High Frequency of *LMAN1* Abnormalities in Colorectal Tumors with Microsatellite Instability

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Abstract

Glycosyl epitopes have been identified as tumor-specific markers in colorectal tumors and various lines of evidence indicate the significance of altered synthesis, transport, and secretion of glycoproteins in tumorigenesis. However, aberrant glycosylation has been largely ignored in microsatellite unstable (MSI-H) colorectal tumors. Therefore, we analyzed mutation frequencies of genes of the cellular glycosylation machinery in MSI-H tumors, focusing on frameshift mutations in coding MNRs (cMNRs). Among 28 candidate genes, *LMAN1/ERGIC53*, a mannose-specific lectin mediating endoplasmatic reticulum (ER)-to-Golgi transit of glycosylated proteins, showed high mutation frequency in MSI-H colorectal cancer cell lines (52%; 12 of 23), carcinomas (45%; 72 of 161), and adenomas (40%; 8 of 20). Biallelic mutations were observed in 17% (4 of 23) of MSI-H colorectal cancer cell lines. *LMAN1* was found to be transcribed but truncated protein remained undetectable in these *LMAN1*-mutant cell lines. Immunohistochemical and molecular analysis of *LMAN1*-mutated carcinomas and adenomas revealed regional loss of LMAN1 expression due to biallelic *LMAN1* cMNR frameshift mutations. In LMAN1-deficient colorectal cancer cell lines, secretion of the LMAN1 client protein α-1-antitrypsin (A1AT), an inhibitor of angiogenesis and tumor growth, was significantly impaired but could be restored upon LMAN1 re-expression. These results suggest that *LMAN1* mutational inactivation is a frequent and early event potentially contributing to MSI-H tumorigenesis. [Cancer Res 2009;69(1):292–9]

Introduction

Microsatellite instability is a hallmark of tumors associated with hereditary nonpolyposis colorectal cancer (HNPCC, Lynch Syndrome) but also occurs in ∼15% of sporadic colorectal carcinomas. It is caused by loss of DNA mismatch repair (MMR) function due to germline or somatic mutations of four MMR genes (*MLH1, MSH2, MSH6,* and *PMS2*) in HNPCC patients or by epigenetic silencing (*MLH1*) in sporadic microsatellite unstable (MSI-H) colorectal tumors (1–4). Clinicopathologic characteristics of MSI-H colorectal cancer differ from their microsatellite stable (MSS) counterpart (5). For example MSI-H colorectal tumors occur at younger age, have better prognosis, are nearly diploid, show predominantly proximal localization, are characterized by strong lymphocytic infiltration and seem to be refractory to 5-fluorouracil chemotherapy.

MSI-H-carcinomas result from a distinctive oncogenic pathway and the detection of selective MSI-H target genes with relevance for carcinogenesis is essential for the understanding of MSI-H tumor development. MSI-H cancers accumulate a large number of mutations in mononucleotide repeats (MNR), and if located in coding regions (cMNR), these frameshift mutations can lead to truncated proteins with altered or complete loss of protein function, thereby affecting cell behavior. Several cMNR containing genes that potentially promote MSI-H tumor development and growth have been verified by functional studies, including *TGFBR2, BAX,* and *ACVR2* (6–8). Woerner and colleagues (9) proposed a statistical model for predicting Selective Target Genes, which in combination with a human MNR mutation database (SelTar*base*)[3] can be applied to browse for putative target genes.

Glycosylation is the most common posttranslational modification of proteins, affecting their function in a variety of physiologic and pathologic events, including cell growth, migration, differentiation, tumor invasion, host-pathogen interactions, cell trafficking, and transmembrane signaling (10). A striking example is the regulation of the biological functions of integrins and cadherins by branched N-glycans in cell-cell and cell-extracellular matrix interactions (11). In the ER and in the early secretory pathway, glycan structures play a pivotal role in protein folding, oligomerization, quality control, sorting, transport, and secretion (12, 13).

Human colon cancer is associated with changes in protein glycosylation (14–17) and altered glycosylation has already been associated with tumorigenesis, tumor growth, and metastatic potential in colorectal cancer. Thus, epigenetic changes in glycosyltransferases have been found to contribute to the malignant phenotype of gastrointestinal cancer (18). Also O-glycosylation was shown to regulate apoptosis, proliferation, and metastatic potential of colorectal cancer cells (19, 20). Recent studies revealed that aberrant glycosylation on a secreted tissue inhibitor of the metalloproteinase-1 reinforces the invasive/metastatic potential of colon cancer (21). Moreover, a functional role of altered N-glycosylation patterns on colorectal cancer cell motility and invasiveness has been described (22).

