Advances in Brief

DETECTION OF CPG METHYLATIONS IN HUMAN MISMATCH REPAIR GENE HMLH1 PROMOTER BY DENATURING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (DHPLC)

DENG Da-jun 邓大君, DENG Guo-ren 邓国仁, ZHOU Jing 周静, XIN Hui-jun 辛慧君
Peking University School of Oncology and Beijing Institute for Cancer Research, Beijing, 100034, China, dengdajun@sina.com

ABSTRACT

Objectives: To develop a novel method to detect CpG methylation by DHPLC. Methods: After DNA was treated with sodium bisulfite, mismatch repair gene hMLH1 promoter was amplified by polymerase chain reaction (PCR). DHPLC was used to separate the PCR products at their partially denaturing temperatures. BstUI digestion assay was also used for comparison study. Results: A 294bp band was obtained by PCR after each DNA samples of colon cancer cell line RKO and gastric cancer cell line PACM82. These two bands could be separated completely by DHPLC at 53°C (retention time 6.7 min for RKO vs. 6.2 min for PACM82). We concluded that the hMLH1 promoter in RKO cells is methylated, while PACM82 is not methylated, since methylation can protect the conversion of C to T and keep higher C/G content after bisulfite treatment, leading to the delayed time. These results consistent with those from BstUI digestion assay. Conclusion: Methylation in CpG islands of hMLH1 could be detected conveniently by DHPLC after bisulfite modification.

Key words: hMLH1,CpG islands, Methylation, DHPLC

Methylation of these CpG islands is associated with silencing of gene transcription and imprinting of genes. Detection of CpG methylation is important for understanding the expression status of target genes. The current major approaches to detect methylation have many defects. We try to detect the methylation with denaturing high-performance liquid chromatography (DHPLC).

MATERIALS AND METHODS

Cell Lines and DNA

Colorectal carcinoma cell line RKO with the silenced hMLH1 was kindly provided by University of California San Francisco. RKO and gastric carcinoma cell line PACM82 were cultured in DMEM medium (Gibco) containing 10% FBS at 37°C with 5% CO_2. Genomic DNA of these cells and one surgical specimen of gastric carcinoma were isolated with phenol/chloroform and modified by sodium bisulfite as described [1,2].

Primers and PCR

Two pairs of primers were designed according to the sequences of the hMLH1 promoter. Upstream and downstream primers in each pair were from the sequences of –318 (1 was indicated as translate start site) to -293 and –48 to –25, respectively. One pair of primers was used to amplify the bisulfite modified hMLH1 (PCR for treated template, tPCR). The sequences of the primer pair are: t-sense, 5'gtatttttgtttttattggttggata; t-antisense, 5'aataccttcaaccaatcac-ctcaata. Another pair was used to amplify the unmodified samples (PCR for wild template, wPCR). Their sequences are: w-sense, 5'gcatctctgctcctattggctggata and w-antisense, 5'agtgccttcagccaatcacctcagtg. Hot-started temperature-decreasing PCR was used (from 65°C to 45°C for tPCR and from 75°C to 58°C for wPCR, -1.0°C per cycle, 35 cycles).

BstUI Digestion Assay for Methylation

1μg PCR of products was digested with 5U of BstUI (New England Biolabs) in 30 μl of total volume at 60°C for 3h. 2.5% agarose gel was used to separate the digested fragments.

Analysis for Methylation by DHPLC

DHPLC was performed with WAVE™ DNA Fragment Analysis System (Transgenomic, Inc.) to detect point mutation in wPCR products and methylation in tPCR products. Chroma-togramphic peaks were detected by UV-detector at 260 nm.

RESULTS AND DISCUSSIONS

A single 294bp band of the hMLH1promoter was observed in both wPCR and tPCR products of templates from RKO and PACM82 cell lines and the tissue sample of gastric cancer. That means the quality of two kinds of PCR products meets the requirement for DHPLC analysis.

Two fragments (88bp and 206bp) were obtained from the 294 bp wPCR products of both the cell lines in the BstUI digestion assay. This agreed with that the hMLH1promoter has a BstUI cleaving site CGCG. It was reported that the methylated

(Continued on the page 191)

by protein-protein interactions. Science 1991; 52:842.

[52] Doria M, Klein N, Lucito R, et al. The hepatitis B virus HBx protein is a dual specificity cytoplasmic activator of ras and nuclear activator of transcription factors. EMBO J 1995; 14:474.

[53] Cross JC, Wen P, Rutter Wi. Transactivation by hepatitis B virus X protein is promiscuous and dependent on mitogenactivated cellular serine/threonine kinases. Proc NatI Acad Sci USA 1993; 90:8078.

[54] Benn J, Schneider RJ. Hepatitis B virus HBx protein activates *ras*-GTP complex formation and establishes a *ras, raf,* MAP kinase signaling cascade. Proc Natl Acad Sci USA 1994; 91:10350.

[55] Su F, Schneider RJ. Hepatitis B virus HBx protein activates transcription factor NF-kB by acting on multiple cytoplasmic inhibitors of *rel*-related proteins. J Virol 1996; 70:4558.

[56] Kim CM, Koike K, Saito I, et al. HBx gene of hepatitis B virus induces liver cancer in transgenic mice. Nature 1990; 351:317.

and other DNA-damaging agents. Blood 1996; Sep 15; 88:1936.

