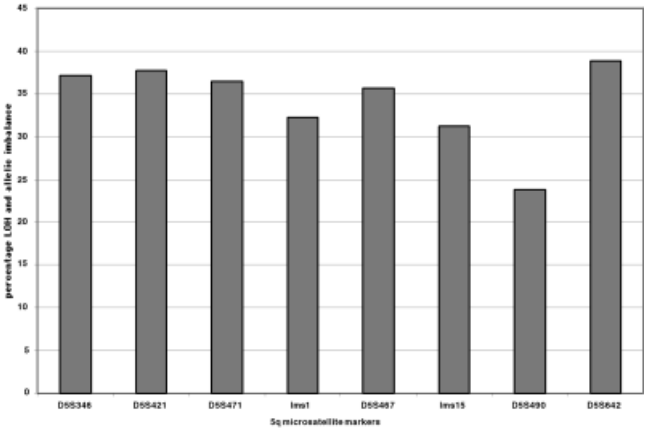

FIGURE 3 – Microsatellite changes at 5q21.3–q23.2 in colon cancer. LOH and allelic imbalance frequencies in tumor DNA samples at the LOX gene locus and flanking centromeric and telomeric markers.

The APC/MCC gene loci at 5q21.3 and the LOX gene loci at 5q23.1 were simultaneously affected in 33.3% (22/66) of tumor samples by LOH, indicating that a relatively large region of chromosome 5q was deleted in these cases. LOH at the APC/MCC gene locus without loss at the LOX gene locus was observed in 3.0% (2/66) of tumors. 10.6% (7/66) of the patients showed loss at the LOX locus in the absence of loss of the D5S346 marker at the APC/MCC locus.

No Stage A tumors tested in our study demonstrated LOH, allelic imbalance or microsatellite instability. LOH and allelic imbalance associated with the LOX gene was observed in Stage B and higher grade tumors. Tumors that demonstrated microsatellite instability were all Stage B.

Reduced LOX mRNA levels in colon tumors

To establish LOX expression levels in tumors relative to normal colonic tissue, an expression array (Clontech) that contained 11 matched normal/colon tumor samples was hybridized with a LOX cDNA probe. All of the tumors were adenocarcinoma, except for 2 which was a benign tumor. All the normal colon samples demonstrated expression of LOX. There was downregulation of LOX in 7 of the 10 adenocarcinoma samples and in the benign tumor (Fig. 4).

RT-PCR was used to evaluate the amount of LOX mRNA in the colorectal tumor tissues that were analyzed for LOH. For this experiment, tumor DNA samples were divided into 2 groups. Group-1 demonstrated either loss of heterozygosity or allelic imbalance at 2 or more 5q21–23 microsatellites. In Group-2 tumor DNA samples, no alterations of these microsatellites were detected. A conserved region of the LOX mRNA, corresponding to sequences encoded by exons 5–6, was amplified using RT-PCR from total RNA extracted from these tumor tissue samples. The LOX mRNA was detected in all Group-2 RNA samples (not shown) but there was a significant reduction of the LOX mRNA in 6 out of 8 tumor tissue samples in Group-1. Two internal controls were used in these experiments: β-catenin mRNA, known to be up-regulated in tumors with 5q loss,[28] was readily amplified in all tumor samples in Group-1 but was not detected in any of the tumor samples in Group-2 (not shown). GAPDH mRNA was abundantly present in all samples (Fig. 5).

