Pathology – Research and Practice 213 (2017) 333–338

Contents lists available at ScienceDirect

Pathology – Research and Practice

journal homepage: www.elsevier.com/locate/prp

Original article

Expression and promoter DNA methylation of MLH1 in colorectal cancer and lung cancer

Yunxia Ma [1], Yuan Chen [1], Iver Petersen *

Institute of Pathology, University Hospital Jena, Friedrich Schiller University Jena, Ziegelmuehlenweg 1, 07740 Jena, Germany

ARTICLE INFO

Article history:
Received 2 December 2016

Keywords:
Colorectal cancer
Lung cancer
MLH1 expression
MLH1 methylation
Tissue microarrays

ABSTRACT

Aims: Aberrant DNA methylation is a common molecular feature in human cancer. The aims of this study were to analyze the methylation status of MLH1, one of the DNA mismatch repair (MMR) genes, in human colorectal and lung cancer and to evaluate its clinical relevance.
Methods: The expression of MLH1 was analyzed in 8 colorectal cancer (CRC) and 8 lung cancer cell lines by real-time RT-PCR and western blotting. The MLH1 protein expression was evaluated by immunohistochemistry on tissue microarrays including 121 primary CRC and 90 lung cancer patient samples. In cancer cell lines, the methylation status of MLH1 promoter and exon 2 was investigated by bisulfite sequencing (BS). Methylation-specific-PCR (MSP) was used to evaluate methylation status of MLH1.
Results: The expression of MLH1 mRNA was detected in 8 CRC cell lines as well as normal colonic fibroblast cells CCD-33Co. At protein levels, MLH1 was lost in one CRC cell line HCT-116 and normal cells CCD-33Co. No methylation was found in the promoter and exon 2 of MLH1 in CRC cell lines. MLH1 was expressed in 8 lung cancer cell lines at both mRNA and protein levels. Compared to cancer cells, normal bronchial epithelial cells (HBEC) had lower expression of MLH1 protein. In primary CRC, 54.5% of cases exhibited positive staining, while 47.8% of lung tumors were positive for MLH1 protein. MSP analysis showed that 58 out of 92 (63.0%) CRC and 41 out of 73 (56.2%) lung cancer exhibited MLH1 methylation. In CRC, the MLH1 methylation was significantly associated with tumor invasion in veins ($P = 0.012$). However, no significant links were found between MLH1 expression and promoter methylation in both tumor entities.
Conclusions: MLH1 methylation is a frequent molecular event in CRC and lung cancer patients. In CRC, methylation of MLH1 could be linked to vascular invasiveness.

1. Introduction

Lung cancer is the leading cause of cancer-related death among both men and women in the United States [1]. Colorectal cancer (CRC) is the second leading cause when both sexes are combined [2]. Despite the progress in modern treatment modalities including surgical, chemotherapy, radiotherapy and targeted therapy, the presence of metastasis remains the major problem of poor prognosis with an overall 5-year survival rate of 18% for lung cancer and 65% for colorectal cancer [2]. To improve diagnosis, treatment and prevention, better understanding of molecular basis of cancer is therefore largely required.

DNA mismatch repair (MMR) is a system for recognizing and repairing erroneous insertion, deletion, and mis-incorporation of bases that can arise during DNA replication [3]. Human MMR genes mainly including MLH1, MSH2, MSH6, and PMS2 are associated with genetic instability. Microsatellite instability (MSI), genetic instability of microsatellite repeat sequences, can be frequently observed in hereditary nonpolyposis colorectal carcinoma (HNPCC) which represents about 5% of the colon cancer cases and is usually caused by germline mutations in MMR genes [4–6]. In up to 26% of sporadic MSI colorectal cancer, somatic mutations in one of the DNA MMR genes have also been reported [7,8]. In the majority of sporadic colorectal cancer, MSI is closely associated with DNA hypermethylation of the MLH1 gene promoter [9,10].

DNA methylation is the most widely studied epigenetic modification restricted to the DNA motif called CpG dinucleotides, i.e. cytosine followed by guanine residues [11]. DNA hypermethylation was proved to be responsible for gene silencing, promoting the initiation and development of carcinogenesis. Recent studies showed that DNA methylation of MLH1 has been found in more than 80% of sporadic colorectal cancer with MSI [12–14]. Compared to CRC, the methylation status of MLH1 in lung cancer has however not yet

* Corresponding author.
E-mail address: iver.petersen@med.uni-jena.de (I. Petersen).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.prp.2017.01.014
0344-0338/

been well investigated and this issue has been addressed in only a handful research articles. Recently it has been reported that DNA methylation of MLH1 was related to clinical response to cisplatin treatment after surgical resection of non-small cell lung cancer (NSCLC), indicating that MLH1 methylation may have a potential to become a biomarker of individualized therapy for NSCLC patients [15].

Here we analyzed the MLH1 expression in CRC and lung cancer cell lines as well as in primary tumor samples, and evaluated the methylation status of MLH1 promoter region in patients with primary CRC and lung cancer.