Despite the obvious significance of altered glycoprotein synthesis, transport, and secretion in colorectal cancer, aberrant glycosylation and glycosylation pathways have not been investigated in MSI-H colorectal tumors. Focusing on this tumor subset, we examined cMNR frameshift mutations in genes encoding
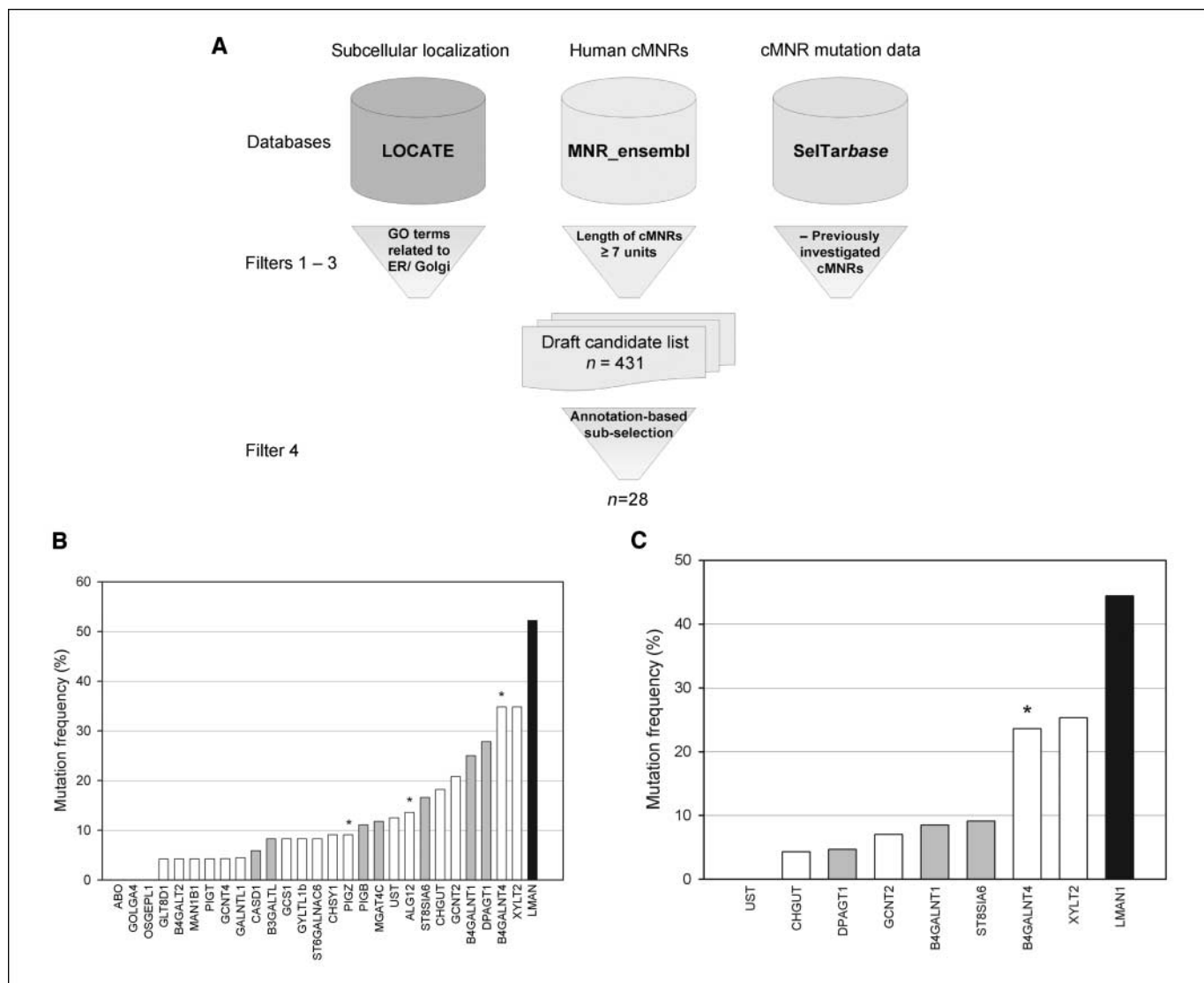
[3] http://www.seltarbase.org

Figure 1. Database analysis and frameshift mutation analysis of candidate genes. *A,* strategy for the identification of candidate genes. Three databases and four filters were used as information sources and selection tools, respectively. For details see Materials and Methods. *B,* frameshift mutation analysis of 28 genes (32 cMNRs) in MSI-H colorectal cancer cell lines. Different lengths (number of repetitive units) of individual cMNRs are indicated (*white, n = 7; gray, n = 8; black, n = 9*). Overall mutation frequencies are depicted for three genes (*) that contain more than one cMNR (*B4GALNT4*, 3 × G7; *ALG12*, 2 × C7; *PIGZ*, 1 × C7, 1 × G7). *C,* frameshift mutation frequencies of a subset of candidate genes in primary colorectal cancers. Gene-specific mutation data were obtained by analyzing 50 MSI-H carcinomas except for *LMAN1* (*n* = 161 tumors), *XYLT2* (*n* = 91 tumors), and *B4GALNT4* (*n* = 93 tumors). Bar characteristics (asterisks, shading) are identical to *B*.

proteins of the cellular glycosylation machinery. Mutational inactivation and expression abnormalities of LMAN1, a carrier that mediates ER-to-Golgi transport of glycoproteins, was found to be a frequent and early event in MSI-H colorectal tumors. Because deficiency of LMAN1 in MSI-H cell lines caused impaired secretion of the antiangiogenic and growth-inhibiting protein α-1-antitrypsin (A1AT), abnormalities of *LMAN1* potentially contribute to MSI-H carcinogenesis.

