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BRIEF ARTICLE

Underexpression of *LATS1* TSG in colorectal cancer is associated with promoter hypermethylation

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Abstract

AIM: To investigate large tumor suppressor 1 (*LATS1*) expression, promoter hypermethylation, and microsatellite instability in colorectal cancer (CRC).

METHODS: RNA was isolated from tumor tissue of 142 CRC patients and 40 colon mucosal biopsies of healthy controls. After reverse transcription, quantitative polymerase chain reaction (PCR) was performed, and *LATS1* expression was normalized to expression of the *ACTB* and *RPL32* housekeeping genes. To analyze hypermethylation, genomic DNA was isolated from 44 tumor CRC biopsies, and methylation-specific PCR was performed. Microsatellite instability (MSI) status was checked with PCR using BAT26, BAT25, and BAT40 markers in the genomic DNA of 84 CRC patients, followed by denaturing gel electrophoresis.

RESULTS: Decreased *LATS1* expression was found in 127/142 (89.4%) CRC cases with the average ratio of the LATS1 level 10.33 ± 32.64 in CRC patients *vs* 32.85 ± 33.56 in healthy controls. The lowest expression was found in Dukes' B stage tumors and G1 (well-differentiated) cells. Hypermethylation of the *LATS1* promoter was present in 25/44 (57%) CRC cases analyzed. *LATS1* promoter hypermethylation was strongly associated with decreased gene expression; methylated cases showed 162× lower expression of *LATS1* than unmethylated cases. Although high-grade MSI (mutation in all three markers) was found in 14/84 (17%) cases and low-grade MSI (mutation in 1-2 markers) was found in 30/84 (36%) cases, we found no association with *LATS1* expression.

CONCLUSION: Decreased expression of *LATS1* in CRC was associated with promoter hypermethylation, but not MSI status. Such reduced expression may promote progression of CRC.

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Key words: Large tumor suppressor 1; Colorectal cancer; Quantitative polymerase chain reaction; Reduced expression; Promoter hypermethylation; Microsatellite

instability; Salvador-Warts-Hippo pathway

Core tip: Searching for new colorectal cancer (CRC) molecular markers is a very important objective, because CRC is one of the most common malignancies in the world and one of the most fatal of human neoplasms. Decreased expression of large tumor suppressor 1 in CRC was associated with promoter hypermethylation, but not microsatellite instability status. Such reduced expression may promote progression of CRC.

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies in the world and one of the most fatal human neoplasms. Almost 1.2 million new cases occur annually, accounting for 608700 related deaths in 2008[1]. Although nearly 90% of patients may be successfully cured with surgery in early stages, CRC is frequently diagnosed in late stages, *i.e.*, Dukes' C and D, when the prognosis is poor[2,3]. Therefore, the search for CRC molecular markers, as well as elucidation of epigenetic factors that are responsible for variability in the expression of putative markers, is very important.

Human large tumor suppressor 1 (*LATS1*, also known as *WARTS*) was discovered in 1999[4] as a highly conserved homolog of the *Drosophila melanogaster* (*D. melanogaster*) lats gene[5]. *LATS1* encodes a serine/threonine kinase, which is involved in the regulation of various cellular processes. Before mitotic division, the presence of LATS1 is crucial for control of the R1 tetraploidy checkpoint[6]. During the early phase of mitosis, LATS1 associates with cell division control protein 2 homolog[7], and the progress of cytokinesis occurs only after association of the MOB kinase activator 1A cytoplasmic protein with LATS1[8,9].

More recently, genetic studies in *Drosophila* have identified LATS as a central mediator in a tumor suppressing pathway called the Salvador-Warts-Hippo (SWH) pathway[10,11]. The SWH pathway is also a critical factor in the regulation of organ size in *D. melanogaster* and mammals[12,13]. Moreover, deregulation of SWH pathway activity has been implicated in the genesis of multiple human cancers[11,14-16]. Several mammalian factors are involved in signal transduction in the SWH pathway, including the tumor suppressor proteins neurofibromin 2, Ras association family member 1-6, serine/threonine kinase 3, LATS1, and an oncogene called Yes-associated protein (YAP). YAP, a transcription coactivator that associates with various transcription factors, is overexpressed in human carcinomas including ovarian, liver, and prostate cancers[13]. LATS1 kinase is a main negative regulator of YAP. LATS1 inhibits the transcriptional activity/function of YAP *via* phosphorylation of Ser 127 in YAP[17]. Moreover, LATS1-phosphorylated YAP is involved in a p53-independent apoptosis pathway in which phosphorylated YAP plays a role in transcriptional activation of the pro-apoptotic gene, p53 up-regulated modulator of apoptosis[18]. Overexpression of *LATS1* in *LATS1*−/− mouse cells (by introducing human *LATS1* with adenovirus-mediated gene transfer) and HeLa cells suppresses tumorigenicity *in vivo* and *in vitro* by inducing apoptosis[18,19].

LATS1 is considered to play a suppressor role in some tumors. Decreased *LATS1* expression is found mainly in soft tissue-derived tumors, including sarcomas[20] and astrocytomas[21]. However, *LATS1* quiescence was also observed in breast[22], cervical[23] cancers and head and neck squamous cell carcinoma[24]. In the gastrointestinal tract, decreased *LATS1* expression has been recently observed in gastric cancer[25], but in a small sample of CRCs, *LATS1* overexpression was found[26].

Hypermethylation of CpG islands (GC-rich sequences) in regulatory portions of a gene is an important epigenetic mechanism responsible for decreased gene expression or gene silencing[27-30]. Aberrant methylation of CpG islands in the promoter region of *LATS1* has been found in breast and ovarian cancers[4,22,31] and soft-tissue sarcomas[20]. Our preliminary results suggested that *LATS1* expression is decreased in CRC and is associated with promoter hypermethylation[32]. In the present study, we used quantitative polymerase chain reaction (QPCR) to determine the expression profile of *LATS1* in a relatively large group of CRC patients. We also examined the hypermethylation status of the *LATS1* promoter as a putative epigenetic mechanism affecting gene expression.

