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ORIGINAL ARTICLE

EGFL7 and *RASSF1* promoter hypermethylation in epithelial ovarian cancer

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Abstract

DNA methylation is one of the epigenetic mechanisms associated with gene expression and plays a key role as in activation and deactivation of oncogenes and tumor suppressor genes, respectively. This study employed DNA methylation array to identify methylated genes which are highly correlated with various phenotypes of epithelial ovarian cancer (EOC) in Thai patients and to quantify promoter CpG-island methylation of candidate genes. Tissues from patients with serous and non-serous EOC showed significantly higher promoter methylation of *EGFL7* and *RASSF1* compared to benign cases. These results indicate the potential of investigating promoter CpG-island methylation of cancer-associated genes as biomarkers of disease progression and even possibly of early detection.

Keywords Bisulfite pyrosequencing, DNA methylation microarray, Epithelial ovarian cancer, Promoter hypermethylation.

Introduction

Ovarian cancer is the deadliest gynecologic cancer in females worldwide [1]. This is because initiation and progression of the disease present nonspecific symptoms making affected females unaware until diagnosed, at which the cancer is at an advanced stage (stage III or IV) and difficult to treat [2]. Currently, there is no specific screening test for early diagnosis in patients with ovarian cancer, and risk factors remain unclear although genetics is believed to have a strong influence [3].

DNA methylation comprises the best-known epigenetic mechanism, which can occur through inheritance and/or environmental exposure, making chromosomal DNA methylation profile unique for every individual [4]. Methylation occurs at C-5 position of cytosine in the so-called CpG island [5]. DNA methylation of various genes is specific to each tissue and cell type. In normal tissue, CpG islands are usually not methylated regardless of the expression status of the gene [6]. Hypermethylation is associated with deactivation of tumor suppressor genes, whereas hypomethylation with activation of oncogenes [7]. Both types of DNA methylation affect ovarian cancer development particularly at the early stage (stage I or II) [8]. No methylation is detected in normal ovary tissues, while in epithelial ovarian cancer (EOC) tissues, promoter methylation levels of a growing number of genes are hypermethylated [9,10].

In this study, DNA methylation microarray was used to identify changes in global DNA methylation of ovarian cancer specimens with subsequent confirmation in genes of interest using bisulfite pyrosequencing. The information can be used as biomarkers for histology-specific and prognosis of ovarian cancer, and to facilitate appropriate treatment regimen to achieve optimal clinical outcome.

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Materials and methods

Study group

Biopsies from 26 ovarian tumors were collected after surgical removal at the presentation before chemotherapy. Tumor tissues were obtained from patients with informed consent, which was approved by the Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine Ramathibodi Hospital, Mahidol University, based on the Declaration of Helsinki (ID 06-59-59). All samples were stored frozen at −80 °C. Data from medical records of the patients were used to retrieve demographic data, International Federation of Gynecology and Obstetrics (FIGO) staging and chemotherapy treatment. Pathology reports, including histologic subtype and grade, were obtained where possible.

DNA methylation microarray assay

DNA from fresh ovarian tissue samples was extracted using QIAamp DNA Micro Kit (QIAGEN, Germany). DNA quality ($A_{260/280\ nm}$ ratio) and quantity were determined using NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). DNA methylation profiles were obtained employing Agilent's Human CpG Island Microarray 1 × 244 K (Agilent, USA). Genomic DNA was sheared into short fragments (200–1,000 bp) and added to antibody-coated magnetic beads. Then, cyanine 3-dUTP and cyanine 5-dUTP were added and the suspension was incubated at 67 °C for 40 hours with shaking. Following washing with stabilization solution and drying, beads were scanned and analyzed using Agilent Feature Extraction 12.0 and Agilent Genomic Workbench 7.0 Methylation (CH3) analysis (Agilent Microarray Analysis of Methylated DNA Immunoprecipitation Protocol, version 2.3.1). The log ratio Cy5 over Cy3 at each probe was used as a measurement.