Materials and Methods

Database analysis. Three databases and four filters were used for candidate gene selection. Information about ER/Golgi resident proteins was retrieved from the LOCATE database,[4] whose XML source was

downloaded (version human_v3_20070620) and prepared for local usage as a MySQL database. Protein subcellular localization in LOCATE v3 was defined by 463 GO terms, of which 30 GO terms were related to ER/Golgi subcellular localization (*Filter 1;* Supplementary Table S1). From this candidate subset, the MNR_ensembl database[5] (version 45.36 g) allowed further specification of candidate genes by restriction of cMNR lengths to a minimum of seven repeat units (*Filter 2*). Subsequent exclusion of all cMNRs previously investigated revealed a list of 431 candidate genes (*Filter 3;* SelTar*base,* 4th release 200707).[3] Final selection by annotation of genes encoding proteins of the cellular glycosylation/deglycosylation and glyoprotein transport system led to 28 genes that were used for subsequent analyses (Filter 4). Automated primer design was performed by a perl script using primer3_core (Primer3 version 0.1)[6] in combination with a self-constructed human mispriming repeat library (containing L1,

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[4] http://locate.imb.uq.edu.au/

[5] http://www.seltarbase.org/?topic=MNR_ensembl
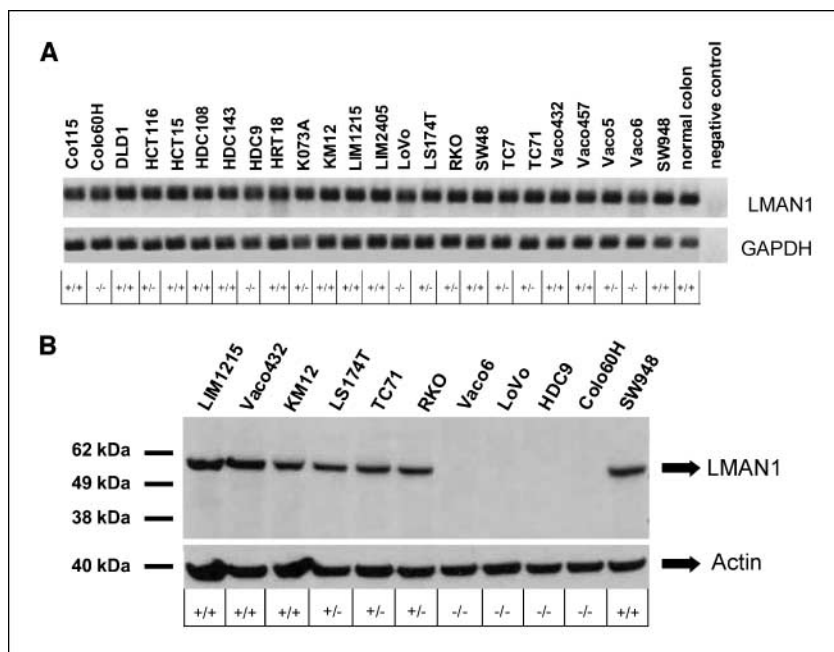[6] http://primer3.sourceforge.net/

Figure 2. Candidate gene expression in colorectal cancer cell lines. *A,* RT-PCR analysis of *LMAN1* expression in colorectal cancer cell lines with homozygous wild-type (+/+), homozygous mutant (−/−), or heterozygous mutant (+/−) cMNR alleles. The expected *LMAN1* PCR fragment of 122 bp could be amplified from cDNA of normal human colon, 1 MSS cell line (SW948), and all MSI-H colorectal cancer cell lines. As a house keeping gene, *GAPDH* was amplified. *B,* Western Blot analysis of MSI-H colorectal cancer cell lines. A single band of 53 kDa corresponding to the LMAN1 protein was detected in MSI-H and MSS colorectal cancer cell lines harboring heterozygous or homozygous wild-type cMNR alleles in MSI-H and MSS cell lines. MSI-H colorectal cancer cell lines with biallelic cMNR frameshift mutations lacked LMAN1 protein expression. No mutated LMAN1 protein (expected size, ~ 36 kDa) was visible in heterozygously and homozygously mutated cell lines. Molecular weight marker bands are depicted. Probing with an antiactin antibody served as loading control.

ALU, etc. sequences; Supplementary Table S2). Our bioinformatics-based approach is outlined schematically in Fig. 1*A*.

cMNR frameshift mutation analysis. Frameshift mutation analyses were performed as described previously (23). Primer design was set up to obtain short amplicons of ~ 100 bp (Supplementary Table S2), thus allowing robust amplification from archival tissues. PCR fragments were analyzed on an ABI3100 Genetic Analyzer (Applied Biosystems). Frameshift mutations were confirmed by DNA sequence analysis.

Human tissues and cancer cell lines. Human tissues were obtained from the local tissue bank established within the German Collaborative Group on HNPCC. Informed consent was obtained from all patients and the study protocol was approved by the local Ethics Committee. The MSI-H status of all colorectal tumor tissues used in the present study (*n* = 161) has been determined previously using the National Cancer Institute/ICG-HNPCC reference marker panel (3) and one additional mononucleotide marker CAT25 (24). Colorectal cancer cell lines have been described previously (23, 25). All cell lines were grown under standard conditions in RPMI 1640 (Invitrogen, Life Technologies, Inc.) supplemented with 10% FCS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin (PAA Laboratories GmbH).

Isolation of genomic DNA and RNA. Genomic DNA was isolated from cancer cell lines and paraffin-embedded archival specimens after manual microdissection of tumor and corresponding normal tissues using the DNeasy Tissue kit (Qiagen). RNA was isolated using the RNeasy Mini kit (Qiagen).

Transient transfection. Colo60H cells (*n* = 10^7) were transiently transfected by electroporation using the Amaxa Nucleofector (Amaxa Biosystems), program T-20 with solution V and 5 μg DNA of pcDNA3.1-*LMAN1* or control vector pcDNA3.1. Subsequently, cells were grown in small plastic flasks (T25) and pelleted cells or conditioned medium were harvested after 24 to 48 h.