MATERIALS AND METHODS

Patients

The study was approved by the local ethics committees, and informed, written consent regarding the use of tissue was obtained before surgery or colonoscopy from all CRC and control patients, respectively. The specimens were obtained from four gastrointestinal endoscopic units and surgical clinics located in geographically different parts of Poland from 2008 to 2011. Clinical and demographic data were collected at the time of enrollment (Table 1). The study included 142 patients with CRC (87 males and 55 females; mean age 68 ± 10.8 years; range, 37-90 years). No CRC patients had a second neoplastic disease. None of the patients had undergone previous chemo- or radiotherapy. Tumors located in the anal canal and anus were not included in this study. The control group comprised 40 healthy individuals (17 males and 23 females; mean age 53 ± 14.2 years; range, 21-76 years) who underwent colonoscopy as part of routine surveillance for CRC. None of the CRC patients or controls

suffered from inflammatory bowel disease or had a family history of CRC. Patients were not on medication at the time of investigation. Before medical examination, blood samples were collected for routine testing from all CRC patients.

Collection of colon samples

All steps of material collection, including patients' clinical data, tissue collection, storage, shipment, and laboratory processing, followed The Cancer Genome Atlas (TCGA) instructions and were standardized in all collaborative clinics[33,34]. Briefly, CRC samples were obtained during surgical hemicolectomy, and control group specimens were collected during colonoscopy. For histopathologic examination and molecular studies, samples (5 mm × 5 mm × 5 mm) from macroscopically altered tumor tissue were taken within 20 min after tumor resection. For control patients, one biopsy (2 mm × 2 mm × 2 mm) was fixed in 10% neutral buffered formalin, and two specimens from the adjacent location to the biopsy site were collected for nucleic acid analyses. The formalin-fixed samples were obtained for the routine histological survey; if the result of histological examination showed pathological condition of the patient's tissue, the adjacent biopsies were excluded from the control group analyzed in this study. Both tumor samples and mucosal biopsies were immediately placed in sterile vials containing RNAlater (Ambion-Life Technologies, Grand Island, NY, United States), incubated for 6 h at 4 ℃, and then stored at -25 ℃ until further analysis.

Nucleic acid extraction and reverse transcription

Total RNA was extracted from a portion of the tumor samples (ca. 3 mm × 5 mm × 5 mm) and the entire mucosal biopsies of control patients using a Total RNA kit (A&A Biotechnology, Gdynia, Poland). Isolated RNA was quantified with spectrophotometry (Nanodrop ND 1000, Thermo Fisher Scientific, Fitchburg, WI, United States). DNA was digested with RNase-free DNase I (Fermentas-Thermo Fischer Scientific, Fitchburg, WI, United States) for 30 min at 37 ℃. Then, the DNase was inactivated by adding EDTA and incubating at 65 ℃ for 10 min. Before storing at -85 ℃, RNA integrity was analyzed with agarose gel electrophoresis. Total RNA (2 μg) was reverse transcribed using 0.5 μg oligo(dT)₁₈ primers (Sigma-Aldrich, Munich, Germany) and 200 U RevertAid M-MuLV Reverse Transcriptase (Fermentas-Thermo Fischer Scientific, Fitchburg, WI, United States) in a total volume of 20 μL, and the resulting cDNA was stored at -25 ℃. In 84 of the CRC cases, 1 mL venous blood that was collected in sterile K2-EDTA vials was used for DNA isolation using a Blood Mini DNA kit (A&A Biotechnology, Gdynia, Poland). From these same patients, DNA was also extracted from a portion of the tumor samples (ca. 3 mm × 5 mm × 5 mm) adjacent to the tumor fragments used for the RNA study using the Genomic Mini AX Tissue kit (A and A Biotechnology, Gdynia, Poland) and stored at -25 ℃.

QPCR assay to determine the LATS1 mRNA level

Quantification of *LATS1* gene expression was carried out using iQ Cycler (Bio-Rad, Hercules, CA, United States) with Sybr®Green I as a fluorophore. *LATS1* expression was determined with Livak's comparative method $2^{-\Delta\Delta Ct}$[35] relative to the geometric mean of the expression levels of two housekeeping genes: β-actin (*ACTB*; GenBank acc. No. NM_001101.3) and ribosomal protein L32 (*RPL32*; NM_000994.3). These genes showed very stable expression in CRC in our previous studies[36,37] and studies of other investigators[38]. Except for the *ACTB* assay[39], all primers were designed by us using GenBank data. QPCR conditions were validated and showed 90%-100% efficiency for all assays. The amplification primer pairs were 5'-TGCACTGGCTTCAGATGGA-CAC-3' and 5'-ATGTGCTAGACATCGCTGGTGC-3'; for *LATS1* (functional transcript, ENSEMBL No. ENST00000543571, GenBank No. NM_004690.2), 5'-TGTGCCCATCTACGAGGGGTATGC-3' and 5'-GGTACATGGTGGTGCCGCCAGACA-3' for *ACTB*[39], and 5'-TGACAACAGGGTTCGTAGAA-GAT-3' and 5'-GTTCTTGGAGGAAACATTGT-GAG-3' for *RPL32*. The reaction mixture (17 μL) included 0.4 μL cDNA, 0.2 μmol/L each forward and reverse primers, and real-time PCR iQ SYBR Green SuperMix (Bio-Rad). All reactions were performed in duplicate. The amplification parameters were denaturation for 5 min at 95 ℃, followed by 38 cycles of denaturation for 15 s at 95 ℃, annealing for 20 s at 55 ℃ for *RPL32*, 57 ℃ for *LATS1*, and 60 ℃ for *ACTB*, extension for 15-25 s at 72 ℃, and fluorescence reading for 5 s at 77 ℃-80 ℃. Dynamic melting curve analysis was performed for all reactions. Data were automatically collected and analyzed with iCycler iQ Optical Software ver. 3.0a (Bio-Rad).