2. Materials and methods

2.1. Cell lines and cell culture

Human normal colonic fibroblast cells CCD-33Co and colon cancer cell lines (HT-29, LoVo, Caco-2, WiDr, SW480, HCT-116, and HRT-18) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). CX-2 was obtained as a gift form Dr. Antje Siegert (Institute of Pathology, Charité Berlin). HRT-18, Caco-2, WiDr and CX-2 were grown in RPMI 1640 medium (Biochrom AG, Berlin, Germany) supplemented with 10% (v/v) fetal bovine serum (FBS) (Biochrom AG). LoVo was cultured in DMEM medium (Biochrom AG) including 10% FBS. HT-29, SW480 and HCT-116 were grown in Leibovitz L-15 medium (Biochrom AG) with 10% FBS and maintained in a humidified atmosphere with 5% CO2 at 37 °C.

Human bronchial epithelial cells (HBECs) were obtained from Clonetics (San Diego, CA, USA) and cultured in BEG media (Lonza, Walkersville, USA). Seven non-small cell lung cancer cell lines including H2170, H1299, H226, H157, H2030 and H23 and A549 as well as one small cell lung cancer cell line COLO677 were purchased from the American Type Culture Collection (ATCC, Rockville, USA). Cells were grown in RPMI 1640 medium supplemented with 10% FBS and maintained in a humidified atmosphere with 5% CO2 at 37 °C.

2.2. RNA extraction and real-time RT-PCR

Total RNA was extracted from cells using the Trizol reagent (peqGOLD TriFastTM, VWR, Germany) according to manufacturer's recommendation. One hundred nanograms of total RNA were reverse transcribed into cDNA using a quantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany).

Real-time PCR was carried out in 0.1 ml tubes on the Rotor-Gene 6000 (Qiagen, Hilden, Germany) in the presence of the FastStart Universal SYBR Green Master (Roche, Mannheim, Germany). Twenty-five nanograms of RNA were used for PCR amplification under the following conditions: 95 °C 15 min; 95 °C 15 s and 60 °C 30 for 45 cycles. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control. Primer sequences are shown in supplementary data 1.

2.3. Western blot analysis

Proteins from cell lysate were isolated and the concentrations were determined by the BCA Protein Assay kit as previously described [16]. Twenty-five μg of protein was used for the analysis. Two antibodies including MLH1 (SC-581, Santa Cruz Biotechnology, CA USA) and β-actin (Millipore, Billerica, MA USA) were used. Signals were visualized with horseradish peroxidise-conjugated goat anti-rabbit and rabbit anti-mouse antibody and ECL plus Western blotting Detection System (GE Healthcare, Munich, Germany).

2.4. Patient samples, genomic DNA isolation and bisulfite modification

A total of 211 tumor specimens (121 from CRC and 90 from lung cancer) were included in the study. All of the patients were undergoing surgical operation at the Department of Surgery of the University Hospital Charité from 1995 to 2000. No adjuvant radiotherapy or chemotherapy was administered before surgery. The study was approved by the local ethical committee.

Genomic DNA from patient samples was isolated by using a QIAamp FFPE kit (Qiagen) according to the manufacturer's instructions. Manual microdissection was carried out before DNA extraction. Bisulfite modification of genomic DNA was performed by using an EZ DNA Methylation kit (Zymo Research, Freiburg, Germany).

2.5. Tissue microarray (TMA) construction and immunohistochemistry

A tissue microarray (TMA) was constructed using a manual tissue arrayer purchased from Beecher Instruments (Woodland, WI, USA) as previously described [17].

Immunohistochemistry (IHC) was performed. Briefly, 3 μm sections from the TMAs were dewaxed with xylene and gradually hydrated. Antigen retrieval was performed by treatment in a pressure cooker for 6 min.

The rabbit anti-MLH1 monoclonal antibody (1:200; Abcam, Germany) was incubated at room temperature for 1 h. Detection was carried out according to the manufacturer's instructions (LSAB-2 kits, DAKO, Germany). IHC was scored semi-quantitatively as negative (<10% positively stained cells; score 0), weak (10–25% positively stained cells; score 1), moderate (26–50% positively stained cells; score 2), or strong (more than 50% positively stained cells; score 3). For statistical analysis, scores 0 and 1 together were considered negative and scores 2 and 3 were for positive.

2.6. Bisulfite sequencing and methylation-specific-PCR

Bisulfite-treated genomic DNA was subjected to PCR amplification. Two pairs of primer from promoter region and exon 2 of the MLH1 gene were designed to amplify bisulfite-modified DNA (supplementary data 1). PCR products were purified using a DNA Clean & Concentrator Kit (Zymo Research) and sequenced by capillary electrophoresis (LGC, Berlin, Germany).

Methylation-specific-PCR (MSP) was carried out as described previously [18]. Primer sequences are listed in supplementary data 1. MSP-PCR amplification is: 95 °C 15 min; 94 °C 1 min, 55 °C 30 s, 72 °C 30 s, 35 cycles; 72 °C 10 min. Each of the PCR amplifications was repeated at least once to confirm the results.

2.7. Statistical analysis

To study the correlation between methylation status/protein expression of MLH1 and clinical parameters, two-tailed chi-square test and Fisher's exact test were performed. Kaplan–Meier analysis was carried out for evaluation of clinical outcome. The statistical analysis was conducted using the software package SPSS 21.0 (SPSS, Chicago, USA). P values less than 0.05 were defined as statistically significant.

3. Results

3.1. Expression of MLH1 in CRC and lung cancer cell lines

We performed real-time RT-PCR and western blotting to analyze the mRNA and protein expression of MLH1 in CRC and lung cancer