Reverse transcription-PCR. One microgram of total cellular RNA was reverse transcribed using Superscript II Reverse Transcriptase according to the manufacturer's instructions (Invitrogen, Life Technologies, Inc.). Primers for the *LMAN1* gene were designed to flank the A9 cMNR (Sense primer, 5′-Fluorescein-GCTCTCGATCTCCTACACTCTCA-3′; antisense primer, 5′-GGAGGAATTTGAGCACTTTCA-3′). *LMAN1*-specific cDNA primers were designed to span exon 8 and exon 9. PCR products were visualized on ethidium bromide–stained 2% agarose gels. All cell lines were examined by frameshift mutation analysis on cDNA for comparison with genomic DNA

mutation data. Mutations were confirmed by DNA sequence analysis. As a control for loading and integrity of mRNA, reverse transcription-PCR (RT-PCR) analysis of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Sense primer, 5′-CCACCCAGAAGACTGTGGAT-3′; antisense primer, 5′-TT-CAGCTCAGGGATGACCTT-3′) was performed.

Western blot analysis. Cell pellets were lysed in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1% sodium desoxycholate, 0.1% SDS, 0.1 mmol/L CaCl₂, and 0.01 mmol/L MgCl₂], sonicated (Bandelin-Sonopuls, Bandelin electronic GmbH & Co. KG), and subsequently ultracentrifuged at 100.000 × *g* for 15 min at 4°C (Beckmann TLA 100.2 rotor). Protein concentration was determined by the Lowry method (26). Sixty-five micrograms of protein was separated on a 4% to 12% Bis-Tris Gel (NuPAGE Invitrogen Life Technologies, Inc.) followed by Western Blot analysis using primary antibody directed against LMAN1 (1:1,000, rabbit-monospecific Sigma Prestige Antibodies; HPA002320) or anti-actin antibody (1:10,000, monoclonal, C4, MP Biomedicals) as control. Horseradish peroxidase (HRP)-conjugated antibodies were used as secondary antibodies [Anti-Rabbit IgG, HRP Conjugate (Promega); Anti-Mouse IgG, peroxidase-linked whole antibody (GE Healthcare Europe)]. Visualization was performed with Western Lighting Chemiluminescence Reagent Plus (Perkin-Elmer LAS, Inc.) on Kodak BioMax light films (Sigma-Aldrich).

Immunohistochemistry. For immunohistochemical staining, 2-μm sections were deparaffinized and rehydrated. For antigen retrieval, slides were boiled in 10 mmol/L citrate buffer (pH 6) for 3 × 5 min and endogenous peroxidase activity was blocked with hydrogen peroxide (0.6%) for 20 min. Slides were rinsed with deionized water and then washed in PBS/0.1% Tween 20 (PBS-T) for 5 min. The slides were then stained by 2-h incubation at room temperature with anti-LMAN1 primary antibody (1:200, rabbit-monospecific; Sigma) in 1% horse serum/PBS-T. A biotinylated anti-mouse/anti-rabbit antibody (1:50; Vector Laboratories, Inc.) in 1% horse serum/PBS-T was used as secondary antibody for 30 min at room temperature and staining procedure was subsequently amplified by the A/B-complex (1:50; ABC-kit; Vector Laboratories, Inc.) for 30 min at room temperature. Immunoreactions were visualized with 3,3′-diaminobenzidine (Dako), followed by counter-staining with hematoxylin. Tumor-infiltrating lymphocytes, muscle cells, and normal colon tissue served as positive controls.

A1AT ELISA. Cells were grown in T25-flasks to ~ 80% confluence (representing 7 μg DNA), and conditioned cell culture medium was harvested after 24 h. For the ELISA 100 μL undiluted conditioned medium

were used. ELISA was established according to manufacturer's instructions (ImmunDiagnostik).

Results

Selection of candidate genes. A bioinformatics-based approach was used to identify cMNR-harboring candidate genes encoding proteins of the cellular glycosylation machinery. Database analysis and specific selection criteria such as subcellular location, cMNR length, and involvement in glycan synthesis, trimming, or export were applied to preselect candidate genes (see Materials and Methods). Based on this search strategy, 28 genes, encoding for proteins of the glycosylation machinery in the ER or Golgi, were selected for further investigation (Fig. 1A).

Frameshift mutation analysis. As a first step, we determined the cMNR frameshift mutation profiles for these 28 genes (32 cMNR) on a panel of 48 colorectal cancer cell lines (MSS, $n = 25$; MSI-H, $n = 23$; Fig. 1B). A wide spectrum of mutation frequencies was observed ranging from 0% to 52%, whereas no frameshift mutations in any of these genes were detectable in MSS colorectal cancer cell lines. LMAN1, harboring an A9 repeat in its coding region, showed the highest frameshift mutation frequency (52%; 12 of 23) in MSI-H colorectal cancer cell lines (Fig. 1B). In most cases, frameshift mutations affected only one allele. However, in some MSI-H colorectal cancer cell lines, biallelic cMNR mutations occurred in LMAN1 (17%; 4 of 23) and/or XYLT2 (8%; 2 of 23).