Microsatellite instability status analysis

Microsatellite instability (MSI) status was determined according to the National Cancer Institute Workshop on Microsatellite Instability for Cancer Detection and Familial Predisposition[40] and was based on polymorphism analysis of three markers: BAT26 for *MSH2*, BAT25 for the *c-kit* oncogene, and BAT40 for the *HSD3B2* suppressor gene. BAT sequences were obtained from the UniSTS database (http://www.ncbi.nlm.nih.gov/unists), and the methodology was based on our previous results[41]. Briefly, the PCR reaction contained 0.5 U Taq polymerase, PCR buffer (Fermentas-Thermo Fischer Scientific, Fitchburg, WI, United States), 200 nmol/L each primer pair, 0.1 mmol/L each dNTP, and 30 ng DNA in a final volume of 15 μL and was performed using the following parameters: denaturation for 5 min at 95 ℃, 35 cycles of denaturation for 15 s at 95 ℃, annealing for 20 s at 49 ℃ (BAT25, BAT26) or 54 ℃ (BAT40), and extension for 30 s at 72 ℃. Denaturing acrylamide gel electrophoresis was performed in a Sequi-Gen Ⅱ Sequencing Cell (Bio-Rad) followed by silver staining (AgNO₃; POCH, Gliwice, Poland) for identification of extra DNA bands, which were considered mutations in the selected BAT markers. Low-

and high-grade MSI (MSI-L and MSI-H, respectively) were confirmed by 1-2 and all 3 mutated markers, respectively. If no mutation was observed in the paired tumor and blood DNA samples, the sample was confirmed as microsatellite stable (MSS).

Bisulfite modification and methylation-specific PCR

Because the method of bisulfite conversion of DNA requires at least 1 μg DNA, we performed this analysis with only 44 tumor samples with sufficient material using the EZ DNA Methylation™ kit (Zymo Research, Orange, CA, United States). Briefly, 1 μg tissue DNA was denatured using 0.2 mol/L NaOH and subsequently incubated with a sodium salt of bisulfite ion (HSO₃⁻) at 50 ℃ for 16 h. Next, the mixture was desulfonated, and DNA was purified on silica-membrane columns to a final volume of 10 μL. Bisulfite-modified DNA was stored at -25 ℃. The methylation status of the *LATS1* promoter region was determined with methylation-specific PCR (MSPCR). Bisulfite-modified DNA was amplified with primers specific for methylated or unmethylated sequences. The methylated DNA was amplified using M primers: sense 5'-TCGTTTTGTCGTTTAGGTTGG-3' and antisense 5'-CGACGTAATAACGAACGC-3', and unmethylated DNA was amplified using UM primers: sense 5'-TAGGTTGGAGTGTGGTGGT-3' and antisense 5'-CCCAACATAATAACAAACACCT-3'. All primer sequences were previously published[20-22] except for the M sense primer, which was redesigned *de novo* using the GenBank database and methPrimerDB online software. For the methylation assay, Human HCT116 DKO Nonmethylated DNA and Human HCT116 DKO Methylated DNA (Zymo Research) after bisulfite modification were used as positive controls in MSPCR. Briefly, 0.6 μL bisulfite-modified DNA was amplified in a total volume of 15 μL containing reagents from the Zymo*Taq*™ DNA Polymerase kit (Zymo Research) and 400 nmol/L each primer. MSPCR reactions were as follows: denaturation for 5 min at 95 ℃, five cycles of denaturation for 30 s at 95 ℃, annealing for 30 s at 52 ℃, extension for 20 s at 72 ℃; 30 cycles of denaturation for 30 s at 95 ℃, annealing for 30 s at 50 ℃, extension for 20 s at 72 ℃; final extension for 5 min at 72 ℃. PCR products (10 μL) were run on a 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet illumination. Images were stored using a Gel Doc apparatus and software (Bio-Rad).

Statistical analysis

Normality of the QPCR data was assessed with the Shapiro-Wilk test. Parametric data such as red blood cells, hematocrit, hemoglobin, and *LATS1* mRNA levels between various groups were evaluated using the Mann-Whitney *U* test. Comparison of *LATS1* mRNA ratios between CRC subgroups with various histological and MSI grades and methylation status was calculated using the Kruskal-Wallis analysis of variance (ANOVA) test. Fisher's exact test was used to assess correlations between the methylation status and clinical-pathological variables.

The statistical analyses were performed using Statistica ver. 10 program (Stat Soft Inc., Tulsa, OK, United States), and the level of significance was set at $P < 0.05$.

RESULTS

Relationship between LATS1 expression and clinical parameters

Clinico-pathological data including tumor stages according to tumor location, Dukes' classification, and G grade[42,43] are presented in Table 1. We found no statistical differences in geographic location of patients, sex, age, tumor location, and disease progression. We found a relationship between tumor location and erythrocyte counts, hematocrit level, and hemoglobin concentration; patients with a tumor on the right side were characterized by decreased values compared with patients whose tumor was on the left side ($P < 0.05$, Table 1). No associations between blood parameters and Dukes classification, TNM, and G grading of CRC were found.

Quantification of *LATS1* mRNA was performed in colorectal tumor samples from 142 CRC patients and compared with tissue samples from 40 healthy persons. Decreased *LATS1* gene expression was found in 127 of 142 (89.4%) tumors in the CRC cases ($P < 0.05$). Because QPCR data were not normally distributed (mean values: 10.33 ± 32.64 *vs* 32.85 ± 33.56, $P < 0.05$), the median expression ratio was 0.075 (range, 0.003-210.672) in CRC patients *vs* 40.097 (range, 0.004-98.228) in controls ($P < 0.05$). Thus, the average expression of *LATS1* was many times lower in tumor tissue than in normal colon mucosa of controls. No correlations between *LATS1* mRNA level and gender, age, or tumor location were found. Also, no statistical differences in the mRNA ratio were observed in patients who lived in different regions of Poland.

Comparison of *LATS1* expression levels with patients' clinico-pathological data revealed 8 times lower *LATS1* levels in Dukes' A stage compared to controls (Figure 1). The lowest *LATS1* expression was observed in Dukes' B stage, which was 42 times lower than in controls, whereas in more advanced CRC cases described as Dukes' C and D stage, *LATS1* expression was 24 and 14 times lower than in controls, respectively. We found a weak negative correlation between tumor progression and the *LATS1* mRNA level ($R^2 = -0.25$, $P < 0.05$, Spearman's test, plot not shown). When the histological G grade of cancer cells was considered, *LATS1* mRNA levels were significantly decreased in both G2 and G3 grades (Figure 1). However, due to the low number of G1 cases (well-differentiated cells) and G4 (undifferentiated cells) cases (three each), no comparison with grades G2 and G3 was possible.

