

Biallelic Inactivation of hMLH1 by Hypermethylation and Loss of Heterozygosity in Non-Small Cell Lung Cancer

Xin Geng
Dong Wang
Guoping Zhu
Liang Zhang
Weiming Zhang

Basic Medical Research Center of Tianjin Medical University, Tianjin 300070, China.

Correspondence to: Weiming Zhang
Tel: 86-22-23542775
E-mail: gengxin111@126.com

OBJECTIVE To investigate the mechanism of hMLH1 deregulation in non-small cell lung cancer (NSCLC).

METHODS A genetic and epigenetic study of the hMLH1 gene was performed using surgical primary tumors from 40 NSCLC patients and their corresponding noncancerous tissues. The molecular alterations examined included promoter methylation by Hpa II/Msp I- based PCR analysis, loss of heterozygosity (LOH) by D3S1621 locus PCR-electrophoresis-silver staining, as well as the loss of protein expression by immunohistochemical analysis.

RESULTS The frequencies of hypermethylation, LOH and loss of protein expression of hMLH1 were 67.5% (27/40), 65% (26/40) and 72.5% (29/40), respectively. Among 26 hMLH1 gene LOH (+) cases, 21 (80.8%) showed hypermethylation, which was significantly higher than the group of LOH (-). The frequency of the double inactivation of the hMLH1 gene by hypermethylation and LOH related to a loss of protein expression of 72.4% (21/29).

CONCLUSION Biallelic inactivation of the hMLH1 gene by hypermethylation and LOH most likely will cause loss of hMLH1 protein expression and play an important role in the development of NSCLC. Therefore, controlling and monitoring for hypermethylation of the hMLH1 gene may be partially useful for treatment and early diagnosis of NSCLC.

KEYWORDS: hMLH1, NSCLC, lung cancer, suppressor gene.

Lung cancer is one of the most common malignancies in the world and is the leading cause of cancer mortality in China. Lung cancer is divided into two major histological categories, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC).^[1] In China, more than 80% of the lung cancers are NSCLC. Lung squamous cell carcinoma and adenocarcinoma are the two major types of NSCLC.^[2]

Hypermethylation of cytosines in CpG-rich islands of the promoter regions of genes is one of the mechanisms of gene silencing.^[3] In cancer, hypermethylation of the promoter regions is associated with transcriptional inactivation and loss of expression of tumor suppressor and other regulatory genes, constituting an alternative, epigenetic way for the loss of gene function.^[4] The hMLH1 gene, one of six human mismatch repair (MMR) genes, is a tumor-suppressor gene that is located in the 3p21.3-22 region. To investigate the mechanism of hMLH1 deregulation in NSCLC, in this research, we performed a genetic and epigenetic study of the hMLH1 gene in resected primary tumors from 40 NSCLC patients and corresponding noncancerous tissues. The molecular alterations examined included promoter hypermethylation, loss of heterozygosity (LOH) as well as immunohisto-

Received April 23, 2006; accepted May 26, 2006.

CJCO <http://www.cjco.cn> E-mail: cocr@eyou.com
Tel (Fax): 86-22-23522919

chemical analysis.

MATERIALS AND METHODS

Samples

The primary tumor samples and their corresponding noncancerous tissues were obtained from 40 Chinese patients who underwent surgery for NSCLC (31 male and 9 female; median age, 61 years, range, 42~70 years). Twenty-eight of the tumors were squamous cell carcinomas and 12 were adenocarcinomas. Eighteen were Stages I+II and 22 were Stages III+IV, according to the Tumor-Node-Metastasis classification.

Genomic DNA extracted

Genomic DNA was extracted from frozen samples of the lung tumors and normal lung tissues using proteinase-K digestion and phenol/chloroform purification followed by ethanol precipitation.