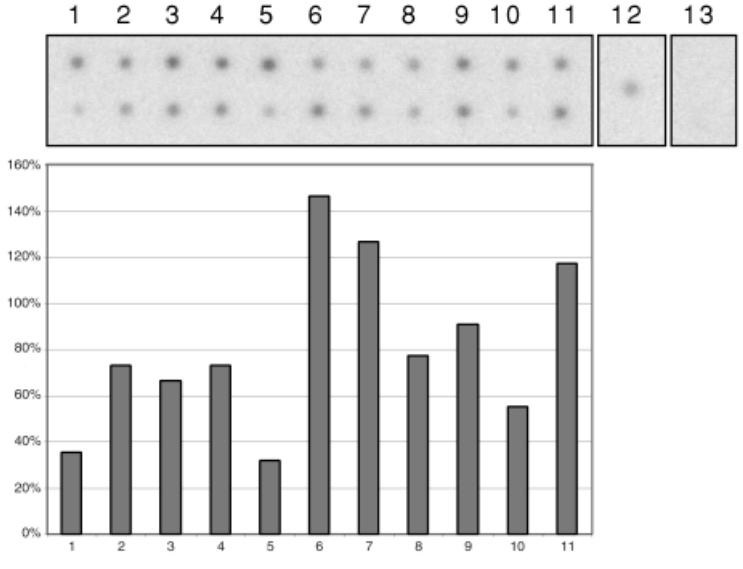

FIGURE 4 – Reduced LOX mRNA levels in matched normal and tumor tissue from colon. A Matched Normal/Tumor Expression Array (Clontech) was analyzed for LOX mRNA expression. The upper row of samples 1–11 are from normal colon tissue and the lower row of samples are from matching colon tumors. *Lane 1*: benign tumor; *lanes 2–11*: adenocarcinoma; *lane 12*: positive control of genomic DNA; *lane 13*: negative control of yeast total RNA. The bar graph demonstrates the amount of LOX expression in colon tumor compared to its matching normal tissue expression.

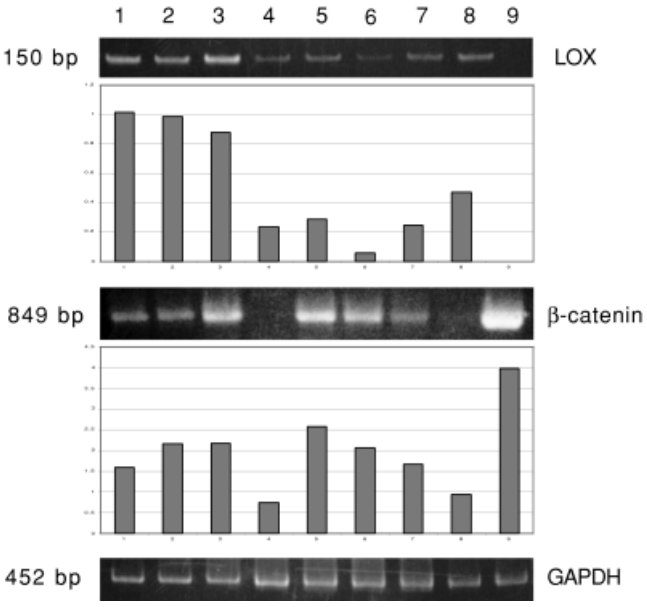

FIGURE 5 – Reduced LOX mRNA levels in colon tumors demonstrating LOH. Total tumor RNA samples were analyzed by RT-PCR for the presence of LOX mRNA, β-catenin and GAPDH mRNA. *Lane 1*: positive control of normal human smooth muscle cell RNA; *lanes 2–9*: RNA samples from colon tumors that demonstrated LOH or allelic imbalance at the LOX gene locus. The sizes of the amplified fragments corresponding to each mRNA are indicated in bp on the left of the panels. The identity of the mRNAs that these RT-PCR products represent are indicated on the right side of the panels.

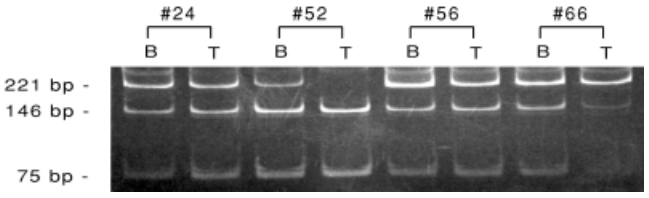

FIGURE 6 – LOX gene allelic deletion detected using a G/A polymorphism. A *Pst*I polymorphic site within exon 1 of the LOX gene was PCR amplified from tumor and blood DNA samples of informative Tumors 24, 52, 56 and 66. The resulting DNA fragments were digested with *Pst*I restriction enzyme and size separated. The major G allele was represented as a 221 bp fragment, the minor A allele was detected as 2 fragments of 146 and 75 bp. B, blood; T, tumor DNA.