MSI status and clinicopathological data

We analyzed 84 of the 142 CRC cases for MSI status. The highest rate of mutation was found in the BAT26 marker ($n = 28/84$; 33%), followed by 26 cases for BAT40 (31%) and 25 for BAT25 (30%) (Table 1). Our analysis revealed

Table 1 Clinical and histopathological characteristics of colorectal cancer patients and results of large tumor suppressor 1 mRNA quantification using quantitative polymerase chain reaction n (%)

Clinical parameter		Blood parameters (mean ± SD)				MSI results (n = 84)					QPCR results
		RBC (10^6/μL)	Ht	Hb (g/dL)	WBC (10^3/μL)	BAT 26	BAT 25	BAT 40	MSI-L	MSI-H	Downregulated cases vs control
CRC 142 cases		4.26 ± 4.58	36% ± 5.2%	12 ± 2.1	7.41 ± 2.71	28/84 (33)	25/84 (30)	26/84 (31)	30/84 (36)	14/84 (17)	127/142 (88)[1]
Sex and age	M (n = 87)	67 ± 10.4 (37-89)									
(mean ± SD, range, yr)		4.36 ± 0.50[2]	36% ± 6.0%	12 ± 2.4	7.26 ± 2.91	21/54 (39)	18/54 (33)	16/54 (30)	22/54 (41)	9/54 (17)	78/87 (90)
	F (n = 55)	69 ± 11.4 (44-90)									
		4.18 ± 0.41	35% ± 4.6%	11 ± 1.8	7.53 ± 2.58	7/30 (23)	7/30 (23)	10/30 (33)	8/30 (37)	5/30 (17)	49/55 (88)
Tumor location	Right side	55 (39)	4.09 ± 0.46[2]	33% ± 4.7%[2]	10 ± 1.9[2]	9/32 (28)	8/32 (25)	7/32 (22)	8/32 (25)	4/32 (12.5)	51/55 (93)
	Left side	87 (61)	4.36 ± 0.43[2]	38% ± 4.8%[2]	12 ± 1.8[2]	19/52 (36.5)	17/52 (33)	19/52 (36.5)	22/52 (42)	10/52 (19)	76/87 (81)
	Ascending colon	46 (32)	4.08 ± 0.46	33% ± 5.0%[2]	11 ± 2.0[2]	9/26 (35)	8/26 (31)	6/26 (23)	7/26 (27)	4/26 (15)	43/46 (93)[1]
	Transverse colon	11 (8)	4.12 ± 0.43	33% ± 4.2%[2]	10 ± 1.5[2]	0/6 (0)	0/6 (0)	1/6 (17)	1/6 (17)	0/6 (0)	10/11 (91)[1]
	Descending/sigmoid colon	41 (29)	4.29 ± 0.53	36% ± 5.2%[2]	12 ± 2.1[2]	6/23 (26)	7/23 (30)	9/23 (39)	9/23 (39)	4/23 (17)	32/41 (78)[1]
	Rectum	44 (31)	4.43 ± 0.33	39% ± 4.5%[2]	13 ± 1.6[2]	13/29 (45)	10/29 (34)	10/29 (34)	13/29 (45)	6/29 (21)	42/44 (95)[1]
Dukes' stage	A	27 (19)	4.23 ± 0.49	38% ± 5.3%	12 ± 2.0	2/19 (11)	2/19 (11)[2]	1/19 (6)[2]	4/19 (22)	0/19 (0)	22/27 (81)[1,2]
	B	41 (29)	4.30 ± 0.47	36% ± 5.5%	11 ± 2.1	9/23 (39)	5/23 (22)[2]	7/23 (30)[2]	8/23 (35)	4/23 (17)	40/41 (98)[1]
	C	54 (38)	4.22 ± 0.47	36% ± 5.0%	12 ± 2.0	12/34 (35)	14/34 (41)[2]	15/34 (44)[2]	14/34 (41)	8/34 (24)	49/54 (91)[1]
	D	20 (14)	4.34 ± 0.26	32% ± 4.4%	10 ± 1.9	5/8 (62.5)	4/8 (50)[2]	3/8 (37.5)[2]	4/8 (50)	2/8 (25)	18/22 (82)[1]
Lymph node metastasis	Negative	68 (48)	4.28 ± 0.46	36% ± 5.5%	12 ± 2.1	11/42 (26)	7/42 (17)[2]	8/42 (19)[2]	12/42 (29)[2]	4/42 (10)[2]	62/68 (91)[1]
	Positive	74 (52)	4.23 ± 0.45	35% ± 5.0%	11 ± 2.1	17/42 (40)	18/42 (43)[2]	18/42 (43)[2]	18/42 (43)[2]	10/42 (24)[2]	65/74 (88)[1]
Histological	Well (G1)	3 (2)	4.17 ± 0.35	36% ± 4.7%	12 ± 1.4	1/3 (33)	1/3 (33)	1/3 (33)	0/3 (0)	1/3 (33)	3/3 (100)[1]
differentiation (G stage)	Moderate (G2)	48 (34)	4.26 ± 0.47	36% ± 5.0%	11 ± 2.0	8/31 (26)	6/31 (19)	8/31 (26)	9/31 (29)	4/31 (13)	46/48 (96)[1]
	Poorly (G3)	88 (62)	4.16 ± 0.45	35% ± 5.5%	11 ± 2.2	18/47 (38)	18/47 (38)	17/47 (36)	20/47 (43)	9/47 (19)	84/88 (95)[1]
	Undifferentiated (G4)	3 (2)	4.36 ± 0.79	37% ± 6.6%	12 ± 2.3	1/3 (33)	0/3 (0)	0/3 (0)	1/3 (33)	0/3 (0)	2/3 (66)[1]

[1]Statistically significant difference between analyzed subgroup and control; [2]Statistically significant difference between subgroups, Kruskal-Wallis analysis of variance test. RBC: Red blood cells count; Ht: Hematocrit; Hb: Hemoglobin; WBC: White blood cells; MSI: Microsatellite instability; BAT26: Marker for MSH2; BAT25: Marker for the c-kit oncogene; BAT40: Marker for the HSD3B2; MSI-L: Low grade MSI, mutation in 1-2 BAT markers; MSI-H: High grade MSI, mutation in 3 BAT markers; CRC: Colorectal cancer; M: Male; F: Female.