Aberrant methylation analysis of the hMLH1 gene

The promoter methylation status of the hMLH1 gene was investigated using Hpa II/Msp I-based PCR analysis. Hpa II is a methylation-sensitive enzyme and Msp I is a methylation-insensitive enzyme. If the promoter region can be amplified after Hpa II digestion and cannot be amplified after Msp I digestion, it was defined as aberrant methylation. Primers of the hMLH1 gene promoter region: 5'-CGC TGC TAG TAT TCG TGC-3' (sense), 5'-TCA GTG CCT CGT GCT CAC-3' (antisense), 603 bp. PCR was performed using the following conditions: 50 ng of DNA template, 10 pmol of each primer, 2.5 mM MgCl₂, 1.5 mM dNTP mix, 1× PCR buffer, and 1 unit of Ampli Taq in a 20-μl final volume. PCR cycles included one cycle of 95°C for 10 min followed by 35 cycles at 95°C for 30 s, 55°C for 45 s, and 72°C for 30 s, 72°C 10 min, in a Perkin-Elmer Gene Amp PCR system 9700.

LOH analysis of the hMLH1 gene

The microsatellite polymorphic marker D3S1621 links together closely with the hMLH1 locus. Cases were defined as LOH when an allele peak signal from tumor DNA was reduced by 50% compared with the normal counterpart. The methodology has been described by Tamotsu K et al.^[5] Primers of D3S1621: 5'-TCT TTT AGT CAG CAG TTA TGT C-3' (sense), 5'-CCC ATA AGA AAT GTT ACT CTA C-3' (antisense), 210 bp. PCR products were loaded on a 7% denaturing gel and then silver stained. PCR was performed on the genomic DNA samples using the following conditions: 200

ng of genomic DNA template, 10 pmol of each primer, 2.5 mM MgCl₂, 1.5 mM dNTP mix, 1×PCR buffer, and 1 unit of Ampli Taq in a 20-μl final volume. PCR cycles included one cycle of 95°C for 10 min followed by 35 cycles at 95°C for 30 s, 55°C for 45 s, and 72°C for 30 s, 72°C 10 min, in a Perkin-Elmer Gene Amp PCR system 9700.

Immunohistochemistry assay of hMLH1 protein expression

The normal staining patterns for hMLH1 are nuclear. Tumor cells that exhibited an absence of nuclear staining in the presence of non-neoplastic cells and infiltrating lymphocytes with nuclear staining were considered to have an abnormal pattern.

Statistical analysis

The statistical analysis was performed with the Student's *t*-test and Chi-square test. *P*<0.05 was regarded as statistically significant.

RESULTS

Aberrant methylation frequency of hMLH1 in NSCLC

Among the 40 cases of NSCLC, there were 27 cases (67.5%) which demonstrated aberrant methylation of the hMLH1 gene promoter region (Fig.1, Table 1). The difference compared to the control group was significant (*P*<0.01).

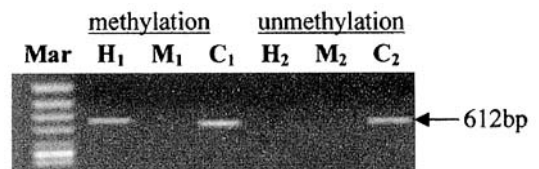


Fig.1. Aberrant methylation of the hMLH1 gene promoter region. H: Hpa II digestion, M: Msp I digestion, C: control, Mar: Marker (ϕX 174-Hinc II). 1: hyper-methylation, 2: un-methylation.

Table 1. Aberrant methylation frequency of hMLH1 in NSCLC

Group	n	Methylation (+)	Frequency (%)	<i>P</i> value
NSCLC	40	27	67.5	<0.01
Control	40	0	0	

LOH frequency in the hMLH1 gene in NSCLC

Of the 40 cases of NSCLC, there were 26 cases (65%) showing LOH in the hMLH1 gene (Fig.2, Table 2). In

Fig.2, the upper band stands for one allele and the lower band stands for the other allele. Cases were defined as LOH when an allele peak signal from tumor DNA was reduced by 50% compared with the normal counterpart. The difference compared to the control group was significant ($P<0.01$).