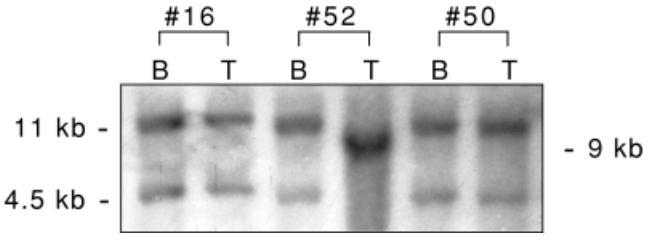

FIGURE 7 – Southern blot analysis of the LOX gene in tumor DNA samples. *Pst*I digested blood and tumor DNA samples that showed LOH at the LOX gene locus 16, 25 and 50, were hybridized to a lysyl oxidase cDNA probe. B, blood; T, tumor DNA. Sizes of hybridizing genomic DNA fragments are indicated in kb.

Somatic mutations of the LOX gene

The results of the LOH studies and reduced LOX mRNA levels in these tumor tissues indicated that the expression of the LOX gene was probably affected by 5q21–23 allelic losses in colorectal tumors. We have used RFLP, Southern blot analysis, exon amplifications, RT-PCR and genomic sequencing to assess the actual deletional status and the spectrum of possible somatic mutations within the LOX gene.

Detection of LOX allelic deletions using an exon 1 Pst*I RFLP*

A previously characterized *Pst*I restriction fragment length polymorphism within the first exon of the LOX gene[25] enabled us to monitor the deletional status of this exon in each allele of the tumor DNA samples of patients that demonstrated microsatellite changes and were informative for this polymorphism. The low frequency of the minor allele of this RFLP in these patients (13%), provided us with only 4 informative patients and 50% of these demonstrated loss of one allele (Fig. 6). The remaining alleles of the LOX gene, allele A represented as 145 and 75 bp bands in Patient 52 and allele G represented as a 221 bp band in Patient 66, were further analyzed by Southern blot hybridization.

Southern blot analysis of the LOX gene

Tumor DNA samples 52 and 66, as well as an additional 12 samples with demonstrated LOH and microsatellite changes at the LOX gene locus were analyzed using Southern blot analysis to detect homo- or heterozygous deletions or rearrangements. *Pst*I digested blood and tumor DNA samples were hybridized to a lysyl oxidase cDNA probe. In one of the tumors (52), a 4.5 kb fragment containing exons 4 and 5 and an 11 kb fragment containing exons 6 and 7 of the LOX gene were absent and an aberrant 9 kb fragment was detected (Fig. 7).

Exon deletions within the LOX gene

The first 5 exons of the LOX gene that encode important functional domains of the LOX protein, including the signal peptide, processing site, copper and metal binding sites, and the catalytic domain, were PCR amplified to test the possibility that intragenic mutations may have affected these exons in colon tumors. The identity of the PCR amplified products was confirmed in each case by DNA sequencing. Exon 1 could not be amplified in 5 out of 6 tumor samples that demonstrated low levels of LOX mRNA and 5q LOH, exon 2 in 3 tumor samples, 2 of which lacked exon 1 as well. All LOX exons amplified from normal DNA samples (Table II).

Mutational analysis of the LOX mRNA

To detect small deletions or point mutations within the LOX gene in colon tumors, total RNA was isolated from the 6 tumor samples that previously demonstrated both 5q21–23 LOH and low LOX mRNA levels, and the 2 tumor samples which demonstrated LOH by exon 1 *Pst*I RFLP. A 5′ domain of the LOX mRNA encoded by exons 1–4 of the LOX gene was RT-PCR amplified from these RNA samples. Subsequent DNA sequence analysis of these RT-PCR products detected a point mutation in one of these samples. This mutation was found in the sequence encoded by exon 4, at codon 332. The mutation replaced a T to an A and changed a TAT codon to a TAA stop codon in Tumor 66. We had already demonstrated alteration of one allele in this patient using a *Pst*I RFLP within exon 1 of the LOX gene (Fig. 8).