MSI-L in 30 cases and MSI-H in 14 cases, which equates to 44 (52%) cases with the MSI genotype and 40 (48%) with MSS. The BAT25 and BAT40 markers were different according to the Dukes' stage (Kruskal-Wallis ANOVA test, $P < 0.05$), followed by higher occurrence of mutations in CRC cases with positive lymph node metastasis (any T, N1-2, any M) vs negative metastasis (Kruskal-Wallis ANOVA test, $P < 0.05$). We found no relationship between MSI status and *LATS1* expression.

MSI status and LATS1 expression

To check if the MSI phenotype influenced the expression pattern of *LATS1*, we compared QPCR results of *LATS1* expression ratios in MSS, MSI-L, and MSI-H cases (Figure 2). Although we found that MSI-L and MSI-H samples were characterized by higher *LATS1* levels than MSS cases, we found no statistical differences among those ratios (Figure 2).

Methylation status of the LATS1 gene promoter and its relationship with mRNA levels, clinical data and MSI data

To assess the methylation profile of *LATS1* during CRC progression, we analyzed 44 CRC cases with different clinico-pathological outcomes. Hypermethylation of the *LATS1* promoter was present in 25/44 cases (57%), whereas in 19 CRC tumor samples, no hypermethylation was found. No correlations were observed between *LATS1* hypermethylation and gender, age, tumor location, Dukes' stage, or G grading. Hypermethylation of the *LATS1* promoter was found in all patients with Dukes' A and D stages, and in 31% and 58% cases with Dukes' B and C stages, respectively (Table 2).

Comparison of *LATS1* promoter methylation status with *LATS1* mRNA levels in the analyzed tumor specimens (Table 2 and Figure 3) revealed a very significant reduction in *LATS1* expression in hypermethylated cases *vs* non-hypermethylated cases ($P < 0.05$). The biggest difference was observed in Dukes' C stage in which the *LATS1*

Table 2 Relationship between large tumor suppressor 1 promoter methylation status, large tumor suppressor 1 mRNA level, and histopathological and microsatellite instability data in colorectal cancer *n* (%)

Clinical parameter		Total	M	Av. *LATS1* mRNA fold change, control *vs* M	UM	Av. *LATS1* mRNA fold change, control *vs* UM	Av. *LATS1* mRNA fold change, UM *vs* M	*P* value between UM and M groups
Tumor total		44	25 (57)	597	19	3.55	162	0.00005
Dukes' stage	A	4	4 (100)	556	0	No data	No data	-
	B	16	5 (31)	699	11	228	3	0.041
	C	19	11 (58)	469	8	1.53	305	0.009
	D	5	5 (100)	1263	0	No data	No data	-
Lymph node	Negative	20	9 (45)	632	11	75	8	0.015
metastasis	Positive	24	16 (67)	586	8	1.53	381	0.0002
Histological	G1	2	1 (50)	801	1	538	1.5	NS
differentiation G stage	G2	11	3 (27)	1216	8	1.5	802	0.018
	G3	28	20 (71)	538	8	166	3.65	0.015
	G4	3	1 (33)	699	2	92	8	NS
MSI status	MSS	27	19 (70)	7	8	1.7	4	NS
	MSI-L	11	7 (64)	8	4	2.4	3.3	NS
	MSI-H	6	4 (67)	12	2	3.5	3.4	NS

M: Hypermethylation of large tumor suppressor 1 (*LATS1*) promoter; UM: Unmethylation of *LATS1* promoter; MSI: Microsatellite instability; MSS: Micro-satellite stable; MSI-L: Low grade MSI, mutation in 1-2 BAT markers; MSI-H: High grade MSI, mutation in 3 BAT markers; NS: Noy significant.
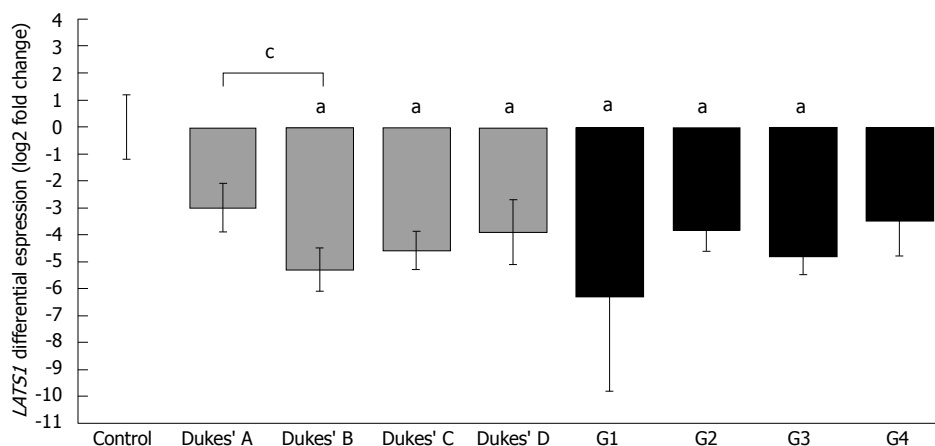

Figure 1 Large tumor suppressor 1 mRNA levels in colorectal cancer and control colon biopsies. Quantitative polymerase chain reaction results of large tumor suppressor 1 (*LATS1*) expression in 142 colorectal cancer (CRC) samples compared with 40 colon biopsies of healthy patients. CRC cases were divided according to clinicopathological data: tumor stage - Dukes' A (*n* = 27), B (*n* = 41), C (*n* = 54), D (*n* = 20); histological differentiation of tumor cells (G staging): G1 (*n* = 3), G2 (*n* = 48), G3 (*n* = 88), G4 (*n* = 3). Vertical bars represent *LATS1* fold ratio calibrated to the average C$_t$ of control ($\Delta\Delta$C$_t^{LATS1}$ = ΔC$_t^{LATS1, sample}$ - ΔC$_t^{LATS1, control}$), error bars: SE. [a]*P* < 0.05 *vs* control group; [c]*P* < 0.05 between subgroups (Mann-Whitney *U* test).

expression level was 305 times lower in methylated CRC samples than in unmethylated samples (*P* < 0.05, plot not shown). The lowest *LATS1* ratio was found in Dukes' D stage (1263 times lower than in control). However, we found that all analyzed samples in this subgroup showed a hypermethylation pattern, and thus, statistical comparison between methylated and unmethylated cases in D stage could not be evaluated. Interestingly, *LATS1* expression in unmethylated CRC cases was not statistically different from that in controls.