[8] Kaufmann SH, Desnoyers S. Specific proteolytic cleavage of poly (ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. Cancer Res 1993; 53:3976.

[9] Kasibhatla S, Brunner T. DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-kappa B and AP-1. Mol Cell 1998; 1:543.

(Connected to the page 171)

cytidines were not transformed to uridines and the unmethylated cytidines were transformed after sodium bisulfite modification.[3] Therefore, the tPCR product of the methylated hMLH1 promoter must be sensitive to BstUI cleaving and that of the unmethylated one must be resistant to. We observed that the tPCR product from RKO was sensitive to BstUI cleaving, but the tPCR product from PACM82 was resistant. These results indicated that the hMLH1promoter of RKO cells was methylated as previously reported [4] and that of PACM82 cells was unmethylated.

Only one strong peak at the retention time 5.3 min could be detected in all the denatured testing wPCR products and the mixture of PACM82 and RKO cells by DHPLC at their partial denaturing temperature 62°C. The phenomenon indicates that all the testing wPCR products have the same sequence. The hMLH1 promoter of RKO cell is wild-type.[5] Therefore, the hMLH1 promoter of the testing gastric carcinoma specimen and PACM82 cell line must be wild-type.

The tPCR products of PACM82 and RKO cells were separated at 53°C completely (retention time, 6.2 min and 6.7 min, respectively). Two peaks were observed on the chromatogram of the denatured tPCR mixture of these cell lines (Figure 1A). The four-peaks typical mutation chromatogram was also obtained from the tPCR products of the testing gastric carcinoma specimen (Figure 1B). This result suggests that the tumor tissue contains both the methylated and the unmethylated hMLH1.

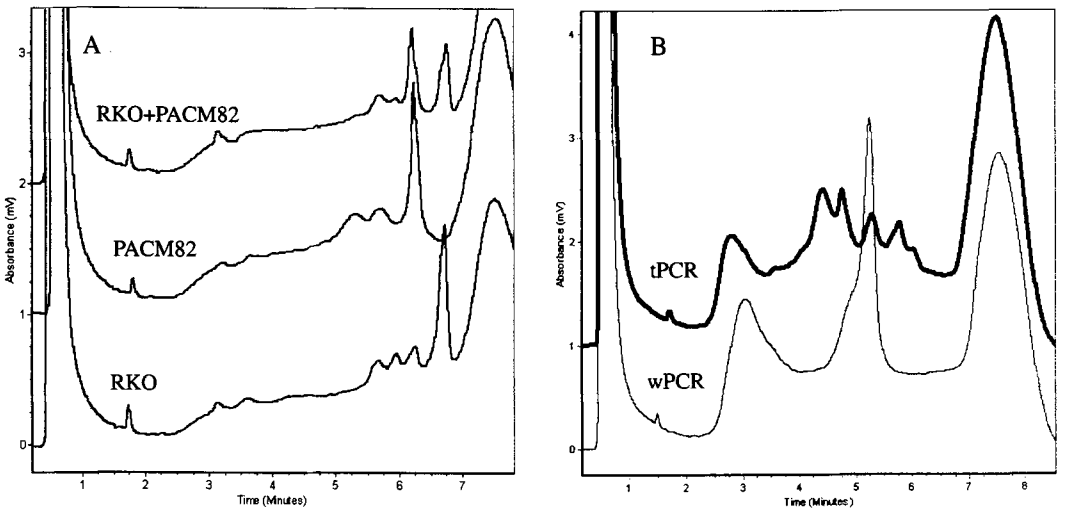

Fig. 1. DHPLC chromatograms of denatured PCR products of the hMLH1 promoter. HMLH1 promoters were amplified by tPCR or wPCR after bisulfite modification or without modification, respectively. The PCR products were partial denatured at 53°C for tPCR or 62°C for wPCR, and separated by DHPLC. A: tPCR products of RKO cells, PACM82 cells, and their tPCR mixture; B: tPCR and wPCR products of gastric carcinoma specimen

In DHPLC analysis, the PCR-amplified region contains several CpG sites, and methylation at any CpG site will lead to the delay of the PCR product. Thus, this analysis can analyze methylation at multiple CpG sites in the promoter region, showing the advantages over the previously existent methods for methylation analysis, such as methylation sensitive enzyme digestion, methylation specific PCR (MSP), etc. In conclusion, the methylated and the unmethylated tPCR products of the hMLH1 promoter could be separated by DHPLC conveniently.

REFERENCES

[1] Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning, A Laboratory Manual. 2nd ed. Beijing: Science Press, 1992; 954.

[2] Herman JG, Graff JR, Myöhänen S, et al. Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996; 93:9821.

[3] Wang RYH, Gehrke CW, Ehrlich M. Comparison of bisulfite modification of 5-methyldeoxycytidine and deoxycytidine residues. *Nucleic Acids Res* 1980; 8:4777.

[4] Deng G, Chen A, Hong J, et al. Methylation of CpG in a small region of the hMLH1 promoter invariably correlates with the absence of gene expression. *Cancer Res* 1999; 59:2029.

[5] Veigl ML, Kasturi L, Olechnowicz J, et al. Biallelic inactivation of hMLH1 by epigenetic gene silencing, a novel mechanism causing human MSI cancer. *Proc Natl Acad Sci USA* 1998; 95:8698.

(Received September 15, 2000; accepted September 30, 2000)