To clarify whether the observed genetic alterations that arise in cultured cell lines also occur in tumors, we performed similar analyses on 50 MSI-H colorectal tumors. For this analysis, we chose those candidate genes, which were most frequently mutated in MSI-H colorectal cancer cell lines. Most of the analyzed genes also showed frameshift mutations in these primary tumors and the observed frequencies were similar to those found for MSI-H colorectal cancer cell lines (Fig. 1C). In particular, in primary MSI-H colorectal cancers, highest cMNR mutation frequencies occurred in 3 genes including LMAN1 (45%), XYLT2 (25%), and B4GALNT4 (23%; Fig. 1C), a finding that was corroborated by extended analysis of these 3 genes on additional MSI-H tumor panels (LMAN1, $n = 111$; XYLT2, $n = 41$; B4GALNT4, $n = 43$). Based on the high mutation frequency of LMAN1 in MSI-H colorectal carcinomas, we asked whether such alterations could also be found in preneoplastic lesions. When we analyzed a subset of 20 MSI-H colorectal adenomas, a significant fraction of these tumors (40%; 8 of 20) showed cMNR frameshift mutations in the LMAN1 gene (data not shown). From these results, we conclude that LMAN1 mutations occur frequently and early in MSI-H tumors.

Figure 3. Immunohistochemical detection of LMAN1 protein on MSI-H colorectal tumors. Representative immunohistochemical staining patterns of MSI-H colorectal tumors using an LMAN1-specific antibody. LMAN1 staining was visualized by using 3,3′-diaminobenzidine (*brown*). Tumor sections were counterstained with hematoxylin (*blue*). In all tumor sections, positive LMAN1 staining of stromal cells and tumor-infiltrating lymphocytes was used as control. *Scale bars,* 100 μm in all panels. *A,* four carcinomas are shown that display either complete or partial loss of LMAN1 expression, sometimes even comprising areas with a heterogeneous speckled pattern. *B,* in two MSI-H colorectal adenomas, complete loss (*left*) as well as focally restricted loss of LMAN1 expression was observed (*right*). *Arrow,* crypt with mixed staining.
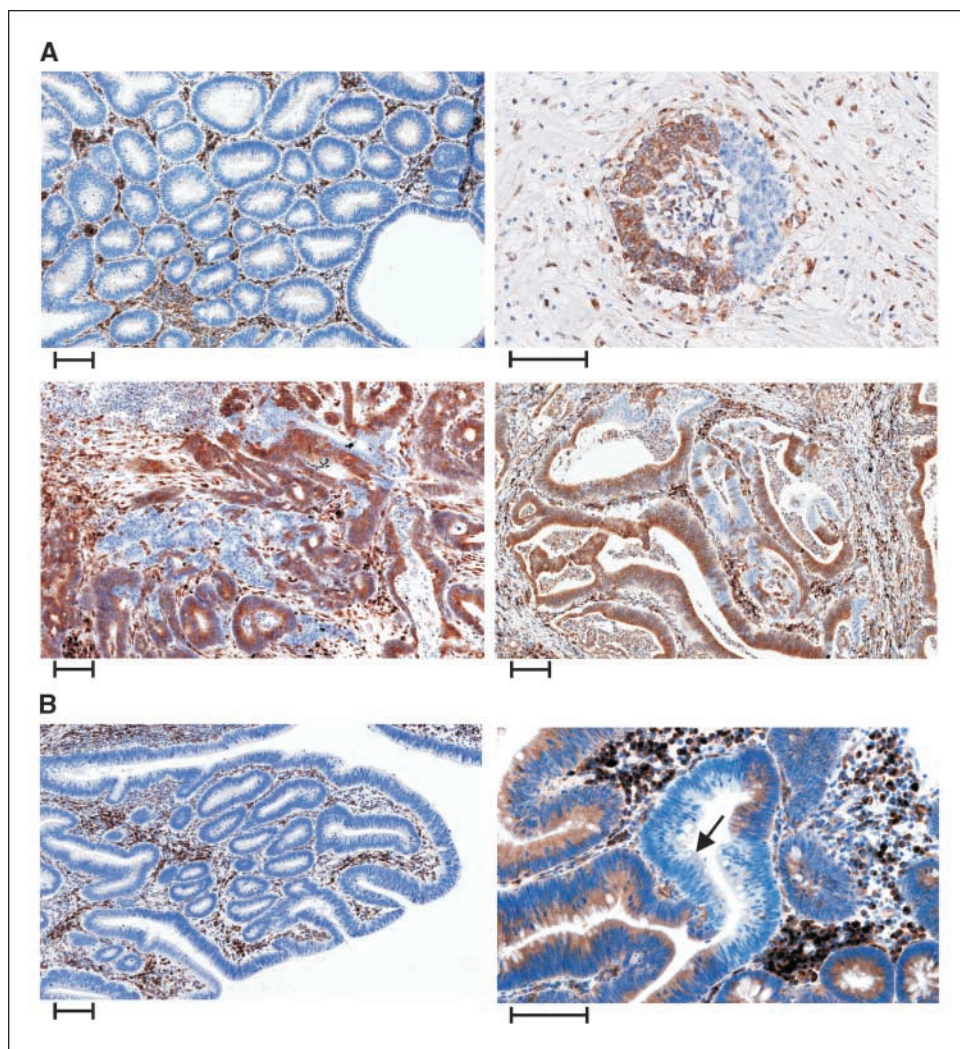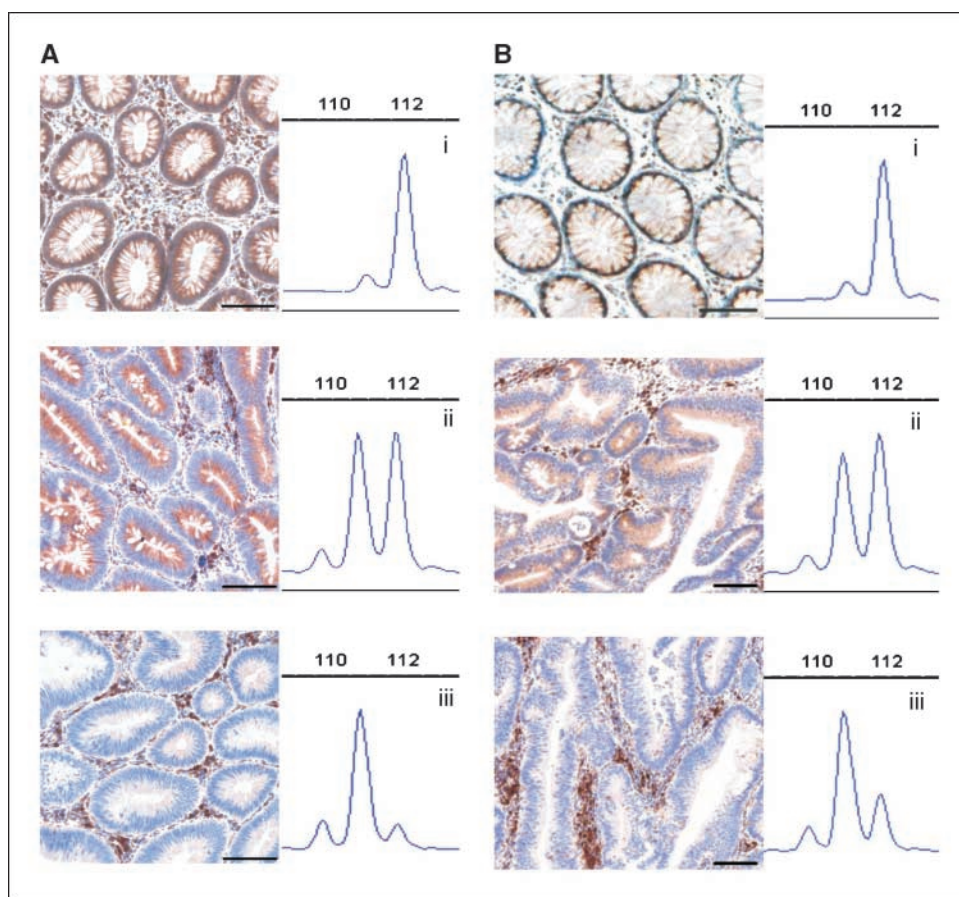
Figure 4. Molecular analysis of intratumoral LMAN1-deficient areas. *A,* regional microdissection of a colorectal carcinoma. Nonmalignant colorectal epithelium strongly expressing LMAN1 only showed *LMAN1* cMNR wild-type allele peaks (+/+) in electropherograms of fragment length analysis (*Ai*). Tumor areas that have retained LMAN1 staining but with reduced staining intensity compared with nontumorous stromal cells exhibited an additional peak in frameshift analysis corresponding to a heterozygous, one bp deletion at the *LMAN1* coding microsatellite (*Aii*). Tumor areas completely lacking LMAN1 staining only displayed homozygous mutant allele and peak pattern (*Aiii*). *B,* regional microdissection of a colorectal adenoma revealed a close correlation of LMAN1 protein staining with mutation status in the corresponding area of the tumor similar to the observations in *A. i,* strong staining, wild-type; *ii,* reduced staining, heterozygous mutation; *iii,* absent staining, homozygous mutation. Low level of wild-type allele retained in this latter electropherogram (*Biii*) results from some remaining nontumorous cells (e.g., tumor-infiltrating lymphocytes) in this microdissected tissue sample. *Scale bars,* 100 μm.

