Abstract

Objectives: Metastasis-associated antigen 1 (MTA1) is implicated in metastasis while 15-lipoxygenase-1 (15-LOX-1) reduces cell motility, when re-expressed in colorectal cancer (CRC). We aimed to understand any potential interplay between MTA1 and 15-LOX-1 in CRC metastasis.

Materials and methods: ALOX15 and MTA1 expression in tumour and normal samples were analysed from TCGA RNA-seq data, microarray data sets and a human CRC cDNA array. Western blots, chromatin immunoprecipitation (ChIP), luciferase assays and electrophoretic mobility shift assays (EMSA) were carried out in HT-29 and LoVo cells re-expressing 15-LOX-1 to determine NF-κB activity at the MTA1 promoter. Functional assays in cells ectopically expressing either 15-LOX-1, MTA-1 or both, were carried out to determine adhesion and cell motility.

Results: Significantly higher expression of MTA1 was observed in tumours compared to normal tissues; MTA1 overexpression resulted in reduced adhesion in CRC cell lines. Re-expression of 15-LOX-1 in the CRC cell lines reduced expression of endogenous MTA1, corroborated by negative correlation between the two genes in two independent human CRC microarray data sets, with greater significance in specific subsets of patients. DNA binding and transcriptional activity of NF-κB at the MTA1 promoter was significantly lower in cells re-expressing 15-LOX-1. Functionally, the same cells had reduced motility, which was rescued when they over-expressed MTA1, and further corroborated by expressions of E-cadherin and vimentin.

Conclusions: Expression of MTA1 and 15-LOX-1 negatively correlated in specific subsets of CRC. Mechanistically, this is at least in part through reduced recruitment of NF-κB to the MTA1 promoter.

INTRODUCTION

Metastasis-associated antigen 1 (MTA1) belongs to a family of proteins (consisting of MTA1, MTA2 and MTA3) that is associated with the nucleosome remodelling and histone deacetylation (NuRD) complex, which couples histone deacetylation with ATP-dependent chromatin remodelling. MTA1, through its association with the NuRD complex, has been shown to hypoacetylate and repress the transcriptional activity of both p53 and oestrogen receptor alpha (ERα) converting the respective parental cells into a more aggressive phenotype. 15-Lipoxygenase-1(15-LOX-1), which has tumour suppressive
properties, was also shown to be repressed by the NuRD complex in colorectal cancer.4

MTA1 is highly expressed in a number of normal tissues including murine liver, testes, brain and kidney where the functions of the protein, albeit currently ill defined, may range from regulation of endocytic traffic, oestrogen receptor signalling, regulation of circadian rhythm, mammary gland development, spermatogenesis, macrophage inflammation and liver regeneration following injury.5 The MTA1 protein is primarily located in the nucleus although it does not have a DNA-binding domain or enzymatic activity.6 Therefore, most of the functions of MTA proteins are through protein-protein interactions with multi protein regulatory hubs such as the NuRD complex.5

High MTA1 levels have been unequivocally correlated with poor prognosis and aggressiveness of many different cancer types.5,6 Human gastric and colorectal cancers showed significantly higher transcript levels of MTA1 compared to paired normal counterparts.7 We have previously shown by immunohistochemistry that the expression of MTA1 protein was statistically significantly higher in all human malignant colon tissue sections compared to the normal tissues.8 A number of in vitro and animal studies have also indicated the importance of MTA1 in the metastatic behaviour of cancer cells.6,9

15-LOX-1 is a highly regulated enzyme, which preferentially metabolizes linoleic acid to 13-hydroxyoctadecadienoic acid (13-HODE).10 Multiple lines of evidence collected from various experimental models in different human cancers suggest that 15-LOX-1 plays an anti-tumourigenic role (reviewed in 11) and re-expression of 15-LOX-1 was shown to reduce cellular motility in colorectal cancer (CRC).12,13

Nuclear factor-kappa B (NF-κB) represents a family of transcription factors of hetero- or homodimers mostly including one subunit of p65 (relA) and another subunit such as p50, c-rel or relB.14 NF-κB is retained in the cytoplasm in an inactive form by binding to the IκB inhibitory proteins.15 In the presence of a stimulus, IκB is phosphorylated, which marks it for ubiquitination and degradation, thereby allowing NF-κB translocation into the nucleus, where it can control the transcription of a variety of genes. NF-κB plays important roles in promoting chronic inflammation and tumorgenesis, especially in colon cancer.16 MTA1 has been shown to be transcriptionally regulated by NF-κB in mice.17 We and others have shown that re-expression of 15-LOX-1 inhibits the transcriptional activity of NF-κB both in vitro and in vivo.18,19

In the current study, we have revisited the role of MTA1 in colorectal cancer. We have analysed publicly available TCGA colorectal cancer RNA-seq data sets as well as microarray data sets and conducted RT-qPCR on human colon cancer cDNA samples to determine the expression of MTA1 and ALOX15. Functionally, MTA1 overexpression in a CRC cell line resulted in reduced cell-cell adhesion. We then examined the hypothesis that the decrease in metastatic potential of 15-LOX-1-expressing CRC cells could be due to the downregulation of MTA1 via 15-LOX-1-mediated inhibition of NF-κB activity. Our data show that the expression of MTA1 was significantly higher in all tumour samples compared to normal samples, irrespective of stage, while the expression of ALOX15 was significantly lower than that of MTA1. A negative correlation between the expression of MTA1 and ALOX15 was seen in two independent microarray data sets as well as in colon cancer cell lines ectopically expressing 15-LOX-1. In the cell lines, this regulation was dependent on NF-κB binding to the MTA1 promoter. Functionally, the re-expression of 15-LOX-1 could reduce the motility of colon cancer cells, through an upregulation of E-cadherin and downregulation of vimentin; this phenotype was rescued when MTA1 was co-expressed.