When the methylation status of the *LATS1* promoter was analyzed in patients with and without the presence of metastatic cells in regional lymph nodes and/or distant organs, we found a strong relationship between the metastatic potency of cancer and reduced expression of *LATS1* and hypermethylation of its promoter. The *LATS1* ratio was 381 times lower in hypermethylated *vs*

unmethylated in metastatic CRC cases (*P* < 0.05, dark grey boxes in Figure 3, Table 2), and only 8 times lower in hypermethylated *vs* unmethylated in non-metastatic CRC cases (*P* < 0.05, light grey boxes in Figure 3, Table 2). Moreover the expression of *LATS1* in unmethylated metastatic CRC samples was 49 times reduced as compared to unmethylated non-metastatic CRC cases (*P* < 0.05, Figure 3, Table 2).

Comparison of the histological grading of CRC (G), methylation status, and *LATS1* mRNA levels revealed the highest proportion of hypermethylation (71% of analyzed cases) in poorly differentiated (G3) CRC cases (Table 2). However, the difference in the *LATS1* expression level in methylated cases was only ca. 4 times lower than in unmethylated cases in the G3 subgroup (*P* < 0.05, Table 2, Figure 4). On the contrary, the difference in the *LATS1* mRNA level between methylated and unmethyl-
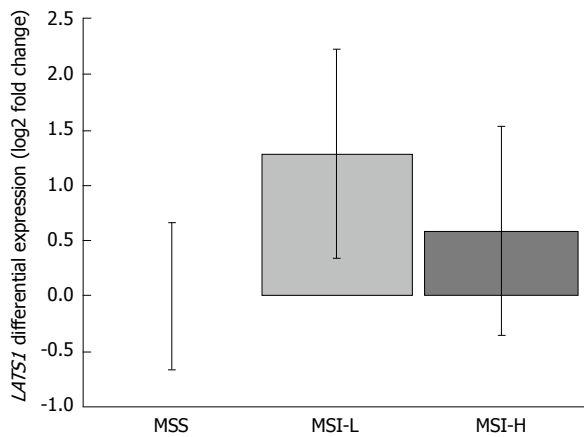
Figure 2 Microsatellite instability status and large tumor suppressor 1 expression. Comparison of large tumor suppressor 1 (*LATS1*) mRNA levels in cases divided by the mutations observed in the BAT26, BAT25, and BAT40 microsatellite markers. Samples were considered to have microsatellite stability (MSS; no mutation), microsatellite instability-low grade (MSI-L; 1-2 mutations, light grey box), and microsatellite instability-high grade (MSI-H; mutations in all three markers, dark grey box). MSS (*n* = 40), MSI-L (*n* = 30), MSI-H (*n* = 14). Vertical bars represent *LATS1* fold ratio calibrated to the average C_t of MSS cases - ($\Delta\Delta C_t^{LATS1} = \Delta C_t^{LATS1, MSI} - \Delta C_t^{LATS1, MSS}$), error bars: SE.

Figure 3 Large tumor suppressor 1 expression in colorectal cancer in relation to promoter methylation status. Comparison between large tumor suppressor 1 (*LATS1*) mRNA expression and epigenetic hypermethylation (M) or absence of hypermethylation (UM) of CpG islands located within the *LATS1* promoter region in a total of 44 colorectal cancer (CRC) cases (black vertical bars, *n* = 25 for M and *n* = 19 for UM). Vertical bars represent the *LATS1* fold ratio calibrated to the average C_t of control ($\Delta\Delta C_t^{LATS1} = \Delta C_t^{LATS1, sample} - \Delta C_t^{LATS1, control}$), error bars: SE. CRC cases were further divided into two subgroups: absence or presence of metastasis in lymph nodes/distant organs: N0M0: Light grey bars (*n* = 20; M: *n* = 9; UM: *n* = 11); N1-2/M0-1: Dark bars (*n* = 24; M: *n* = 16; UM: *n* = 8), respectively. [a]*P* < 0.05 *vs* control group; [c]*P* < 0.05 between subgroups (Mann-Whitney *U* test).

ated CRC tissue was much more pronounced in moderate-differentiated G2 cells (*P* < 0.05, Figure 4). Moreover, the *LATS1* ratio in G2 unmethylated cases was not statistically different from that in control healthy patients. Interestingly, G3 unmethylated cases showed much lower (ca. × 100) *LATS1* expression than G2 unmethylated biopsies (*P* < 0.05, Figure 4). Finally, we did not observe any statistically significant correlation between G grading and the *LATS1* mRNA level or hypermethylation of its promoter.

When we focused on MSI and the *LATS1* methylation status, we did not find any significant relationship because the statistical distribution of the results was very broad (Table 2, figure not shown). Most methylated cases (27/44) were considered MSS with no significant difference between methylated and unmethylated cases. MSI-L and MSI-H samples were also characterized as having relatively small differences in *LATS1* expression between methylated and unmethylated cases.

