Available online at www.sciencedirect.com
SCIENCE DIRECT
Cancer Letters 241 (2006) 213–220
CANCER Letters
www.elsevier.com/locate/canlet

Methylation and expression analysis of 15 genes and three normally-methylated genes in 13 Ovarian cancer cell lines

Masayoshi Imura [a,b], Satoshi Yamashita [a], Li-yi Cai [a], Jun-ichi Furuta [a], Mika Wakabayashi [a], Toshiharu Yasugi [b], Toshikazu Ushijima [a,*]

[a] *Carcinogenesis Division, National Cancer Center Research Institute, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan*
[b] *Department of Obstetrics and Gynecology, Graduate School of Medicine, The University of Tokyo, 3-1 Hongo 7-chome, Bunkyo-ku, Tokyo 113-0033, Japan*

Received 18 August 2005; received in revised form 11 October 2005; accepted 13 October 2005

Abstract

Aberrant methylation of CpG islands (CGIs) in promoter regions of tumor-suppressor genes causes their silencing, and aberrant demethylation of normally methylated CGIs in promoter regions causes aberrant expression of cancer-testis antigens. Here, we comprehensively analyzed aberrant methylation of 15 genes and demethylation of three normally methylated genes in 13 ovarian cancer cell lines. *RASSF1A* was most frequently methylated (complete methylation in 7 and partial methylation in 4 cell lines), followed by *ESR1* (5 and 2, respectively), *FLNC* (4 and 4), *HAND1* (4 and 2), *LOX* (3 and 2), *HRASLS* (3 and 2), *MGMT* (3 and 0), *CDKN2A* (3 and 0), *THBD* (2 and 1), *hMLH1* (2 and 0), *CDH1* (1 and 1) and *GSTP1* (1 and 0). *hTERC* and *TIMP3* were only partially methylated in 7 and 2 cell lines, respectively. *BRCA1* was not methylated at all. Aberrant demethylation of *MAGE-A3*, *-B2* and *-A1* was detected in 8, 4 and 3 cell lines, respectively. Gene expression was consistently absent in cell lines without unmethylated DNA molecules. Aberrant methylation was frequently observed in MCAS, RMUG-L (mucinous cell carcinomas), RTSG (poorly-differentiated carcinoma) and TYK-nu (undifferentiated carcinoma) while infrequent in HTOA, JHOS-2, and OV-90 (serous cell carcinomas). Aberrant demethylation was frequently observed in OV-90, OVK-18, and ES-2 cell lines. It was shown that aberrant methylation and demethylation were frequently observed in ovarian cancer cell lines, and these data will provide a basis for further epigenetic analysis in ovarian cancers.

Keywords: Ovarian cancers; DNA methylation; Epigenetics; Demethylation; Gene expression

1. Introduction

Epigenetic alterations are deeply involved in carcinogenesis [1,2]. Methylation of a CpG island (CGI) in a promoter region leads to repression of its downstream gene, and when this happens to a tumor-suppressor gene, it is involved in its inactivation [1,2]. Methylation of CGIs in promoter regions has been expected to be potentially reversible, and a successful study showing that a demethylating agent, 5-aza-2′deoxycytidine, was effective in some hematological malignancies was recently reported [3]. In addition to methylation of CGIs that are kept unmethylated in normal cells, some CGIs are known to be normally methylated and to be aberrantly demethylated in cancer cells [4,5]. Some of these genes, such as melanoma-associated genes (*MAGE*s), are cancer-testis antigens, and are expected to be targets of cancer immunotherapy [6].

* Corresponding author. Tel.: +81 3 3547 5240; fax: +81 3 5565 1753.
E-mail address: tushijim@ncc.go.jp (T. Ushijima).

0304-3835/$ - see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2005.10.010

Aberrant methylation in ovarian cancers, one of the most common gynecological malignancies [7], include methylation of *BRCA1* (8–15%) [8–13], *ESR1* (3 of 7 cell lines) [14], *hMLH1* (48%) [15], *CDKN2A* (*p16*) (0–40%) [16–19], and *RASSF1A* (10–40%) [20,21]. Although it is important to analyze multiple genes to have an overall picture of epigenetic alterations, there are only limited reports on such analysis in ovarian cancers [22,23]. Moreover, there are no reports on aberrant demethylation of normally methylated CGIs in ovarian cancers, although severe hypomethylation of repeat sequences is reported to be associated with a poor prognosis [24].