2 | MATERIALS AND METHODS

2.1 | RNA-seq and microarray data analyses

The RNA-seq by expectation-maximization (RSEM) normalized RNA-seq data of colon adenocarcinoma and rectal adenocarcinoma were downloaded from FireHose database FireBrowse facility.20 The RSEM normalized RNA-seq data of normal tissue were downloaded from the TCGA data portal website (http://cancergenome.nih.gov/) using the data for colon adenocarcinoma and rectal adenocarcinoma. Log2 transformation was used to standardize the data. Robust Multiarray Analysis (RMA) normalized data set for GSE39582 was obtained from the NCBI GEO.21 Data for the C5 subgroup from this data set also were extracted and used for further analysis. Raw data of GSE41258 were downloaded from the NCBI GEO22 before performing RMA normalization using gcrma package of Bioconductor.23 Probesets used for expression data analyses for microarray data sets were as follows: ALOX15: ‘207328_at’; MTA1: average of ‘202247_s_at’ and ‘211783_s_at’. A built-in Matlab function was used to calculate Pearson’s correlation between expression values of ALOX15 and MTA1. plotSpread toolbox of Matlab was used to plot the expression data. Matlab was used for ANOVA and F-test analysis.

2.2 | TissueScan array and Real-time qRT-PCR

Human TissueScan Colon Cancer Tissue qPCR Panel IV (HCRT304), containing first-strand cDNA from 48 samples covering 8-normal, 5-Stage I, 8-IIA, 1-II, 1-IIIA, 6-IIIB, 3-IIIIC, 6-III, 10-IV patients was purchased from Origene(Rockville, MD, USA). Three identical plates were used to determine the expressions of ALOX15, MTA1 and ACTB (calibrator gene). Prior to the experiment, the TissueScan plate was removed from storage at −20°C and allowed to warm to room temperature. A SYBR Green pre-mix was prepared using 2X SYBR Green Master Mix, 10 pmol/μL of gene-specific forward and reverse primers and PCR grade water, making up the reaction mixture to 30 μL/well. After dispensing 30 μL of the PCR pre-mix into the wells, the top of the plate was covered with an adhesive cover sheet and left on ice for 15 min to allow the dried cDNA to dissolve. Finally, the plate was mounted into the heating unit of 96-well ABI7500 Real-Time machine for the PCR reaction. The Ct values were calculated using relative standard curve method and the fold change in expression was calculated by the Pfaffl method.24
2.3 | Cell culture and transient transfections

Human CRC cell line HT-29 was obtained from ŞAP Enstitüsü (Ankara, Turkey), LoVo cells were obtained from ATCC (Middlesex, UK) and HCT-116 cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). All cells were grown in RPMI-1640 medium (Biochrom, Berlin, Germany) supplemented with 10% foetal bovine serum (FBS) (Thermo Scientific, Waltham MA, USA), 2 mmol/L L-glutamine and 1% penicillin/streptomycin. The cells were grown in a humidified atmosphere containing 5% CO₂ at 37°C.

Cells were either stably (HCT-116) or transiently (LoVo and HT-29) transfected with a mammalian 15-LOX-1-pcDNA3.1 vector. Additionally, where indicated, these cells were transiently transfected with an MTA1-pcDNA3.1 vector. Before transfection, the complete culture medium was changed with 1X antibiotic-free OptiMEM medium. X-tremeGENE HP (Roche, Mannheim, Germany) was used as a transfection reagent in a ratio of 3:1 (X-tremeGENE HP to plasmid) for the transfection of 80%–90% confluent cells according to the manufacturer’s instructions. Each transfection was confirmed for the expression of the 15-LOX-1 or MTA1 protein by Western blot. Stable transfection of HCT-116 cells with the 15-LOX-1 plasmid has been described previously.

2.4 | Hanging drop assay

To establish whether the overexpression of MTA1 resulted in alterations in cellular adhesion, a hanging drop assay was carried out as described previously with some modifications. HCT-116 cells were transfected with either the MTA1 expression vector (500 ng and 1 μg) or the empty vector for 24 hours, harvested and 2 × 10⁶ cells/ml were suspended in complete RPMI-1640 medium as single cells. Drops of 27 μL were pipetted onto the inner surface of a lid of 10 cm low attachment sterile Petri dish. The lid was placed upright on the plate and 5 mL of PBS was placed in the Petri dish to maintain humidity. The plate was cultured for 48 hours and the hanging drops were transferred to regular microscope glass slides, covered with coverslips and photographed. Images of each aggregate were taken (n=20 for each condition) with a 4X objective and the size of the aggregate was measured.