DISCUSSION

LATS1 is a tumor suppressor gene involved in several important mitotic processes, which are crucial in the development of CRC[7,44]. The most recent data suggest that the SWH pathway may play a very important role in CRC progression[45]. LATS1 is a key transducer of this pathway, and reduced expression of *LATS1* is connected with deregulation of SWH, thus activating the *YAP* oncogene[18]. Moreover, p53, a "genome guardian" protein, is indirectly regulated by LATS1[46]. MDM2, the regulator of p53 ubiquitination, is sequestered by native cellular LATS1, so that in the case of reduced *LATS1* expression, degradation of p53 cannot be triggered by MDM2[46]. Those observations suggest that studies on the role of *LATS1*
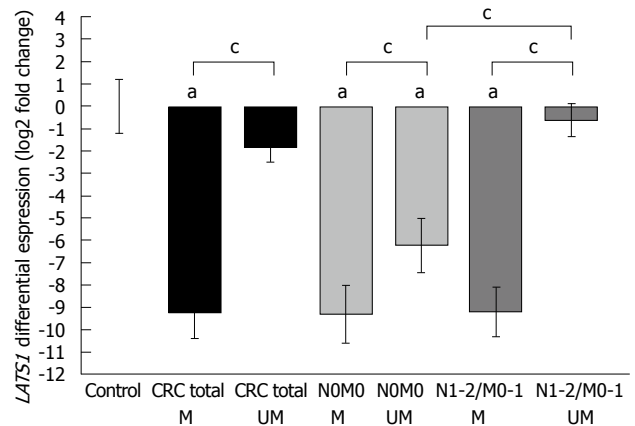
in CRC should be intensified. Our investigation provides the first analysis of the *LATS1* expression profile in a relatively large group of CRC patients compared with 40 healthy persons as well as analysis of *LATS1* promoter hypermethylation as a putative quiescence factor for *LATS1* expression in CRC. The results of our quantitative study, which demonstrated decreased *LATS1* expression in 89% of CRC patients, are consistent with the decreased *LATS1* expression found in other tumors[20-22]. However, Bianchini *et al*[26] reported 3.11-fold increased expression of *LATS1* in 25 CRC patients compared with 13 non-cancerous adjacent tissue samples from the surgical margin. This discrepancy may be due to important methodological differences between the two studies. First, Bianchini *et al*[26] compared their CRC data to 13 non-cancerous adjacent tissue samples from the surgical margin, whereas in our study of 142 CRC (Dukes' stages A-D) patients, the histologically normal mucosa of 40 healthy controls was used as a reference sample. Second, Bianchini *et al*[26] used the microarray technique to generate expression profiles of 19200 different transcripts normalized to glyceraldehyde 3-phosphate dehydrogenase expression in only Dukes' B and C stage CRC patients. Seven transcripts are generated from *LATS1* (Ensembl database), however, Bianchini *et al*[26] did not specify the isoform they analyzed. Our QPCR assay was designed to amplify the functional isoform of *LAST1* that was also analyzed in other tumors[20-22]. Hence, our data cannot be directly compared with the contradictory results of Bianchini *et al*[26]. Moreover, we are not aware of any other reports suggesting increased *LATS1* expression in cancer. Immunohistochemical analysis of LATS1 protein expression in gastric cancer revealed lower expression
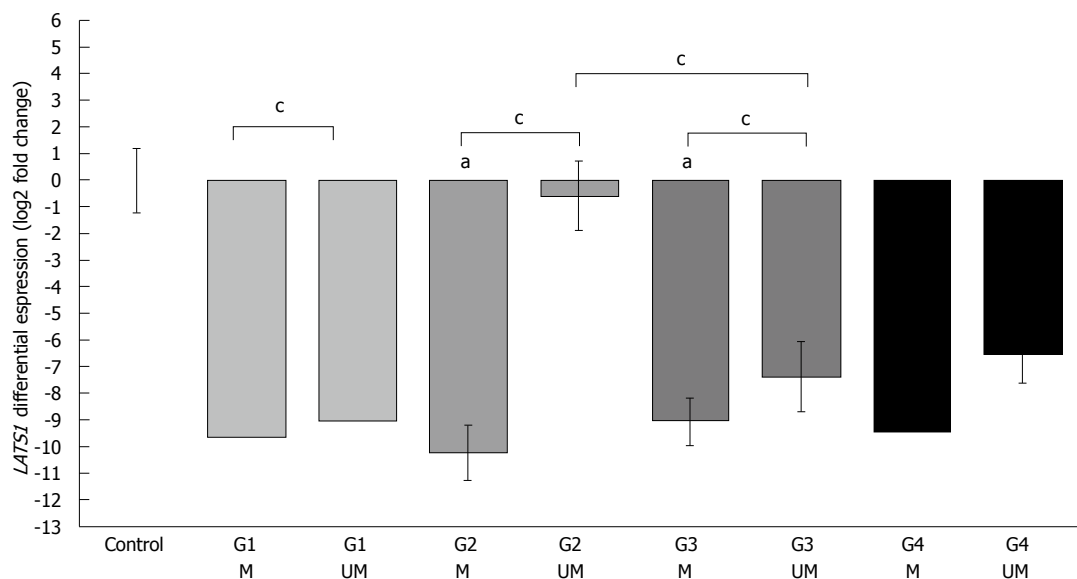
Figure 4 Methylation status of large tumor suppressor 1 in relation to the expression ratio and histological staging of cells. Forty-four colorectal cancer cases were classified according to histological examination: G1: Well-differentiated cells, *n* = 2 (light grey bars), G2: Moderately differentiated cells, *n* = 11 (grey bars), G3: Poorly differentiated cells, *n* = 28 (dark grey bars), G4: Undifferentiated cells, *n* = 3 (black bars). G1 epigenetic hypermethylation (M) (*n* = 1), G1 absence of hypermethylation (UM) (*n* = 1), G2 M (*n* = 3), G2 UM (*n* = 8), G3 M (*n* = 20), G3 UM (*n* = 8), G4 M (*n* = 1), G4 UM (*n* = 2). Vertical bars represent the large tumor suppressor 1 (*LATS1*) fold ratio calibrated to the average C_t of control ($\Delta\Delta C_t^{LATS1} = \Delta C_t^{LATS1, sample} - \Delta C_t^{LATS1, control}$), error bars: SE. [a]*P* < 0.05 *vs* control group; [c]*P* < 0.05 between subgroups (Mann-Whitney *U* test).

levels in 40 of 78 tumor lesions compared with normal gastric mucosa. The expression of LATS1 protein was significantly lower in gastric cancer with lymph node metastases than in cases without lymph node involvement[25]. Furthermore, in a group of 117 breast cancer patients, LATS1 mRNA was significantly decreased in the tumor tissue, and its decreased level was associated with a large tumor size, high lymph node metastasis rate, and poor prognosis[22]. In 30 astrocytoma cases, the level of LATS1 was 2-10 times lower as quantified by QPCR compared with 10 samples from normal brain tissue[21]. The most recent data showed that reduced expression of *LATS1* was correlated with the occurrence of metastatic glioma and poor survival of patients in a group of 17 cases[47]. Hence, decreased expression of *LATS1* in tumor tissue may suggest a suppressor role in CRC and other tumors.