In this study, we analyzed methylation and demethylation profiles of promoter CGIs in 13 ovarian cancer cell lines. Methylation was analyzed for 15 genes, including five genes, *BRCA1*, *ESR1*, *hMLH1*, *CDKN2A*, and *RASSF1A*, known to be methylated in ovarian cancers and 10 genes, *CDH1* [25], *GSTP1* [26], *MGMT* [27], *hTERC* [28], *TIMP3* [29], *LOX*, *HRASLS*, *FLNC*, *HAND1*, and *THBD* [30], known to be frequently methylated in other cancers. Demethylation was analyzed for three normally methylated genes, *MAGE-A1*, *MAGE-A3*, and *MAGE-B2* [4,5]. Further, association between methylation and loss of gene expression was confirmed for all the genes.

2. Materials and methods

2.1. Cell lines

HTOA and JHOS-2 were obtained through the RIKEN Cell Bank (Ibaraki, Japan) by the courtesy of their originators, Drs I. Ishiwata and H. Ishikawa. OV-90, TOV-112D, ES-2 and TOV-21G were purchased from the American Type Culture Collection (Manassas, VA). MCAS, RMUG-L, RMG-I, RTSG, TYK-nu and KURAMOCHI were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan). OVK-18 was obtained through the Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan). HOSE6-3 was established by immortalizing normal ovarian epithelial cells with HPV-E6 and E7 [31,32].

2.2. Methylation-specific PCR (MSP)

One microgram of genomic DNA, digested with *Bam*HI, underwent sodium bisulfite modification [33], and was suspended in 20 μl of TE. One microliter of a solution containing bisulfite-modified DNA was used for PCR with primers specific to the methylated (M) or unmethylated (U) sequence. An annealing temperature for each set of primers was determined using the DNA from HOSE6-3 and DNA that had been methylated with *Sss*I methylase. A minimum number of PCR cycles to yield a visible band for a positive control was determined, and four cycles were added for test samples. The primer sequences and PCR conditions are listed in Supplementary Table 1.

2.3. Quantitative RT-PCR

Total RNA was extracted by ISOGEN® (Nippon Gene, Tokyo, Japan), and treated with DNase I (Ambion, Austin, TX). cDNA was synthesized from 5 μg of total RNA using a SuperScript II reverse transcriptase (Invitrogen, Groningen, The Netherlands) and oligo(dT)$_{12\text{-}18}$ primer (Invitrogen). Quantitative PCR was performed using SYBR Green PCR Core Reagents (Applied Biosystems, Foster City, CA) and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The number of cDNA molecules in a sample was measured by comparing its amplification with the amplification of standard samples that contained 10^1–10^7 molecules. The number of cDNA molecules was normalized to that of *GAPDH*. The primer sequences and PCR conditions are listed in Supplementary Table 2. The absence of non-specific amplification was confirmed by analyzing the melting temperature of PCR products, and their agarose gel electrophoreses.

2.4. Clustering analysis

For clustering analysis, M, M/U, and U were converted into 2, 1, and 0, respectively. Average-linkage hierarchical clustering of an uncentered Pearson correlation similarity matrix was applied with the program Cluster, and the figures were generated with the TreeView program [34]. Similarities of methylation profiles among ovarian cancer cell lines were assessed by the Pearson correlation coefficient.

3. Results

3.1. Aberrant methylation of 15 genes and demethylation of 3 genes

Methylation statuses were analyzed by MSP in 13 ovarian cancer cell lines and a control cell line, immortalized ovarian surface epithelial cells (HOSE6-3) (Fig. 1). Aberrant methylation was most frequently observed for *RASSF1A* (complete methylation in 7 and partial methylation in 4 cell lines), followed by *ESR1* (5 and 2, respectively), *FLNC* (4 and 4), *HAND1* (4 and 2), *LOX* (3 and 2), *HRASLS* (3 and 2), *MGMT* (3 and 0), *CDKN2A* (3 and 0), *THBD* (2 and 1), *hMLH1* (2 and 0), *CDH1* (1 and 1) and *GSTP1* (1 and 0). Only partial methylation was observed for *hTERC* and *TIMP3* in 7 and 2 cell lines, respectively. *BRCA1* was not methylated in any of the cell lines analyzed. As for the three normally methylated genes, demethylation of *MAGE-A1*, *MAGE-*