2.5 | Nuclear/cytoplasmic and whole protein extraction

Total protein was isolated using the M-PER protein isolation kit (Pierce, Rockford, IL, USA) containing protease and phosphatase inhibitors (Roche) according to the manufacturer’s guidelines. For the isolation of nuclear and cytoplasmic proteins, cells were collected in pre-chilled 1.5 ml Eppendorf tubes and centrifuged at 300 × g for 5 min at 4°C. The supernatant was discarded and the cells were resuspended in PBS/phosphatase inhibitor solution and centrifuged at 300 × g for 5 min at 4°C. This process was repeated twice. Following the removal of the supernatant, 500 μL ice-cold 1X hypotonic buffer, prepared from a 10X stock solution (100 mmol/L HEPES (pH 7.5), 40 mmol/L NaF, 100 μmol/L Na₂MoO₄·1 mmol/L EDTA) in the presence of protease and phosphatase inhibitors, was added and after gentle pipetting, the cells were incubated on ice for 15 min. About 100 μl of 10% Nonidet P-40 was then added and mixed gently, centrifuged for 30 s at 4°C and the supernatant (cytosolic fraction) was transferred into a new tube and stored at −80°C. The pellet was resuspended in ice-cold 1X complete nuclear extraction buffer, prepared from 2X nuclear extraction buffer (20 mmol/L HEPES (pH 7.9) 0.2 mmol/L EDTA, 3 mmol/L MgCl₂, 840 mmol/L NaCl and 20% glycerol) in the presence of protease and phosphatase inhibitors as well as DTT (10 mmol/L), vortexed for 15 seconds at the highest setting and gently rocked on ice for 5 min using a shaking platform. The lysate was then centrifuged at 14000 × g for 10 min at 4°C and the supernatant was collected as the nuclear fraction. The protein content was measured using the modified Bradford Assay using a Coomassie Plus protein assay reagent according to the manufacturer’s instructions.

2.6 | Western blot

Whole-cell extracts (50–80 μg) and pre-stained Page Ruler protein ladder (Fermentas, Lithuania) were separated in a 10% polyacrylamide gel and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) at 4°C for 1.5 hours. The membrane was blocked in 5%-10% skim milk and probed with appropriate antibodies at pre-optimized dilutions. The 15-LOX-1 antibody was obtained from Abnova (Taipei City, Taiwan), MTA1, p65 (polyclonal and monoclonal), kBα, E-cadherin, vimentin, β-actin, GAPDH, Topolβ and α-tubulin antibodies were obtained from Santa Cruz (Dallas, TX, USA). Primary antibody incubation was followed by incubation with horseradish peroxidase-conjugated appropriate secondary antibodies (1:2000 dilution). The bands were visualized using an enhanced chemiluminescence kit ECL Plus (Pierce) according to the manufacturer’s instructions. The images were taken with a BioRad Chemidoc station.

2.7 | Chromatin immunoprecipitation (ChiP) assay

ChiP assay was carried out as described previously. Briefly, HT-29 or LoVo cells were grown in 10 cm dishes until confluent and crosslinked with 0.75% paraformaldehyde at room temperature for 7 minutes. Crosslinking was stopped by addition of 2 mmol/L glycine and the cells were collected and washed. Chromatin was sheared to an average size of 500–1000 bp by probe sonication (7 cycles of 30 seconds pulses for LoVo cells or 9 cycles of 30 seconds pulses for HT-29 cells at 20% output). An aliquot was collected and treated with RNase A (1 hour, 37°C) or proteinase K (O/N, 60°C) followed by the isolation of DNA with a Roche High Pure PCR purification kit (Roche) according to the manufacturer’s instructions and referred to as input DNA. According to the input DNA amount obtained, lysates corresponding to 25 μg chromatin for each sample was immunoprecipitated with 10 μg
of p65 (polyclonal) antibody or isotype-matched rabbit IgG by rotating overnight at 4°C. Protein A/G sepharose beads saturated with bovine serum albumin (BSA) and single-stranded calf thymus DNA were added to the lysate to isolate the antibody-bound complexes. The beads were washed several times to remove non-specific binding, and the antibody-bound chromatin was eluted. The eluate was treated with RNase A for 1 hour at 37°C and with proteinase K, overnight at 60°C, and finally, the DNA was extracted using Roche High Pure PCR purification kit and used in PCR reactions. Of the final PCR products, 10 μL was electrophoresed on a 2% agarose gel at 100V and photographed under UV light. Primer sequences are shown in Table S1.

2.8 | Bioinformatic analysis of the MTA1 promoter

The promoter region of MTA1 (2000 bases upstream of the +1 site) was analysed with the Alibaba 2.1 program (http://www.gene-regulation.com/pub/programs/alibaba2/index.html), and based on the putative NF-κB-binding sites, the promoter was divided into two parts. Region I had one NF-κB-binding site, while Region II had five binding sites (Fig. 3a).

2.9 | Construction of reporter plasmids

Region I was cloned as a double-stranded oligonucleotide directly into the multiple cloning site of the pLuc-MCS vector (Stratagene, Santa Clara, CA, USA) and named pLuc-RI. Region II (600 bp) was PCR amplified and cloned into pLuc vector and named as pLuc-RIL (oligonucleotide and primer sequences are given in Table S1). Both sequences were cloned into the HindIII and XhoI restriction sites (oligonucleotide and primer sequences are given in Table S1). Both sequences were cloned into the HindIII and XhoI restriction sites and confirmed by sequencing (Sequencing Core Facility, METU Central Lab).

2.10 | Luciferase assays

Luciferase assays were carried out using Dual-Glo® Luciferase Assay System according to the manufacturer’s instructions. Cells pre-transfected with either 15-LOX-1 or pcDNA3.1 empty vector (EV) plasmids were separately transfected with either pLuc-RI, pLuc-RIL or pLuc-empty vector plasmids together with phRL-TK (Renilla) plasmid for 24 hours. The Dual-Glo® Luciferase reagent, which includes a cell lysis reagent and acts as a substrate for firefly luciferase, was added onto the cells and luminescence of the lysate was measured in white plates. Then Dual-Glo® Stop & Glo® Reagents, which provide substrate for Renilla luciferase, was added and luminescence was measured again. Signals obtained from the cells transfected with empty pLuc-empty vector and phRL-TK plasmids were used for normalization of the data.