Bisulfite pyrosequencing

Genomic DNA (250 ng) was treated with bisulfite using EpiTect Fast Bisulfite Conversion Kit (QIAGEN, Germany) and 50 ng of DNA were subjected to PCR in a total volume of 25 µL using the PyroMark PCR Master Mix (PyroMark PCR Kit, QIAGEN, Germany) containing primer pairs *EGFL7* _FP 5′-TTTGTTGTTAGTAGTGGGGTATGT-3′/*EGFL7*_RPB 5′-Biotin-CCTCCTAAAACCCCACCACACACTCA-3′ and *RASSF1*_FP 5′-GAAGAGGTAAGTTTGGGAAGATG-3′/*RASSF1*_RPB 5′-Biotin-ACCACAAATAAACAAAAATAACTACTTATA-3′. Thermocycling was conducted in Applied Biosystems ProFlex 3 × 32-well PCR System (Thermo Fisher Scientific) as follows: 45 cycles of 94 °C for 30 s, 60 °C for *EGFL7* or 56 °C for *RASSF1* for 30 s, and 72 °C for 30 s. Amplicons (311 and 141 bp for *EGFL7* and *RASSF1,* respectively) were analyzed by 2% agarose gel-electrophoresis and staining with GelRed Nucleic Acid Gel Stain (Biotium, California, USA). Then 20 µL aliquot of PCR solution was subjected to pyrosequencing in the PyroMark Q24 instrument (QIAGEN) and 0.3 µM sequencing primers (*EGFL7*_SP 5′-TTAGGTTTATTTTTTTATTTTTAA-3′ and *RASSF1*_SP 5′- GGTTAAGGTATTTTAAAGATAAGA-3′).

Table 1 Patients' epithelial ovarian cancer (EOC) characteristics and age.

DNA pool	Sample ID	Age	Histology	Stage
1	RA001	62	Serous EOC	IIIC
1	RA002	67	Serous EOC	IIIA
1	RA003	59	Serous EOC	IIIC
1	RA004	48	Serous EOC	IIIC
1	RA005	50	Serous EOC	IIIC
1	RA006	60	Serous EOC	IIIC
2	RA007	72	Serous EOC	IVA
2	RA008	58	Serous EOC	IIIA
2	RA009	67	Serous EOC	IV
2	RA010	78	Serous EOC	IV
2	RA011	65	Serous EOC	IV
2	RA012	70	Serous EOC	IV
3	RA013	44	Clear cell	II
3	RA014	57	Serous, Mucinous, Endometrioid	IC2
3	RA015	84	Mucinous	III
3	RA016	65	Serous, Clear cell	IIIB
3	RA017	70	Clear cell, Endometrioid	IC1
3	RA018	50	Clear cell	IV
4	RA019	48	Endometriotic cyst	–
4	RA020	76	Benign; Brenner tumor	–
4	RA021	57	Benign; Mucinous cyst	–
4	RA022	57	Benign	–
4	RA023	44	Endometriotic cyst	–
4	RA024	41	Endometriotic cyst	–
4	RA025	19	Endometriotic cyst	–
4	RA026	61	Fibroadenoma	–

Statistical analysis

Mean age was used for analysis. Percent methylated markers from pyrosequencing′ were compare using IBM SPSS Statistics 20 and Kruskal-Wallis test with p-value < 0.05 considered significant.

Results

Patients' clinical picture and age

Histology of ovarian cancer as classified by World Health Organization (2014) guidelines was as follows: 12 (46%) cases of serous EOC, 6 (23%) non-serous cases and 8 (31%) benign cases (Table 1). Mean age (years) at time of diagnosis of patients with serous, non-serous and benign was 63 (range 48 to 78), 62 (range 44 to 84) and 50 (range 19 to 76), respectively. The stages of ovarian cancer (18 cases) as defined by FIGO (2014) were: 2 (11%) cases at stage I, 1 (6%) case at stage II, 9 (50%) cases at stage III, and 6 (33%) cases at stage IV.

Microarray analysis of DNA methylation

DNA of the 26 ovarian samples were divided into four pools: pool 1 and 2 were from serous EOC which had similar characteristics, including the histology and the stage of disease,