Wild-type and mutant *LMAN1* transcript and protein expression in MSI-H colorectal cancer cell lines. *LMAN1* mutations would be of importance only if the affected gene is expressed. Hence, we analyzed *LMAN1* expression, both at the transcript and protein level. *LMAN1* mRNA was found to be expressed in normal human colon mucosa as well as in all tested cell lines, including those harboring monoallelic or biallelic *LMAN1* cMNR frameshift mutations (Fig. 2*A*).

When LMAN1 protein was traced by Western Blot analysis, a band of 53 kDa corresponding to the expected size of wild-type LMAN1 protein was detectable in all 19 heterozygously mutated cell lines. However, this band was missing in all four cell lines Colo60H, LoVo, HDC9, and Vaco6, harboring biallelic *LMAN1* mutations (Fig. 2*B*). Moreover, no truncated LMAN1 protein (expected size of 36 kDa) was detected in cell lines with monoallelic or biallelic cMNR frameshift mutations although the LMAN1-antibody recognizes an NH_2-terminal epitope.

These results indicate that *LMAN1* frameshift mutant alleles are stably expressed at the transcript level but—in contrast to the wild-type alleles—remain undetectable at the protein level. The identification of LMAN1-deficient cell lines provides a valuable tool for studying the functional consequences of LMAN1 inactivation.