2.11 | Electrophoretic mobility shift assay (EMSA)

A non-radioactive EMSA kit (Pierce) was used for this assay. Nuclear proteins were extracted from 15-LOX-1- or empty pcDNA3.1 vector-transfected HT-29 and LoVo cells as described above. NF-κB DNA-binding sequences were designed as three repeats together with their mutated oligos (mainly Gs were converted to As in the original oligo sequences, please see Table S1 for sequences) and obtained commercially (Iontek, Istanbul, Turkey). Oligos for the forward and reverse strands containing the binding or mutated sequences were biotinylated at their 3’ ends using Biotin 3’ End DNA Labeling Kit (Pierce) according to the manufacturer’s instructions. The products were subjected to phenol/chloroform extraction and equal amounts of forward and reverse oligos were annealed by heating to 95°C and gradually cooling at the rate of 1°C/minute until ambient temperature was achieved.

For binding reactions, 10 μg of protein was mixed in a 20 μL reaction volumes with 2 μL 10X binding buffer, 1 μL of 50% glycerol, 1 μL of 100 mmol/L MgCl₂, 1 μL of 1 μg/μl poly (dI•dC), 1 μL of 1% NP-40, 20 fmol labelled oligo and ultrapure water. The reaction specificity was confirmed by the inclusion of 200-fold molar excess (4 pmol) of unlabelled oligos (cold probe), which would lead to a loss of the gel shift, whereas for the supershift assay, a p65 monoclonal antibody was used. After mixing with 5X loading dye, the reactants were separated in 8% polyacrylamide gels at 100 V for 1 hour at 4°C and transferred on to Biodyne A or B membranes (Pierce) according to the manufacturer’s instructions using electro blotting in 0.5X TBE buffer at 4°C. Subsequently, the membrane was crosslinked with UV and then blocked in streptavidin containing nucleic acid blocking buffer and finally detection was achieved using luminol-enhanced hydrogen peroxide substrate and signals were collected on films by the use of Kodak X-ray film developer machine.

2.12 | Motility assay

To understand whether the regulation of MTA1 expression via 15-LOX-1 was functionally relevant, we carried out a Transwell migration assay and a wound healing assay. For the migration assay, three groups of cells were used: HCT-116 cells stably expressing 15-LOX-1, HCT-116 cells stably expressing 15-LOX-1 that were transfected for 24 hours with 500 ng of the MTA1 plasmid and wild-type HCT-116 cells transfected with 500 ng MTA1 plasmid. The plasmid was pcDNA3.1 for all transfections. The migration assay was carried out as described previously. Briefly, 5x10⁴ cells were counted and pipetted into Transwell inserts in 1% serum containing medium and the inserts were then placed in medium containing 10% serum. The cells were allowed to migrate for 48 hours, after which the inserts were taken and the non-migrated cells were removed by scrubbing with sterile cotton swabs. The inserts were fixed in 100% methanol for 10 minutes, stained with modified Giemsa stain for 2 minutes and washed twice in distilled water. The membranes from the inserts were then cut out, mounted on slides and the number of migrated cells was counted using a Leica light microscope with 20X objective.

For the wound healing assay, LoVo cells at 80% confluency were transfected with either 1.5 μg of the 15-LOX-1 expression vector, 500 ng of the MTA1 expression vector or a combination of the
15-LOX-1 and MTA1 expression vectors. In all cases, the final plasmid concentration was adjusted to 2 μg by adding the empty pCDNA3.1 vector. The cells were transfected for 24 hours in serum containing medium, washed and a scratch was made as described below. For the HCT-116 cells, the same three groups of cells as the migration assay were used. The cells were scratched with a 200 μL pipette tip, the plate was washed with PBS to remove any debris and complete medium was added. The plates were photographed immediately afterwards as well as every 24 hours for the next 48 hours. The cells were then collected and total proteins were extracted for Western blot. The total experiment duration was restricted to 72 hours to ensure that the transient expression of MTA1 continued until the end of the experiment.

2.13 | Statistical analysis

Results are expressed as the mean ± SD. One-way ANOVA with Tukey’s post hoc test or Student’s t-test were used for statistical analysis, and differences at P<.05 were considered significant. Experiments were repeated independently at least three times with at least three technical replicates.

3 | RESULTS

3.1 | MTA1 expression is upregulated in tumours compared to normal colon cancer samples

MTA1 has a number of important functions in normal tissues; nonetheless, a number of studies have shown that MTA1 is upregulated in many tumours. To reconfirm this upregulation in colorectal cancer, we analysed MTA1 expression using mRNA-sequencing data for colorectal tumours (TCGA COADREAD cohort n=416 with known stages, and normal samples n=50). We observed a significant increase in MTA1 mRNA expression in tumours (Fig. 1a, left panel)

![Figure 1](image)