Mutation detection by genomic sequencing

To confirm the mutational status of the first 5 exons of the LOX gene, these exons were PCR amplified using primers based on flanking intronic sequences and the resulting DNA fragments were sequenced. In 8 tumors DNA tested, only 1 mutation was found in Tumor 66, confirming the result of cDNA sequencing.

Alterations or deletions of both LOX alleles were detected in 12.1% (8/66) of the tumors. These results and LOH at markers flanking the LOX gene within the same tumor samples is summarized in Table II.

TABLE II – SPECTRUM OF SOMATIC MUTATIONS WITHIN THE LOX GENE AND FLANKING MARKERS IN COLON TUMORS[1]

Tumor sample no.	Ims1	D5S467	LOX							Ims15
			Exon 1	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7	
5	HET	NI	HOM	+	+	+	+	NT	NT	HET
9	HET	HET	HOM	HOM	+	+	+	NT	NT	HET
19	HET	HET	HOM	HOM	+	+	+	NT	NT	HET
43	NI	HET	+	HOM	+	+	+	NT	NT	HET
51	NI	HET	HOM	+	+	+	+	NT	NT	HET
63	HET	NI	HOM	+	+	+	+	NT	NT	HET
66	INS	INS	HET	+	+	MU	+	NT	NT	INS
52	NI	NI	HET	+	+	+	HOM	HOM	HOM	NI

[1]Imx1, D5S467, and Ims15 are LOX gene flanking microsatellites at 5q23.1. HET, heterozygous deletion; HOM, homozygous deletion; INS, microsatellite instability; MU, mutation; NT, not tested; NI, non-informative; +, exon present.

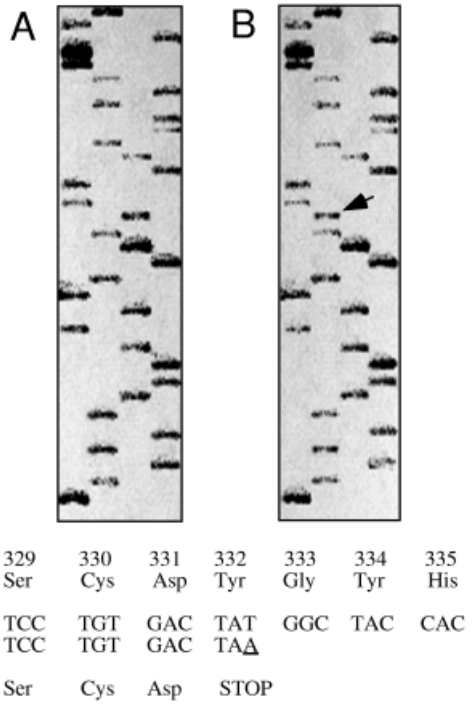

FIGURE 8 – Inactivating point mutation within the LOX gene. Tumor DNA sample from Patient 66 who demonstrated microsatellite instability at the LOX gene was analyzed by direct exonic sequencing. (*a*) Exon 4 sequence from a normal individual. (*b*) A T to A change in colon Tumor 66. The nucleotide change is indicated by an arrow, numbers refer to codons within the LOX mRNA.

DISCUSSION

Previous studies reported LOH and mutational analysis of chromosome 5q21–22, including the APC and MCC genes[29–32] and the more telomeric myeloid leukemia locus at 5q31.[33] Observations of high frequency losses of markers along 5q, including the 5q23 region, indicated that additional important putative tumor suppressor genes may also be affected in several malignancies. No further LOH or mutational studies, however, were directed to the region between 5q22 and q31. Similarly, there are no previous reports addressing the significance or clinical relevance of mutations affecting other genes located within this critical region. The results reported here have confirmed the loss of allelic markers in the 5q23 region and identified somatic mutations within surviving alleles of LOX, a putative tumor suppressor gene.