LMAN1 shows loss of expression in MSI-H colorectal carcinomas and adenomas. In cultured cells, biallelic mutation of *LMAN1* caused loss of expression at the protein level. To investigate the expression of LMAN1 in primary tissues, we performed immunohistochemical analysis on a set of 50 MSI-H colorectal carcinomas. A significant fraction of these carcinomas showed either local (38%; 19 of 50) or complete loss (6%; 3 of 50) of LMAN1 expression. Representative examples of this heterogeneous staining pattern are outlined in Fig. 3*A*, including complete loss or local loss of LMAN1 protein expression. Moreover, when a small number of MSI-H colorectal adenomas was subjected to immuno-histochemical staining, a similar pattern (Fig. 3*B*) was observed. We hypothesized that this regional loss of LMAN1 expression might be attributable to biallelic mutational inactivation of the *LMAN1* gene in specific tumor areas. To address this issue, we isolated DNA from areas with or without LMAN1 expression by regional microdissection on a few MSI-H carcinomas (*n* = 4) and MSI-H adenomas (*n* = 3; Fig. 4*A*). DNA fragment analysis revealed biallelic cMNR frameshift mutations in the *LMAN1* gene (Fig. 4*Aiii*) and concomitant loss of LMAN1 protein expression in these tumor areas. Areas with decreased LMAN1 expression compared with normal tissue (Fig. 4*Ai*) showed heterozygous mutations for *LMAN1* (Fig. 4*Aii*). Correlation between protein expression and frameshift mutation pattern was also observed in adenomas (Fig. 4*B*).

From these results we conclude that primary MSI-H colorectal carcinomas and adenomas frequently show heterogeneous LMAN1 expression abnormalities caused by regional biallelic cMNR frameshift mutations in MSI-H tumor cells.

LMAN1 deficiency causes decreased A1AT secretion. Because LMAN1 is a mannose specific lectin involved in ER/Golgi transport, LMAN1-deficiency could vastly influence protein distribution or secretion and, hence, cell homeostasis. One well-known client

protein of LMAN1 is A1AT, an inhibitor of several serine proteases (27) that is involved in local neoangiogenesis and growth inhibition. Notably, a strong correlation between lower local levels of A1AT and enhanced tumor growth has been described (28). We therefore chose to compare A1AT-secretion in LMAN1-deficient *versus* LMAN1-proficient cell lines. When we examined conditioned medium of these colorectal cell lines by using an A1AT-specific ELISA, significantly decreased levels of A1AT were measured in LMAN1-deficient (~ 5 µg/L) compared with LMAN1-proficient cell lines (~ 15 µg/L; Fig. 5*A*). These lower medium levels are not due to lower expression levels of A1AT as RT-PCR analysis revealed similar *A1AT* transcript levels in all cell lines (data not shown). A moderate but nonsignificant decline in A1AT concentration was also observed in MSI-H colorectal cancer cell lines harboring heterozygous *LMAN1* mutations (data not shown). To test whether LMAN1 reconstitution can overcome this secretion defect, LMAN1-deficient Colo60H cells were transiently transfected with *LMAN1* cDNA. Indeed, there was a 10-fold increase in A1AT concentration in the medium of Colo60H cells overexpressing LMAN1 compared with the vector control (Fig. 5*B*). No effect of *LMAN1* transfection on *A1AT* transcript levels was observed (data not shown).

In conclusion, the decreased A1AT secretion in LMAN1-deficient cell lines and the increased A1AT secretion after transient expression of LMAN1 in a deficient background indicate a potential role of LMAN1-mediated A1AT secretion in tumor progression.

Discussion

Our screening approach for frameshift mutations in cMNRs of genes that encode proteins of the cellular glycosylation machinery identified *LMAN1/ERGIC53* as a major mutational target in MSI-H colorectal cancer cell lines and tumors. LMAN1 is a marker for the ER/Golgi intermediate compartment, a mobile membrane structure that carries secretory proteins along microtubules from the ER to the Golgi (29). LMAN1 binds as a mannose-specific lectin to its substrates by a luminal carbohydrate-recognition domain in the neutral and Ca^{2+}-rich ER (30) and Man_9 seems to be the preferred glycan structure for binding (31). LMAN1 can also act as a transport receptor for the serine protease inhibitor A1AT (27) and is involved in the ER-export of the lysosomal glycoproteins cathepsin C and Z as well as the blood coagulation factors V and VIII (27, 32–34). Genetic alterations in *LMAN1* are associated with a human disease termed combined deficiency of coagulation factors V and VIII (F5F8D).

Our results suggest that genetic alterations in *LMAN1* also might be involved in human tumorigenesis. Several lines of evidence support this hypothesis.

First, the high mutation frequency observed in tumor tissues predicts *LMAN1* as a MSI-H target gene whose mutational inactivation seems to be positively selected during MSI-H carcinogenesis. Second, biallelic mutations occurred frequently in MSI-H colorectal adenomas and carcinomas and, although regionally restricted, were associated with complete loss of LMAN1 protein expression. Such biallelic mutational inactivation usually points to a tumor suppressor function of the affected gene. Our results on cell lines with biallelic *LMAN1* mutations also provide some clue about the mechanism that leads to loss of LMAN1 protein expression. *LMAN1* mutant transcripts harbor a premature termination codon and, hence, are predicted to be NMD-sensitive i.e., they should be recognized and degraded by the nonsense-mediated mRNA decay (NMD) system (35). However, *LMAN1* transcripts were easily detectable by RT-PCR analysis in cell lines with biallelic frameshift mutations and hence seemed NMD resistant. Because no truncated protein band of ~ 36 kDa could be detected by Western blotting, mutant *LMAN1* transcript is probably an additional member of a recently described new class of NMD-escape transcripts affected by nonsense-mediated translational repression (35).

Third, LMAN1-deficient cell lines show loss of LMAN1 transport function as shown by impaired A1AT secretion. Our data on A1AT secretion support the results of Nyfeler and colleagues (27) on mouse embryonic fibroblasts (MEF) derived from *LMAN1* ($-/-$) knockout mice. LMAN1-deficient MEFs secreted only $\sim 25\%$ A1AT in a given time period compared with LMAN1 (+/+) MEFs. Our results show a significant decrease of A1AT secreted into the cell culture medium reaching $\sim 20\%$ of the level secreted by the LMAN1-proficient control cell line Caco2.