**FIGURE 1** Expression analysis of MTA1 and ALOX15 in colon tumour data sets. (a) Analysis of MTA1 expression in tumour (n=416) and normal samples (n=50) in the TCGA colon adenocarcinoma and rectal adenocarcinoma data (left panel). MTA1 expression in a colon cancer cDNA RT-qPCR array consisting of tumour (n=40) and normal samples (n=8) (right panel). Significantly higher expression was seen in the tumour compared to the normal samples. (b) Hanging drop assay showing significantly lower cell-cell adhesion in HCT-116 colon cancer cells transfected with 500 ng and 1 μg of an MTA1 vector when compared to the empty vector-transfected cells. (****P<0.0001) (c) Comparative expressions of ALOX15 and MTA1 in the TCGA colon and rectal tumour data set. Lower expression with a significantly greater variability was seen in the expression of ALOX15 compared to MTA1 (n=408). (d) Correlation analysis showing a significant negative correlation in the expressions of ALOX15 and MTA1 in the GSE39582 data set showing patient stratification into subgroups C1-C6 (left panel) (n=566 for tumour, n=19 for normal). Stronger negative correlation in the expression of MTA1 and ALOX15 in the patient subgroup C5 (n=152 right panel). (e) Correlation analysis showing a significant negative correlation in the expressions of ALOX15 and MTA1 in the GSE41258 microarray data set including 54 normal and 186 primary tumour samples. P value is based on Pearson's correlation coefficient.
irrespective of the stage (Fig. S1A) compared to the normal samples. Corroborating this, an increased expression of MTA1 was observed in tumours compared to normal samples in a human colon cancer cDNA array by RT-qPCR (Fig. 1a, right panel) and two large microarray data sets GSE39582 and GSE41258 (Fig. S1B).

To detect whether MTA1 overexpression showed any functional effects in colon cancer cell lines, a hanging drop assay was carried out (Fig. 1b). HCT-116 cells transfected with the empty vector (control cells) efficiently aggregated in hanging drops and generally formed one large cell cluster per drop. On the other hand, HCT-116 cells transfected with 500 ng MTA1 showed small, irregularly shaped, loosely associated cell clusters. When the cells were transfected with 1 μg of the MTA1 plasmid, there was a significant inhibition in the formation of compact aggregates and all of the 20 drops analysed formed dispersed and very small cell aggregates.

Analysis of the same TCGA RNA-seq data set indicated very low expression of ALOX15 in the tumour samples when compared to MTA1 along with a considerably higher variation in ALOX15 expression indicating dysregulation (Fig. 1c; One-tailed F-test for variation: F=18.6, df=407; pVal= 1.2908e-147). However, variation based on microarray expression measurements was greater for MTA1 in comparison with ALOX15, suggesting platform-specific differences in the measurement of expression (F-test for variation: GSE41258, F=0.33, df=185, pVal= 6.6756e-14; GSE39582, F=0.33, df=565, pVal= 6.9920e-38). Additionally, a downregulation in ALOX15 expression in tumours compared to normal was seen in the data set GSE39582 (P=0.052, Supplementary data 1C). Correlation analyses indicated that MTA1 and ALOX15 were negatively correlated with statistical significance in the two microarray data sets examined (Fig. 1d). In particular, for the data set GSE39582, a specific subgroup of patients defined as C5 showed a stronger negative correlation compared to the other subgroups. No significant negative correlation could be detected between the expression of the two genes in the TCGA RNA-seq data set or the cDNA array (data not shown).

3.2 | 15-LOX-1 re-expression downregulates the expression of MTA1 in CRC cell lines

The negative correlation observed in the expression of ALOX15 and MTA1 in human colon tumour samples (Fig. 1d) indicated the presence of a potential crosstalk between 15-LOX-1 and MTA1. To examine this further, we used colon cancer cell lines (HT-29 and LoVo) that we previously found to highly express MTA1.

We have transiently expressed 15-LOX-1 in these cell lines and have observed a reduction in the expression of MTA1 compared to empty vector carrying cells (Fig. 2).

3.3 | 15-LOX-1 expression reduces the nuclear translocation of NF-κB

We and others have previously shown that 15-LOX-1 expression leads to an inhibition in NF-κB activity. MTA1 is also known to be transcriptionally regulated by NF-κB. We therefore aimed to understand mechanistically whether the inhibition in MTA1 expression observed in the presence of 15-LOX-1 was related to the inhibition of NF-κB. We have previously reported that HT-29 cells ectopically expressing 15-LOX-1 showed reduced nuclear translocation and activity of NF-κB. To confirm whether the same was true in LoVo cells, we carried out a Western blot and observed reduced nuclear translocation of p65 in the cells expressing 15-LOX-1 compared to the empty vector transfected cells. This was accompanied by increased stability of IκBα, a protein that restrains p65 in the cytoplasm, in the 15-LOX-1-expressing cells (Fig. 3). The results confirmed that expression of 15-LOX-1 in both HT-29 and LoVo (current study) cells could reduce NF-κB translocation when compared to the empty vector carrying cells. The nuclear translocation of p65 in HT-29 cells was also examined in the course of the current study and found to be reproducible (data not shown).

3.4 | 15-LOX-1 expression regulates p65 binding to the MTA1 promoter

To identify the NF-κB-binding sites on the MTA1 promoter, ~2 kb sequence upstream of the start site was extracted from the NCBI database and analysed using the Alibaba 2.1 transcription factor-binding site detection program. The program offered several binding sites between the regions −1874 and −730 upstream of the MTA1.
transcription start site (Fig. 4a). We have also carried out a multiple alignment analysis of the same genomic region on the MTA1 promoter in order to understand whether binding sites are conserved within different species. The results of comparative analysis of sequences from Pan troglodytes, Gorilla gorilla, Pongo abelii, Macaca mulatta, Mus musculus, Rattus norvegicus, Bos taurus and Canis familiaris species interestingly showed that binding sites were conserved within the primates but not with the other species (data not shown).

A number of assays were carried out to determine the binding of NF-κB on the putative sites in the human MTA1 promoter in the presence and absence of 15-LOX-1 expression.