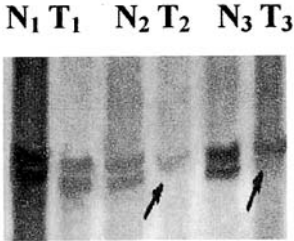


Fig. 2. LOH in the hMLH1 of the D3S1621 locus. N:normal, T:tumor.

Table 2. LOH frequency of the hMLH1 gene in normal tissue and NSCLC

Group	n	LOH (+)	Frequency (%)	P value
NSCLC	40	26	65	<0.01
Control	40	0	0	

The relationship between LOH and methylation of hMLH1 in NSCLC

In 26 of the hMLH1 gene LOH (+) cases, 21 cases (80.8%) had hypermethylation of promoter regions, which was significantly higher than the group of LOH (-) ($P<0.05$, Table 3). This result suggested that aberrant methylation was related to LOH of hMLH1.

Table 3. Correlation between methylation of hMLH1 and LOH in NSCLC

LOH	Cases	Methylation (+)	Frequency (%)	P value
LOH (+)	26	21	80.8	<0.05
LOH (-)	14	6	42.9	

Protein expression of hMLH1 in NSCLC

The hMLH1 protein expression in the control group was 100% (40/40). Among the 40 cases of NSCLC, 29 (72.5%) demonstrated loss of hMLH1 expression. The difference with the control group was significant ($P<0.05$, Table 4). Of these 29 cases, there were 20 cases (69%) of squamous cell carcinoma and 9 cases (31%) of adenocarcinoma.

Table 4. Loss of hMLH1 expression in NSCLC

Group	n	Loss of expression	Frequency (%)	P value
NSCLC	40	29	72.5	<0.05
Control	40	0	0	

Upon statistical analysis, in 27 hMLH1 gene aberrant methylation cases, 26 cases (96.3%) had loss of protein expression of hMLH1, which was significantly higher than the un-methylated group ($P<0.05$). This result indicated that aberrant methylation was related to loss of protein expression of hMLH1. In 26 hMLH1 gene-LOH (+) cases, 23 cases (88.5%) had loss of protein expression of hMLH1, which was significantly higher than the group of LOH (-) ($P<0.05$). These results also suggested that LOH was related to loss of protein expression of the hMLH1 gene.

Among the 29 cases showing loss of hMLH1 protein expression, 21 cases (72.4%) displayed LOH and aberrant methylation. On the other hand, of the 11 cases showing positive hMLH1 expression, 7 cases (63.6%) had neither LOH nor aberrant methylation. These results suggested that aberrant methylation combined with LOH was related to loss of hMLH1 protein expression.

DISCUSSION

The development of human cancer is generally thought to be a multiple process that involves multiple genetic and epigenetic changes. Recently, several studies have shown that methylation of CpG islands located within the promoter regions of tumor suppressor genes is a frequent event in the development of several human malignancies.^[6] This epigenetic modification has been proposed to be an alternative way of inactivation of tumor suppressor genes in cancer.^[7] Since gene promoter hypermethylation and chromatin structure alteration are comparable with genetic mutations or deletions of tumor suppressor genes in cancer, it is very urgent to find some target genes that are inactivated by hypermethylation. In fact, there are many examples of CpG-rich islands methylation-mediated transcriptional silencing of tumor suppressor genes, such as p15, p16, APC, Rb, hMLH1, E-cad etc. In our study, we have found that hMLH1 is a target gene for hypermethylation in NSCLC. This aberrant methylation can cause cancer by inactivation of the hMLH1 gene and lead to a series of molecular events.

The hMLH1 gene, one of the six human mismatch repair (MMR) genes, is a tumor-suppressor gene that is located in the 3p21.3-22 region. MMR can correct base mismatches during the DNA replication progress, eliminating the unpaired base sequence.^[8,9] Inactivation of the hMLH1 gene leading to loss of protein expression plays an important role in NSCLC carcinogenesis. Chromosome 3p allelic losses are frequent events in

many types of cancers including lung cancer. LOH at certain chromosomal loci accumulates during tumor progression.^[10] Previous studies have shown that chromosomal 3p21 regions were found to be hemizyously deleted in 55% of the patients^[11] and that LOH of hMLH1 plays an important role in NSCLC carcinogenesis. At the present time, the mechanism of hMLH1 gene transcriptional inactivation in NSCLC is still unclear.