MSI status has been regarded as one of the most important genetic markers and is strongly associated with molecular data, clinical findings, medical treatment, and patient outcome[48,49]. Our finding based on three BAT markers showed that more than half of the CRC patients had MSI tumors. Because we obtained samples from various clinics in different locations in Poland, our findings add to the observations by Smigiel *et al*[50] who observed MSI-L and MSI-H in 20% and 20.1% of cases, respectively, in a group of 143 CRC patients in the Lower Silesia region, which was not included in our analysis. The MSI phenotype may affect expression patterns of different proteins[51], and thus, we tried to estimate if decreased expression of *LATS1* was associated with MSI. Our findings excluded MSI-L and MSI-H as factors that may affect *LATS1* expression in the studied sample of CRC patients.

Inactivation of a typical tumor suppressor gene is

generally induced by epigenetic factors such as mutation of one allele and/or loss of heterozygosity (LOH) of the other allele[52,53] or hypermethylation of CpG islands in the regulatory region of the gene[27-30]. Such factors may lead to a complete loss of gene function in cancer[54,55]. Expression of the *LATS1* transcript can be epigenetically decreased by hypermethylation of CpG islands located within the 5' upstream regulatory region of the gene[20-22]. Because *LATS1* was reduced in several malignancies, we decided to assess the hypermethylation status of the *LATS1* CpG island. Our study is the first report of the hypermethylation status of *LATS1* in CRC, showing an association between hypermethylation and decreased *LATS1* expression. *LATS1* hypermethylation was observed in 17/30 (56%) breast cancers and was associated with decreased *LATS1* expression; methylated cases showed a 3-fold decreased expression compared with unmethylated cases[22]. *LATS1* hypermethylation was found in 13/54 (24%) cases of head and neck cancer[24] and in 64% (56/88) of astrocytomas[21]. Moreover, in astrocytomas, the methylation status was associated with decreased *LATS1* expression[21]. A similar relationship between decreased *LATS1* expression and its hypermethylation was observed in our study in 57% of analyzed CRC cases. Interestingly, other known epigenetic factors do not seem to be involved in reduced *LATS1* expression in cancer. In a group of 25 breast cancers, LOH at 6q24-25.1 (*LATS1* locus) was found in only one case (4%), whereas no mutation was found and only two gene polymorphisms were observed. However, neither polymorphism caused amino acid substitution[31]. As further support that hypermethylation may be the major epigenetic factor in *LATS1* silencing, the expression of *LATS1* in the

hypermethylated cell lines U251 (an established glioma cell line) and SHG-44 (a human malignant glioma cell line) was restored by addition of 5-aza-deoxycytidine, and apoptosis of cancer cells results[21].

Decreased expression of *LATS1* that is associated with promoter hypermethylation may contribute to suppression of the SWH pathway[10,11,13,18]. This pathway is prone to deregulation because few proteins involved in signal transduction are both tumor suppressors and oncoproteins. Altered expression of *YAP*, *RASSF1A*, *LATS1*, and *MST2* in cancer cell lines leads to higher resistance of the cells to apoptosis[10,13,17,18]. Moreover, reduced expression of other genes that are not directly involved in the SWH pathway, such as WW and C2 domain containing 1 (*KIBRA*) and salvador homolog 1 (*SAV1*), may contribute to the quiescence of this pathway[11,16,56]. Such suppression of the SWH pathway is related to epithelial-to-mesenchymal transition features and poor prognosis in breast cancer[16].

In conclusion, this is the first study to show decreased expression of *LATS1* in CRC, confirming its tumor suppressor function and linking its downregulation to the epigenetic hypermethylation of the *LATS1* promoter region.

ACKNOWLEDGMENTS

COMMENTS

Background
Searching for new colorectal cancer (CRC) molecular markers is a very important objective, because CRC is one of the most common malignancies in the world and one of the most fatal of human neoplasms. The molecular mechanisms of CRC are still unknown, but deregulation of mitotic division as well as apoptosis resistance are clearly associated with CRC progression.

Research frontiers
Human large tumor suppressor 1 (*LATS1*) encodes a serine/threonine kinase, which mediates a tumor suppressor pathway called the Salvador-Warts-Hippo (SWH) pathway. Abnormal expression of *LATS1* was observed in some tumors, and its expression in CRC has not been analyzed quantitatively.

Innovations and breakthroughs
This is the first study of a large group of CRC patients that shows quantitatively reduced *LATS1* expression at the mRNA level. Decreased levels of *LATS1* were strongly associated with hypermethylation of its promoter, particularly in metastatic tumors.

Applications
With knowledge regarding the decreased expression of *LATS1* in CRC, focusing on its intracellular signaling pathways in CRC and the probable involvement of this gene in CRC pathogenesis as a molecular marker may be possible.

Terminology
LATS1 is a putative tumor suppressor gene that shows reduced expression in

several malignancies. LATS1 is important in karyo- and cytokinesis and is part of the SWH pathway. Hypermethylation of the *LATS1* promoter is a common epigenetic factor responsible for downregulation and silencing of this gene.

Peer review
The authors collected and processed samples of CRC tumors and control colon biopsies from four collaborative clinics from four different regions of Poland. Molecular quantitative assays based on quantitative polymerase chain reaction revealed strongly reduced expression of *LATS1* in CRC tumors. Furthermore, this downregulation was strongly associated with the occurrence of hypermethylation of the *LATS1* promoter but not with microsatellite instability. This observation confirms the suppressor role of *LATS1* in carcinogenesis in this first study on a large group of CRC patients.

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