3.5 | Chromatin immunoprecipitation (ChIP) assay

ChIP followed by PCR was carried out to amplify the region between −1874 to −1725 (Region I, containing one binding site for NF-κB) and −1331 to −730 (Region II, containing five different binding sites for NF-κB) on the MTA1 promoter. The primer sequences for the amplification are given in Table S1. Sheared chromatin from 15-LOX-1- or EV-transfected HT-29 and LoVo cells were immunoprecipitated with a polyclonal p65 antibody. Amplification of immunoprecipitated DNA from EV-transfected control cells, along with IgG immunoprecipitated (negative control) and input DNA (positive control) showed the presence of p65 binding in both Region I and Region II on the MTA1 promoter (Fig. 4b). As an additional negative control, immunoprecipitated DNA was subjected to PCR amplification by a primer set that amplifies a gene desert region on chromosome 12 and therefore is expected not to have any transcription factor binding (control primer sequences are given in Table S1). 15-LOX-1-transfected HT-29 cells showed reduced recruitment of p65 onto both Regions I and II, while LoVo cells showed reduced recruitment only in Region I when compared to the empty vector transfected cells.

3.6 | Reporter gene assays

To analyse the transcriptional activity of NF-κB in the MTA1 promoter, we carried out luciferase assays. The single binding sequence in Region I was cloned as an oligonucleotide containing five repeats into the Agilent pLuc-MCS reporter plasmid (pLuc-MCS-RI). Region II, with five binding sequences, was PCR amplified and cloned into the pLuc-MCS vector (pLuc-MCS-RII; sequences for Region I oligo and cloning primers for Region II are given in Table S1). HT-29 and LoVo cells pre-transfected with either 15-LOX-1 or its EV were co-transfected either with pLuc-MCS vector and renilla (phRLTK) vectors or the pLuc-MCS-RII and phRLTK vectors. Luciferase and renilla signals were measured and normalized to Renilla luciferase activity. The representative data shown in Fig. 4c indicate significantly (*P<.05 and **P<.01) reduced activity in 15-LOX-1-transfected cells in both cell lines compared to the corresponding empty vector-transfected cells.

3.7 | Electrophoretic mobility shift assay (EMSA)

Non-radioactive EMSA was carried out to establish the binding of NF-κB p65 to the individual sequences identified in Regions I and

FIGURE 3 15-LOX-1 expression results in a loss of NF-κB nuclear translocation in LoVo cells. Western blot analysis of nuclear and cytoplasmic extracts isolated from 15-LOX-1-expressing LoVo cells. The lysates were probed with a p65 or an IκBα antibody. Control cells included the LoVo cells transfected with the empty pcDNA3.1 vector. Equal protein loading and lack of cross contamination between nuclear and cytoplasmic extracts was shown by TopoIIβ (180 kDa) and α-Tubulin (55 kDa).

FIGURE 4 Analysis of NF-κB binding to the promoter of MTA1. (a) Schematic diagram of NF-κB-binding sites in the region between chr14: 105884100-105886185 on the MTA1 promoter. Two regions were identified: Region I with one and Region II with five NF-κB-binding consensus sequences. (b) ChIP assay showing decreased NF-κB p65 recruitment on the MTA1 promoter at Region I and Region II in the presence of 15-LOX-1 expression in HT-29, and LoVo cells. 15-LOX-1 re-expression is shown by Western blot in the ChIP lysates from HT-29 and LoVo cells. (c) Luciferase assays showing reduced MTA1 promoter activity in HT-29 and LoVo cells, respectively after re-expression of 15-LOX-1. The data are normalized to pLuc-MCS-EV (empty vector)/phRLTK ratios. (d) EMSA showing the loss of p65 binding to the consensus sequences 1 and 6 on the MTA1 promoter in the presence of 15-LOX-1 expression. Nuclear lysates from pcDNA3.1 (empty vector)-transfected HT-29 and LoVo cells served as controls and showed binding to these regions. Incubation of the nuclear extracts from EV transfected cells with the mutated oligos resulted in a complete loss of binding, further confirming the specificity of the reaction. Three independent experiments were performed and statistical comparisons were carried out using paired t-test (*P<.05, **P<0.01)
II on the MTA1 promoter. Equal amounts of nuclear extracts from HT-29 cells were incubated with biotin-labelled oligos covering the six binding sequences identified (oligo sequences are given in Table S1). The biotin-labelled oligonucleotides 1, 2, 3 and 6 were able to form complexes with proteins from the nuclear extract (Fig. S2). On further addition of corresponding unlabelled oligonucleotide (cold probe), we observed the disappearance of the shift as evidenced by the ability of excess unlabelled probe to compete
with complex formation. The presence of p65 in the shifted complex was confirmed by the addition of a monoclonal p65 antibody, which resulted in a super shift of the complex.

As maximum binding was observed with oligos 1 and 6, the NF-κB binding sequences in these oligos were mutated (see Table S1 for the mutated oligo sequences) and the binding was further validated with nuclear extracts from both HT-29 and LoVo cells (Fig. 4d). As expected, 15-LOX-1 expression resulted in reduced p65 binding to the oligo sequences 1 and 6 in HT-29 cells. For LoVo cells ectopically expressing 15-LOX-1, reduced binding was observed to oligo 6, while binding to oligo 1 could not be detected within the detection limits of this assay. Of note, incubation of the nuclear extracts from EV-transfected cells with the mutated oligos resulted in a complete loss of binding, further confirming the specificity of the reaction.