New microsatellites, that we have characterized, enabled us to position the LOX gene to D5S467 within the 5q23.1 region and proved to be valuable for the microdeletional analysis of the LOX gene locus. Using these microsatellites, we were able to detect a high frequency of deletions at the 5q23.1 region in tumor DNA of colon cancer patients. The results of these studies also demonstrated a spectrum of changes of the LOX gene locus that included loss of heterozygosity, allelic imbalance and the presence of new alleles.

Further mutational analysis revealed that, in addition to these changes, the LOX gene was affected by a range of somatic mutations that included 5′ intragenic alterations or deletions, a 3′ end rearrangement, possibly due to deletion, and a point mutation resulting in a premature stop codon. Alterations within the LOX gene affected exon 1 and in fewer tumor samples, exon 2, but not exon 3.

To monitor changes in the expression of the LOX gene within tumor samples we have used a quantitative approach. An expression array with matched normal colon and colon tumor samples allowed us to demonstrate the expression of LOX mRNA in normal colon tissue and the reduction of this mRNA level in most of the matched colon tumors. The LOH status of the LOX gene region was not known for this randomly selected panel. In tumors that demonstrated 5q LOH, significantly reduced amount of RT-PCR amplified LOX mRNA was detected. Though significant variations existed among individuals, the reduction of LOX levels were consistent in both panels. In some of the tumors, the small amount of LOX mRNA we have detected could also be derived from normal cells present in these tissues.[34,35] Based on our hypothesis that loss or reduction of LOX function is associated with colon tumor pathogenesis, further mutation detection focused on tumors that had significantly reduced amount of the LOX mRNA. Therefore, it is possible that mutations that did not result in dramatic reduction of the mRNA were not detected in our study.

In addition to allelic losses, alterations, a rearrangement and a point mutation of the LOX gene were noted. In one tumor, one of the LOX alleles was absent based on RFLP analysis and the other carried a point mutation in exon 4 resulting in a premature stop codon and consequently a null allele. This was 1 of the tumors representing about 15% of our patient population with an RER+ phenotype.[27] In this tumor, we have detected 3 different mutational mechanisms including a replication error, a deletion and a point mutation, in contrast to an earlier report by Lengauer *et al.*[36] who concluded that these mutational mechanisms may be mutually exclusive.

Most of the patients on our panel that demonstrated LOH or mutation of the LOX gene also had LOH of the D5S346 marker located between the APC and MCC genes. Though loss of function of neither the APC gene nor the MCC gene was established by our study, it is likely that in some of the cases with LOH at the D5S346 marker, the APC or the MCC gene are also inactivated. Therefore, the contribution of LOX gene deletions relative to the APC and MCC genes in colorectal tumor pathogenesis could not be determined. There was only 1 patient (43) on our mutation panel, however, that demonstrated loss of both alleles of the LOX gene that did not show LOH of the D5S346 marker.

The results presented here demonstrate that the LOX gene is affected by a spectrum of alterations and mutations in colon carcinoma and have established that reduction or loss of function of the LOX gene in colon tumors is due, at least partly, to these somatic mutations. Therefore, the LOX gene is part of a cascade of mutations affecting chromosome 5q[37,38] and this loss of LOX expression is associated with the pathogenesis of colon carcinoma, supporting the hypothesis of the role of this gene as tumor suppressor possibly involved in several malignancies.[11,13]

It is not known, however, how alterations in LOX levels lead to a tumorigenic phenotype. Specific transport of the fully processed 32 kD LOX protein from the extracellular space to the nucleus has been recently demonstrated.[39] Furthermore, this nuclear form of LOX was shown to exhibit intra-nuclear catalytic activity.[5] That LOX could affect nuclear function was confirmed by the observation of a less tight chromatin packing state directly induced by LOX transfection,[40] which may be a mechanism that influences the expression of many genes and thus the growth and differentiated state of colonic epithelial cells. Alternatively, LOX might affect a main cell growth control mechanism. Indeed, LOX has been shown to block the ras-MEK1 pathway downstream of Erk1, and may contribute to downstream inhibition of *ras*-triggered processes such as mitogenesis and transformation reported by DiDonato *et al.*[3] Mutations of the LOX gene could result in loss of this ras-inhibitory function and lead to tumor pathogenesis.

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