In the present study, the hypermethylation in the promoter region and loss of heterozygosity (LOH) of the hMLH1 gene were frequent (65% and 67.5%, respectively) in NSCLC. We found that 72.4% (21/29) of the cases with negative hMLH1 protein expression showed hypermethylation and LOH at the same time. Our results suggested that hMLH1 may be inactivated in NSCLC in accordance with the Knudson's two-hit inactivation model,^[12] involving deletion of one allele and hypermethylation of the other. Previous work has shown that LOH at the hMLH1 gene region was observed frequently in NSCLC. The present study demonstrated that hMLH1 hypermethylation significantly correlated with LOH at the hMLH1 region in NSCLC. These results suggest that hMLH1 gene alterations may play a role in the NSCLC carcinogenesis via a two-hit mechanism, including epigenetic changes for tumor suppressor-gene inactivation. Biallelic inactivation of the hMLH1 gene by hypermethylation and LOH most likely will cause loss of hMLH1 expression and play an important role in the development of NSCLC. Therefore, controlling and monitoring for hypermethylation of the hMLH1 gene may be useful for treatment and early diagnosis of NSCLC.

REFERENCES

1 Park SH, Lee GY, Jeon HS, et al. A polymorphism of

hMLH1 and risk of primary lung cancer. *Int J Cancer* 2004;112:678-682.

- 2 Chan EC, Lam SY, Tsang KW, et al. Aberrant promoter methylation in Chinese patients with non-small cell lung cancer: patterns in primary tumors and potential diagnostic application in bronchoalveolar lavage. *Clin Cancer Res* 2002;8:3741-3746.
- 3 Wilson IM, Davies JJ, Weber M, et al. Epigenomics: mapping the methylome. *Cell Cycle* 2006;5:155-158.
- 4 Teodoridis JM, Strathdee G, Plumb JA, et al. CpG-island methylation and epigenetic control of resistance to chemotherapy. *Biochem Soc Trans* 2004; 32 :916-917.
- 5 Safar AM, Spencer H, Su X, et al. Methylation profiling of archived non-small cell lung cancer: a promising prognostic system. *Clin Cancer Res* 2005;11:4400-4005.
- 6 Tamotsu K, Francesco T, Sai Y, et al. Allele loss and promoter hypermethylation of VHL, RAR- β , RASSF1A, and FHIT tumor suppressor genes on chromosome 3p in esophageal squamous. *Cancer Res* 2003;63:3724-3728.
- 7 Okuda T, Kawakami K, Ishiguro K, et al. The profile of hMLH1 methylation and microsatellite instability in colorectal and non-small cell lung cancer. *Int J Mol Med* 2005;15:85-90.
- 8 Ellenson LH. hMLH1 promoter hypermethylation on microsatellite instability-positive endometrial carcinoma. Cause or consequence? *Am J Pathol* 1999;155:1399-1402.
- 9 Hansen LT, Thykjaer T, Orntoft TF, et al. The role of mismatch repair in small-cell lung cancer cells. *Eur J Cancer* 2003;39:1456-1467.
- 10 Martinez R, Schackert HK, Appelt H, et al. Low-level microsatellite instability phenotype in sporadic glioblastoma multiforme. *J Cancer Res Clin Oncol* 2005;131:87-93.
- 11 Benachenhou N, Guiral S, Gorska-Flipot I, et al. High resolution deletion mapping reveals frequent allelic losses at the DNA mismatch repair loci hMLH1 and hMSH3 in non-small cell lung cancer. *Int J Cancer* 1998;77:173-180.
- 12 Knudson AG. Hereditary cancer: two hits revisited. *J Cancer Res Clin Oncol* 1996;122:135-140.