3.8 Motility in HCT-116 cells expressing 15-LOX-1 and MTA1

To understand whether the low MTA1 expression observed in CRC cell lines ectopically expressing 15-LOX-1 was functionally relevant, a Transwell migration assay was carried out using HCT-116 cells (Fig. 5a). HCT-116 cells were used for this purpose as we observed these cells to be more motile than LoVo or HT-29 cells under identical conditions. We have previously reported a reduction in endogenous MTA1 levels as well as a reduction in NF-κB activity in HCT-116 cells stably expressing 15-LOX-1.13,18 The migration data indicate that HCT-116 cells stably expressing 15-LOX-1 showed reduced motility compared to the empty vector-transfected cells. As expected, HCT-116 cells transfected with the MTA1 expression vector alone showed significantly higher migration. On the other hand, in a rescue experiment where we transfected the 500 ng of the MTA1 plasmid to cells stably expressing 15-LOX-1, a significantly higher motility was seen when compared to cells expressing 15-LOX-1 alone, but significantly lower than cells expressing MTA1 alone. These functional changes were also reflected in the protein levels of vimentin, a mesenchymal marker that has been reported to be important for epithelial to mesenchymal transition (EMT) and metastatic dissemination of colorectal cancer cells.28 Thus, we observed lower expression (P=.09) of vimentin in cells expressing 15-LOX-1 alone, when compared to the empty vector-transfected cells. In the HCT-116 cells stably

![Figure 5](image-url)
re-expressing 15-LOX-1, co-expression of 500 ng MTA1 retained the inhibitory effect of 15-LOX-1 on the expression of vimentin (P=0.0013). On the other hand, transfection of 1 µg of the plasmid resulted in a rescue of the phenotype with the cells expressing higher amounts of vimentin, irrespective of whether 15-LOX-1 was expressed or not. We did not observe significant changes in the expression of E-cadherin in HCT-116 cells with any of the transfections.

To observe whether these functional and molecular changes were also valid in the LoVo cell line model used in the current study, a scratch assay was carried out in these cells transiently expressing 15-LOX-1, MTA1 or a combination of the two plasmids (Fig. 5c). Although the LoVo cells were considerably less motile than HCT-116 cells, transfection with the MTA1 plasmid resulted in a faster closure of the scratched area compared to transfection with the 15-LOX-1 plasmid. In the rescue experiment, we observed intermediate motility of cells, indicating some but not complete recovery of motility in cells expressing both 15-LOX-1 and MTA1. Following the scratch assay, cells were collected and processed for protein extraction and Western blot (Fig. 5d). A robust increase in the expression of E-cadherin, a junctional protein that is essential for epithelial cell-cell interactions, was observed in LoVo cells ectopically expressing 15-LOX-1 compared to the empty vector transfected cells. Cells transfected with the MTA1 plasmid did not show a significant change in E-cadherin expression in LoVo cells. E-cadherin has previously been shown to be reduced in plasmid did not show a significant change in E-cadherin expression.

MTA1 is a part of the multi-subunit NuRD chromatin remodeling complex which, in a context-dependent manner, can repress or activate the expression of many different target genes. A repressive function was ascribed to NuRD in the regulation of expression of ALOX15 in colorectal cancer. As 15-LOX-1 re-expression in colon cancer cell lines was shown to reduce cellular motility, we explored the presence of any potential negative crosstalk between MTA1 and 15-LOX-1 in colorectal cancer. Of the two independent microarray data sets that showed a statistically significant negative correlation between the expression of the two genes, the negative correlation in the GSE39582 array was mostly driven by data from patients who were subcategorized into the group C5. This subcategorization, which was carried out by the authors for better stratification of colon tumours, divided the colon tumours into six categories (C1-C6) based on their transcriptomes. According to KEGG analysis, each of the different subcategories had unique pathways and signaling mechanisms. It was particularly interesting to note that in C5, a significant downregulation in arachidonic and linoleic acid metabolism was seen, along with a significant upregulation of genes involved in the regulation of EMT and cellular communications. In the cohort studied, 27% of the patients belonged to C5; therefore, it is possible that the negative correlation seen between ALOX15 and MTA1 is valid for a specific subset of patients with a specific gene expression signature. The lack of significant correlation from the RNA-seq data or the cDNA RT-qPCR array may have resulted from over-representation of patients from the other subcategories in those cohorts as well as from measurement differences between the RNA-seq and microarray experimental platforms.

We next aimed to determine the mechanistic implications of the negative correlation observed between ALOX15 and MTA1 in the patient cohort. For this, we used two colon cancer cell lines HT-29 and LoVo cells (both cell lines express robust amounts of MTA1), in which re-expression of 15-LOX-1 resulted in a significant reduction in the expression of endogenous MTA1. Although this reduction was relatively modest, it is likely to be functional. We have previously observed that shRNA-mediated silencing of MTA1 by just 35% was enough to almost completely abrogate migration and invasion through Transwells as well as anchorage-independent growth in CRC cell lines.