Figure 5. A1AT secretion in LMAN1-proficient and LMAN1-deficient cell lines. *A,* A1AT concentration in conditioned medium was measured by an A1AT specific ELISA. MSS and MSI-H cell lines only harboring wild-type *LMAN1* cMNR alleles (+/+) showed ~ 3-fold higher levels of secreted A1AT than MSI-H cell lines with homozygous mutations for *LMAN1* ($-/-$). The relative A1AT concentration was adjusted to the DNA amount (7 µg) in 80% confluent cells. Lactate dehydrogenase measurement excluded distortion by dead cells. *Columns,* mean of three independent experiments; *bars,* SD. *B,* reconstitution of A1AT ELISA secretion upon *LMAN1* cDNA transfection into a LMAN1-deficient cell line. Colo60H cells were transiently transfected with a *LMAN1* cDNA expression vector (*pcDNA3.1-LMAN1*) or empty control vector (*pcDNA3.1*). Forty-eight hours after transfection, there was a significant increase of A1AT concentration in conditioned medium compared with the same cell line transfected with the empty vector.
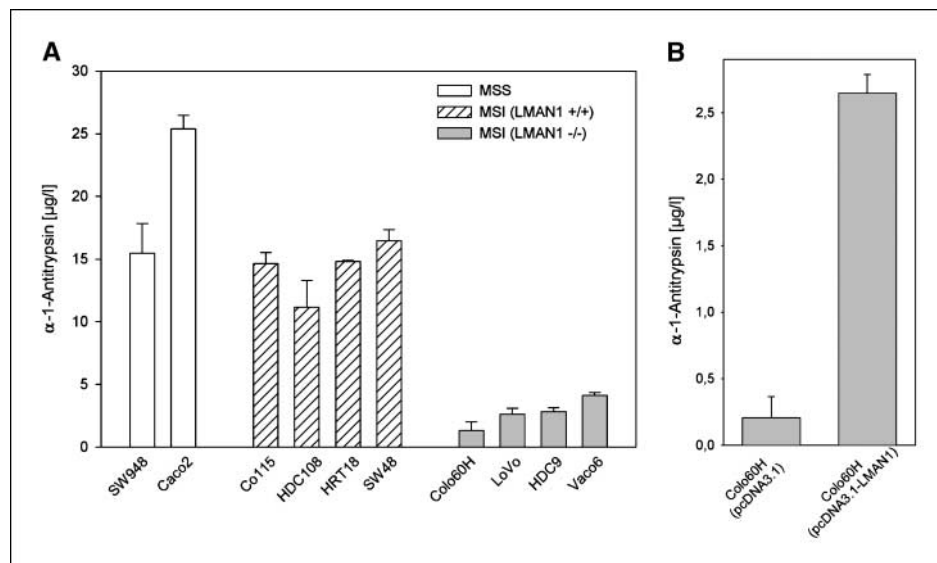
How might LMAN1 deficiency contribute to MSI-H carcinogenesis? One possible answer to this question might come from the biological function. As a protein shuttle, LMAN1 mediates intracellular transport of several client proteins from the ER to the Golgi. One of its recently identified client proteins is A1AT, a serine protease inhibitor in human plasma. A1AT expression is found in many different tissues including the human colon (36, 37). A1AT not only exerts antiproteolytic function but also confers angioinhibitory activity. Studies on tumor cell xenografts in nude mice have shown that systemic administration of A1AT delayed tumor progression and reduced microvessel density (28). Comparative cDNA microarray expression analyses of human normal and tumor tissues revealed that lower levels of A1AT transcripts correlated with larger tumor size (28) and higher levels of noncirculating A1AT within tumors tend to be associated with better prognosis (38). Interestingly, it was hypothesized that A1AT is protective against MSI-H–colorectal cancer development (39). The work by Huang and colleagues (28) also shows significant variations in local A1AT levels in human tumor tissues. Our observation of intratumoral heterogeneity of local LMAN1 deficiency might provide a molecular explanation for this finding (27).

Apart from A1AT secretion defects, alternative explanations may be considered. For example, LMAN1 deficiency might promote MSI-H colorectal tumorigenesis by affecting the secretion of other known client proteins such as cathepsin C and Z or by interference with interacting proteins such as WHAMM and MCFD2 (40, 41). Moreover, it is reasonable to assume that additional and yet unknown LMAN1 cargo and binding proteins

exist, which might be affected by a loss of LMAN1 function. Although only secreted proteins have been identified as LMAN1 client proteins, one might speculate that membrane-bound glycoproteins are also transported by this mechanism. Support for this hypothesis is gained from our preliminary observation that changes in cell surface glycoprotein pattern occur in LMAN1-deficient cell lines upon reconstituted LMAN1 expression.[7]

Overall, our combined bioinformatics/molecular biology approach has identified a member of the cellular glycoprotein transport machinery as a putative novel MSI-H target gene frequently mutated in MMR-deficient colorectal tumors. Our work also points to genetic alterations in other genes of the cellular glycosylation machinery such as *XYLT2* and *B4GALNT4*. Mutational inactivation of these genes and their encoded proteins is expected to directly change glycosylation of substrate proteins at or beyond the surface of affected MSI-H tumor cells, thereby offering novel diagnostic and therapeutic strategies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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