To determine the mechanism behind the decrease in MTA1 levels in HT29 and LoVo cells where 15-LOX-1 was restored, we examined the activity of NF-κB, a master regulator of inflammation. In a high-throughput study where gene expression in MTA1 knockout mouse embryonic fibroblasts (MEFs) was compared with the wild-type MEFs,
the genes regulated by MTA1 were primarily involved in the inflammatory response.\textsuperscript{39} MyD88 expression and NF-κB activation in LPS-stimulated macrophages also involved MTA1 as a co-regulator with enhanced recruitment of MTA1, RNA polymerase II, and p65 RelA complex to the NF-κB consensus sites in the MyD88 promoter.\textsuperscript{40} Mouse model studies have shown that MTA1 can be transcriptionally upregulated directly by NF-κB\textsuperscript{17}; however, there is very little conservation of the regulatory sequences at the MTA1 promoter between humans and mice. The human MTA1 gene is at a different location from the mouse genome and the human MTA1 promoter has not been characterized yet for NF-κB binding. We have first re-confirmed decreased nuclear translocation (indicating inhibition in activity) of NF-κB p65 in the presence of 15-LOX-1 in the cell lines used in this study. Next, we identified possible binding sites for p65 on the MTA1 promoter. When 15-LOX-1 was expressed in HT-29 and LoVo cells, a reduction in the recruitment and transcriptional activity as well as DNA binding of p65 was observed in these regions. We have therefore identified novel NF-κB binding sites in the promoter of the human MTA1 gene along with a potential contribution of 15-LOX-1 signalling in regulating the expression of MTA1. NF-κB, while an essential transcription factor for the induction of inflammatory responses in immune cells, is also widely hyperactivated in gastrointestinal cancers with an inflammatory component.\textsuperscript{41} Treatment of mice that also harboured targeted expression of human 15-LOX-1 in the gut with the carcinogen azoxymethane (AOM) resulted in alteration of 22% of NF-κB regulating genes in the normal colon epithelia compared to wild-type AOM-treated mice. In the same study, LPS- or TNFα-stimulated primary colonic epithelial cells isolated from the mice expressing the 15-LOX-1 transgene also showed reduced nuclear translocation of p65 as well as reduced expression of a number of important NF-κB target genes.\textsuperscript{19} Mechanistically, we have previously shown that an upregulation in the activity of peroxisome proliferator-activated receptor gamma (PPARγ) through the production of the 15-LOX-1 metabolite, 13(S)-HODE, mediated the inhibition in NF-κB.\textsuperscript{18} As a consequence of reduced NF-κB signalling, we have identified here a reduction in the expression of the MTA1 gene containing several conserved NF-κB-binding sites.

Functionally, two different cell lines (HCT-116 and LoVo) showed enhanced motility using different techniques when overexpressing MTA1, while the same models showed reduced motility when the cells ectopically expressed 15-LOX-1. In the rescue experiments where cells expressed both 15-LOX-1 and MTA1, a modest but significant recovery in motility was seen compared to cells expressing 15-LOX-1 alone. In the HCT-116 model, the motility data were supported by the expression of the mesenchymal marker vimentin, while in the LoVo model, the motility data were reflected in the expression of the junctional protein E-cadherin. Vimentin is an intermediate filament protein that is expressed when epithelial cells start gaining mesenchymal characteristics and has been considered to be an important marker for EMT.\textsuperscript{42} On the other hand, loss of E-cadherin is associated with a loss in cell-cell contact and is frequently associated with not only the metastatic dissemination of cancer cells but can also lead to uncontrolled proliferation through loss of contact inhibition.\textsuperscript{43} Reflecting the motility data, we showed for the first time a significant increase in E-cadherin in LoVo cells re-expressing 15-LOX-1 compared to the empty vector-transfected cells which was retained even when the cells co-expressed MTA1. On the other hand, the reduced expression of vimentin in the 15-LOX-1-expressing HCT-116 cells was completely rescued when the cells co-expressed MTA1. Although both models (HCT-116 and LoVo) reveal that 15-LOX-1 re-expression reduced the motility of cancer cells, which could be rescued (albeit partly) with the overexpression of MTA-1, the mechanisms appear to be different. It should be remembered here that cellular motility is an intricate mechanism and can involve complex signalling mechanisms and crosstalk between many factors such as extra-cellular matrix components, accessory fibroblasts and pro-inflammatory cells. Moreover, the cell line models have different mutational landscapes and cell behaviour, which may have resulted in differences in signalling mechanisms activated. Reduced motility in cells re-expressing 15-LOX-1 has previously been ascribed to reduced secretion of the growth factor VEGF.\textsuperscript{12} Here, we have established that re-expression of 15-LOX-1 may lead to reduced motility through several important cellular changes including a reduction in the expression of MTA1 and vimentin and an enhancement in the expression of E-cadherin.

Re-expression of 15-LOX-1 in MTA1-overexpressing tumours may be of therapeutic relevance. It is important to note that the expression of 15-LOX-1 is lost in tumours and cell lines primarily through epigenetic mechanisms and re-expression of the enzyme has been possible through treatment with histone deacetylase inhibitors, drugs such as celecoxib, white tea extract and Honokiol.\textsuperscript{10} It remains to be seen whether the re-expression of 15-LOX-1 with such agents can also lead to a concomitant loss in MTA1 in vivo models.

In conclusion, in this study, we have shown a new mechanistic explanation of the reduction in motility of colon cancer cells ectopically expressing 15-LOX-1. The expression of MTA1 in specific subsets of colorectal cancer samples was negatively correlated with the expression of 15-LOX-1, which may be one of several mechanisms that may predispose the cells to metastasize. Re-expression of 15-LOX-1 in such cells can lead to an inhibition of NF-κB and thereby a reduction in the levels of MTA1 as well as re-expression of the cell-cell junctional protein E-cadherin. We believe these data further highlight the mechanisms behind the tumour suppressive functions of 15-LOX-1 and help us to understand novel strategies to regulate the expression MTA1, a master regulator of tumourigenesis, in colorectal cancer.

**ACKNOWLEDGEMENTS**

Dr Uddhav Kelavkar and Dr Ansgar Brüning are acknowledged for sharing plasmids. Dr Elif Erson-Bensan and Dr Mesut Muyan from Biology Department, METU are acknowledged for useful discussions. This work was supported by the Scientific and Technological Research Council of Turkey (TÜBİTAK, Project 113S063) and the Turkish Academy of Sciences Young Investigators Award (TÜBA-GEBİP) to SB.
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